Developing an Animal Model of Single Sided Deafness (SSD)

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Abstract

There remains a large gap in the literature regarding the mechanisms behind single-sided deafness (SSD) in humans. To begin our analysis of this issue, it is necessary to first develop an animal model to represent the phenotype witnessed among human patients. We used a transgenic mouse that expresses the human diphtheria toxin receptor (DTR) in cochlear hair cells and injected different concentrations of diphtheria toxin (DT) into the posterior semi-circular canal (PSCC) of the left ear of the adult mouse to ablate hair cells and thereby potentially model the rapid onset typically seen in the human condition of SSD. Using auditory brainstem response (ABR) testing, we identified appropriate hearing thresholds for 8 kHz, 16 kHz, and 32 kHz tones and considered cochlear damage to be successful when the threshold was greater than 90 dB SPL. Mice exposed to 0.1 mg/ml and 0.05 mg/ml concentrations of DT during PSCC surgery produced waveforms representing unilateral deafness. With the development of this novel model, we subsequently tested the effects of SSD on central auditory circuit plasticity. One measure of that plasticity is the expression of muscarinic acetylcholine receptors, a neurotransmitter receptor thought to be involved in cerebral cortex plasticity following sensory deprivation in other animal models. Using radio-ligand binding techniques, we measured the expression of muscarinic receptors in the primary auditory cortex (A1) and found a strong trend towards a difference in receptor expression ($p = 0.0865$) between the contralateral and ipsilateral hemispheres in the 0.1 mg/ml DT dosed mouse. This knowledge of neuroplasticity in A1 after SSD onset will eventually contribute to better treatment outcomes.

Keywords: acetylcholine, auditory brainstem response, diphtheria toxin, muscarinic receptors, radioligand binding, single-sided deafness

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The person(s) who contributed to the data collection and production of each illustrated figure is indicated in this table:

Figure #	Person(s) who was responsible for the illustrated experiment
1	Luis Rivera collected ABR data on three experimental mice, including this one. I collected data on all other mice and analyzed all waveforms.
2	Diagram from Shaneen et al. (2010).
3	Diagram designed by Dr. Gregory Basura. Brain image processed and inserted by me.
4	Tim Desmond and I developed and edited these brain images.
5	Luis Rivera and I collected data. I analyzed and produced the figure.
6	All data in this figure was collected and analyzed entirely by me.
7	All data in this figure was collected and analyzed entirely by me.

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Introduction

Sudden onset, profound unilateral sensorineural hearing loss, or single-sided deafness (SSD) is common (60,000 cases annually in US). Permanent SSD leads to listener disability and long-term challenges with sound localization and speech perception.

SSD affects 3-8% of the general population. This life-span deficit occurs in 0.5/1000 newborns (Kral et al., 2013; Watkin & Baldwin, 2012), 2–5/1000 children (Hassepass et al., 2013), and 1/10,000 adults (Vincent et al., 2015). In the US, nearly 60,000 people acquire SSD each year (Weaver, 2015). The etiology is unknown and may result from sudden inner ear viral inflammation or ischemia. Untreated children are at risk for speech delays and cognitive deficits (Sharma et al., 2016). Usami et al. (2017) reported SSD to impact approximately 7.9-13.3% of the population in the United States. This number has even been classified as an underestimate in some cases, as many of these studies are based on self-reporting (Agrawal et al., 2008). Both children and adults can experience unilateral hearing loss so profound as to be classified as SSD. Furthermore, considering the aging population and the increase in the use of personal listening devices, this number is expected to increase over time.

Hearing loss can be a disabling condition. It has become a societal problem as it impacts overall health care costs and general quality of life for the sufferer. Sudden and rapid presentations of deafness also characterize SSD, making gradual adjustment to the new sensation and stimulus presentation extremely challenging. This change in stimulus presentation can be frustrating and impact social well-being and confidence.

Currently, there exists an incomplete understanding of the underlying etiology of SSD as well as optimized treatments (Van Zon et al., 2015; Kitterick et al., 2015; Bishop et al.; 2010). We attempted to contribute to the growing literature investigating SSD to improve prevention strategies, accurate diagnosis, effective treatments, and overall quality of life. Tertiary prevention is especially of interest in our efforts as we attempted to track changes in brain plasticity resulting from the unique presentation and long-term impact of this pathology. By producing an effective animal model that may mimic the human phenotype, we will create a platform for further research into SSD to increase our understanding and ultimately improve health outcomes.

Clinical Presentations of SSD

SSD is a complete, or near-complete, unilateral sensorineural hearing loss that is often sudden in onset and permanent. SSD is typically diagnosed as an isolated problem, yet may be associated with other otologic signs or symptoms (Schreiber et al., 2010). The standard threshold of characterizing SSD in patients is evidence of audiometric thresholds that meet or exceed 90dB sound pressure level (SPL) in one ear. However, some also consider diagnoses of SSD patients to be more complex and that it should be a comparison of the hearing thresholds of both ears (Lucas et al., 2018). Hearing loss in general usually begins at higher frequencies, and severity can be classified as mild, moderate, and severe to profound (Agrawal et al., 2008). SSD is often described as sudden and profound in nature versus other forms of unilateral deafness that may be progressive or insidious in onset or nature.

Despite the prevalence of this pathology most patients are not routinely screened for SSD (US Department of Health and Human Services, 1997). Rather, given the typical sudden nature of SSD onset, patients often present to the emergency room or primary care provider who eventually refer them on to an audiology or ear nose and throat specialist. It is important to distinguish the sudden onset (minutes to hours) nature that typically, but not always, defines SSD, versus the gradual onset of hearing loss that can manifest over months to years to decades. As such, patients suffering from SSD are usually immediately aware of the loss that is sudden and profound, which typically prompts them to seek medical attention. Commonly associated symptoms with SSD include rapid-onset tinnitus (phantom perception of sound in the absence of a bonafide sound stimulus), ear (aural) fullness, or feelings of blockage or fullness within the middle ear (Schreiber et al., 2010). Vertigo (sense of rotational movement in the absence of actual physical rotation) may also be reported by patients, suggesting the presence of a concurrent peripheral vestibular insult (Lee & Baloh, 2005) that may impact gait and general mobility and stability.

Causes of SSD

The etiology of SSD is unknown and may result from an inner ear viral inflammation or ischemia. Permanent SSD leads to long-term difficulties with sound localization and speech perception in noise. Untreated children are at risk for speech delays and cognitive deficits (Sharma et al., 2016). Commonly, these pathologies are attributed to dysfunction within the

cochlea, although damage to the middle or external ear can also result in this phenotype (Agrawal et al., 2008). Damage to the auditory nerve or cerebral cortices less commonly results in hearing loss, especially in loss so profound as to be quantified as deafness (Lee & Baloh, 2005). Still, causes of hearing loss may be classified as either sensorineural (involving the inner ear or auditory nerve), conductive (mechanical cause leading to prevention of sound from reaching the inner ear) or both, termed mixed hearing loss (Bogardus et al., 2003). Patients do not need to have a prior history of risk factors to experience SSD, including noise exposure, head trauma, meningitis, ototoxic drug use, or infectious causes (i.e., syphilis).

Causes of SSD greatly differ based on age of onset (Usami et al., 2017). Early-onset SSD was commonly cited to result from cochlear nerve deficiency (CNV). Other causes in early-onset SSD include cytomegalovirus (CMV) infection, mumps infection, and inner ear malformations (Simons et al., 2006). Adult SSD cases, classified as post-lingual, represent different etiologies. In one study, idiopathic (etiology unknown) sudden sensorineural hearing loss (SSNHL) was cited to be the most common etiology within adult patients (REF). Other causes include chronic otitis media, cholesteatoma, and ANCA-Associated Vasculitis (OMAAV). Functional hearing loss and cerebellar tumors were cited as less-likely causes but are still of importance as they require a much different approach in intervention. Finally, while a majority of early-onset bilateral hearing loss diseases are attributed to genetic causes, most cases of unilateral hearing loss are not and ultimate etiology is unknown (Morton & Nance, 2006).

Prevalence of SSD

Unique clinical presentations of SSD have encouraged the design of demographic studies to understand its prevalence, further improving health care delivery, screening, and rehabilitation. Agrawal et al. (2008) aimed to contribute to the knowledge by further understanding the demographics of deafness and cited differences in unilateral and bilateral hearing loss. They estimated that 29 million Americans between the ages of 20 to 69 experienced some form of hearing loss, whether unilateral or bilateral. This exceeded the cited estimate of 28 million Americans at the time of the report, which they hypothesized was attributed to an aging population and the increase in exposure to damaging noise through personal devices. These authors also note the increased prevalence of hearing loss in adults over 70 years and recognized

a disproportionate level of hearing loss among males and white participants, especially in the third and fourth decades of life.

Niskar et al (1998) analyzed the influence of sex, race/ethnicity, and socioeconomic status on hearing loss in children. While a correlation between demographics and the condition was inconclusive, they encouraged further research be conducted as variations in environmental exposure is still a likely modifying factor. Severity of hearing loss was only slight in this population, with unilateral hearing loss being most prevalent. Similar to older populations, more children were cited to experience hearing loss at higher frequencies than lower frequencies. Overall, the presence of deafness among a range of ages may suggest that this divergence from the normal phenotype is initiated at early life stages.

Approximately 50% of the participants in the Agrawl et al. study demonstrated phenotypes akin to SSD (2008). Again, male, white, and less educated participants were more likely to present with unilateral deafness. Despite the overlapping results in susceptible populations, these authors hypothesized that there is a distinction between the pathophysiological processes that cause unilateral and bilateral deafness.

A unique characteristic of SSD is its sudden presentation. Similarly, SSNHL is defined by an increase in hearing threshold of more than 30dB SPL over three consecutive frequencies within 72 hours. Hwang et al. (2017) focused on outcomes after treatment from patients presenting with SSNL. They determined that age may be a predictor of non-recovery after treatment, indicating the possible influence of demographics on onset and treatment outcomes of unilateral deafness.

It is particularly difficult to find demographic data on unilateral deafness cases; bilateral deafness and sudden hearing loss were among the most prominent topics of study (Agrawal et al., 2008; Niskar et al. 1998). The severity of the symptoms of the latter conditions may encourage more resources directed towards developing the literature. However, the impact of SSD on the daily lives of patients are more profound than a unilateral increase in threshold.

Effects of SSD

Patients with unilateral hearing loss experience major effects in the processing of the sound that they can recognize with the unaffected, only-hearing ear. The symmetric skull of humans has made binaural cues across a horizontal plane vital in sound localization (Kumpik &

King, 2018). Distinguishing whether a sound is in front of or behind the listener can be extremely difficult without intact bilateral hearing. These comparisons are also frequency dependent, with higher frequency sounds being more difficult to localize in space. Unilateral hearing loss and SSD also impairs target detection of speech, or the ability to filter background noise in "cocktail-party" situations. Interfering sounds are especially prominent without bilateral hearing, which impacts patients' ability to multitask.

While these incidents are especially prominent during initial periods of SSD, and especially with sudden SSD, patients have employed short-term strategies to improve their auditory experiences, even before diagnosis. Examples of this include head-turning to achieve direct listening in conversations and speech-reading to decipher conversations through a different sense (Wie et al., 2010). Patients have also been cited to eventually modify their reliance on binaural cues over time to improve their abilities. In both early- and late-onset SSD, neuroplasticity is important in calibrating neural circuits based on stimuli presentation (Kumpik & King, 2018). By re-weighting different spatial cues through experience and/or training, sensitivity to binaural spatial cues can be improved and diminish the effects of SSD. This evidence of neuroplasticity has fueled interest in further investigation of the impact of SSD on the brain. The dramatic and long-term change in stimuli presentation has allowed the research community to postulate this to be an important effect of the initial clinical symptoms used for diagnosis. The literature, and current research gaps will be discussed in a later section.

The untoward effects on communication following SSD may significantly impact social interaction. The impact of their reduced abilities is a subjective experience and varies across the patient population (Wie et al., 2010). Still, difficulty fixating on a sound when background noise is present or difficulty processing speech in general can be exceedingly disabling. SSD patients have reported feeling excluded in conversations with multiple speakers, experiencing reduced well-being in social settings, and avoiding social gatherings all together. As such, SSD may lead to increased rates social isolation leading to or resulting from clinical depression and anxiety.

Other consequences of SSD have also been explored. Lucas et al. (2018) found common themes in processing SSD within a small cohort of patients. These include worrying about losing their hearing in their unaffected ear, experiencing strong negative emotions such as embarrassment and frustration, low self-esteem, and negative coping strategies such as lack of motivation to complete situations that challenged their hearing ability. Interestingly, the study

also identified some positive impacts of the condition on patient mentality. Participants experienced reduced disturbance from unwanted sounds, pride in their ability to successfully communicate by other means, happiness in receiving support from others, and satisfaction with their ability to develop coping strategies. Nevertheless, the array of negative physiological and social consequences of SSD is substantial, especially considering the fact that patients still experience normal hearing in one ear.

Treatments of SSD

For permanent SSD, auditory rehabilitation options include hearing aids with contralateral routing of signal (CROS-HA) or bone-anchored hearing devices (BAHA) (Bishop & Eby, 2010). These route signals to the non-affected ear and have limited success with speech perception and sound localization. Only the cochlear implant (CI) offers ear-specific and central auditory pathway rehabilitation. CI is not consistently available for SSD in the US as insurance authorization criteria currently require bilateral deafness. For patients who receive a CI for SSD, there is large variability in hearing rehabilitation performance that may be a direct result of age, deafness duration and timing of implantation, all of which, may impact central auditory plasticity (Tokita et al., 2014). Most patients with SSD go without treatment for years and so understanding the potential window to intervene is highly important.

While a cure for SSD does not currently exist, some treatments have been developed and analyzed for efficacy. The effects of head shadowing have been reduced by devices which re-route sound that arrives at the impacted ear to intact, only-hearing ear (Bishop & Eby, 2010). Similarly, background noise can be processed to exist at a normal ratio compared to the actual signal through contralateral routing of signals (CROS). Cochlear implants are also available to support this reestablishing of a normal environment for signal processing. Only cochlear implants truly rehabilitate the ear and the useful aspects of binaural hearing to increase ability to process intensity and improve sound localization for improved speech understanding (Kitterick et al., 2015).

While some CROS hearing aids may improve quality of life in SSD patients as measured by the HRQOL (Kitterick et al., 2015), they do not reverse symptoms or reduce difficulties with speech or sound localization. Furthermore, the sparse research in this area has provided limited

understanding into the optimal timing of treatments after SSD onset. Our work could contribute to understanding key mechanisms of SSD that may eventually apply to optimal treatments.

SSD and Neurobiology: Long-Term Effects

In addition to the short-term physiological changes and social disadvantages of SSD, this change in stimuli presentation and overall experience can result in long-term impact. As previously discussed, perception of auditory stimuli following unilateral deafness can change overtime the brain becomes acclimated to the new sensation; patients may begin to overcome the loss of binaural cues through other techniques subconsciously (Kumpik & King, 2018). This phenomenon has prompted increased investigation into changes in the brain or neuroplasticity as a potential primary underlying feature.

Neuroplasticity is generally defined as the restructuring of neural connections via synaptic strengthening, generation, and degradation. Different mechanisms of neuroplasticity exist (Glick & Sharma, 2017). Cross-modal plasticity is a form of neuroplasticity that can be a consequence of decreased or abnormal sensory input. Cerebral cortical regions that lose significant amounts of sensory input due to unilateral hearing loss become vulnerable to other non-auditory sensory inputs that may still be intact and arrive at that hemisphere. In addition, as one sensory system is partially lost, patients may begin to rely on another sense as compensation, as discussed by the use of speech-reading to disambiguate speech. Another form of neuroplasticity is intra-modal plasticity, where a particular cortical area of the brain is impacted by the increase or decrease in sensory input. Studies have explored which form of neuroplasticity is the result of auditory deprivation. Sharma & Glick (2016) collected data that supported the former, hypothesizing it to be a result of recruiting visual or somatosensory processing. However, considering the drastic change in stimuli presentation and the streamlined nature of the auditory pathway, intra-modal plasticity and impact to the auditory cortex is still a possibility of SSD (Kim et al., 2018).

Studies have attempted to track neuroplasticity in animal models and human patients to understand the impact of SSD on neural pathways. In humans, normal hearing is classified by symmetric auditory stimuli presentation in both ears, which sends signals to the brainstem and projects ipsilaterally and contralaterally within the auditory pathway (Chang et al., 2016). The final step of this signal transduction pathway is the accumulation of these inputs into the bilateral

auditory cortices. Disrupting the paired function of the two ears is what makes the short-term effects of SSD so drastic and is potentially what promotes reorganization of the central auditory pathway. Adults with normal hearing demonstrate bilateral hemisphere activation with dominance of the contralateral pathway (Maslin et al., 2013). In contrast, SSD and asymmetric hearing loss can result in shorter latencies and larger amplitudes in contralateral hemisphere activation compared to the ipsilateral hemisphere. Similar results of hemispheric asymmetry have been demonstrated thorough additional analytical tools including electroencephalography (EEG).

Animal studies have explored other techniques to demonstrate neuroplasticity of the auditory cortex after hearing loss. Robertson & Irvine (1989) utilized a technique to damage the cochlea within guinea pigs to induce partial unilateral deafness and analyzed the impact to a topographical map of sound frequency within the adult auditory cortex. They discovered significant reorganization, where neurons responded to frequencies outside of their traditional range. This suggested that an expansion of representative frequencies in the adult auditory cortex had occurred as both the lower and upper boarders of frequency range had significantly expanded following the trauma. Kim et al. (2018) analyzed the impact of a unilateral cochlear ablation surgical technique in a mouse model on neural activity using manganese-enhanced magnetic resonance imaging (MEMRI). The decrease in manganese on both the ipsilateral side (within the cochlear nucleus of the auditory cortex) and contralateral side (within the superior olivary complex, lateral lemniscus, and inferior colliculus) represented significantly decreased neural activity in their respective regions. They also witnessed a partial recovery of this response as the duration of the SSD model increased. Overall, these studies support the possibility of neuroplasticity as a modifying factor in the experience of SSD as well as the use of animal models in further exploring the impact of this pathology.

As alluded to by Kim et al. (2018) the impact of SSD can change over time. Therefore, it is necessary to explore the ways in which the symptoms and neural changes within the auditory pathway adapt overtime. Furthermore, the fact that SSD occurs in both children and adults makes the age of onset important in analyses. As children develop, they encounter sensitive periods where extrinsic sensory stimulation profoundly shapes synaptogenesis, therefore impacting the organization of the cortex. For the auditory cortex, peak synaptogenesis occurs between 3.5 to 4 years of age (Sharma & Glick, 2016). Extrinsic factors will continue to refine the auditory

pathway for the remainder of their lives, making adult-onset cases important to study as well. Overall, human and animal studies have shown that reorganization of neural connections can occur during critical periods of development and well into adulthood when neural differentiation and development is complete (Kim et al., 2018; Robetson & Irvine, 1989; Glick & Sharma, 2017; Chang et al., 2016).

Understanding the impact of SSD and unilateral hearing loss on cortical regions is crucial as it impacts treatment outcomes. As noted previously, treatments of this pathology have extended to cochlear implants in an attempt to restore the functionality of the impacted ear rather than rerouting stimuli to the intact ear. The success of this treatment approach is dependent on whether the impacted ear is salvageable after dormancy. Studies have already begun to demonstrate the disappearance of contralateral dominance in the auditory cortex after unilateral hearing loss (Kim et al., 2018). With this knowledge, the question now lies in what extent this reorganization occurs and whether there is an optimal time window for intervention. Furthermore, understanding whether reorganization occurs within the auditory cortex alone or if it is accompanied by other cortical areas that process sensory information (i.e. visual cortex, somatosensory cortex, etc.) is necessary to account for the impact of sudden restoration of bilateral hearing to our general neurobiology and sensory systems.

Creating an Animal Model of SSD

Despite the existence of these data, there still exists a large gap in the literature regarding the impact of SSD on neuroplasticity and long-term effects that would ultimately influence the development of treatments and their outcomes (Kim et al., 2018).

The Kresge Hearing Research Institute at the University of Michigan has recognized the vitality of an intact auditory system on quality of life. Efforts through the laboratory of Dr. Gregory Basura have previously worked to understand the mechanisms of tinnitus through a collection of anatomical and physiological studies within animal models. Recognizing the prevalence of SSD cases in the Otolaryngology Department in the University of Michigan Health System, we utilized similar methodologies to gain insight into neurological characteristics impacted by SSD.

We attempted to contribute to the understanding of the impact of SSD on the primary auditory cortex (A1) as it is a crucial point in the auditory processing pathway. We focused on

A1 as there is an obvious change to auditory presentation with this disorder and ambiguity in the changes in other senses after unilateral deafness.

An animal model that mimics the human condition of sudden onset SSD is needed to better characterize the changes in central auditory circuits after the insult. Our study was designed to provide a reliable representation of SSD to the field using diphtheria toxin receptor (DTR) mediated targeted cell ablation in a mouse model. Destroying hair cells within the inner ear through this technique allowed us to avoid confounding variables cited in other animal models (Kim et al., 2018; Robertson & Irvine, 1989). Ultimately, future studies will be able to better define etiology of SSD and its effects. This will improve current outcomes and inspire the design of new, more effective treatments.

Evaluating A1 Plasticity After SSD

Cerebral cortex plasticity is often mediated by acetylcholine. Receptors of this neuromodulator can be classified as either nicotinic or muscarinic and both exist on excitatory and inhibitory neurons in the central auditory system, including within the auditory cortex (Deng et al., 2015). Nicotinic acetylcholine receptors are ionotropic, while muscarinic acetylcholine receptors are metabotropic members of the G protein-coupled receptor family. While both receptor types are present in the cerebral cortex, nicotinic receptors have been recorded at constant levels throughout cortical development while muscarinic receptor expression peaks at specific intervals (Shideler & Yan, 2010). This has led many to classify muscarinic acetylcholine receptors as especially critical for development, maturation, and ongoing modification the cortex.

Our study used [^{3}H]scopolamine, a muscarinic acetylcholine receptor antagonist, in radioligand binding assays as it has the ability to pass through the blood brain barrier and bind to muscarinic receptors in the central nervous system (Klinkenberg & Blokland, 2010). This strategy allowed us to identify changes in muscarinic receptor density within A1 resulting from sudden and rapid unilateral deafness.

Materials and Methods

Mice Preparation and Background

Mice are an ideal species to develop a possible model for SSD as they have undergone many genetic manipulations that provide opportunities to mimic human conditions. As such, we used a mouse model and technique developed by Golub et al. (2012) to produce transgenic mice with the ability to conditionally and selectively destroy hair cells in the adult mouse cochlea with the injection of diphtheria toxin (DT) via surgery protocol. In this mouse model, expression of the human diphtheria toxin receptor (DTR) gene is driven by the promoter for Pou4f3, a gene selectively expressed in cochlear hair cells. Systemic injection of diphtheria toxin (DT) in these mice results in profound loss of cochlear hair cells and bilateral deafness, without inducing damage to spiral ganglion neurons or other parts of the cochlea.

To breed Pou4f3^{DTR} mice, we crossed heterozygous Pou4f3^{DTR} mice (Jackson Labs stock # 028673) with wild-type C57BL6/J mice. In total, we used five (5) DTR mice and six (6) control mice (sham mice) for our study. DTR mice were congenic with the C57BL6/J background and sham mice were of C57BL6 background. Ages of the mice (based from first ABR reading) ranged from P45 to P58 (exception of Treatment 1 mice (2) being P139), with sham mice being less variable and complications in breeding resulting in a more variable age range within DTR mice. Sham mice were also exclusively male in an attempt to reduce confounding variables. The difficulty in breeding with DTR mice resulted in breeding of female models to mediate effects of the DTR gene (DTRxC57BL6/J). DTR mice also exhibited higher mortality rates and different behavioral patterns, including difficulty in anesthetization prior to surgery. Otherwise, this model was cooperative with our surgical methods, specifically with our use of DT. All mouse experiments were approved by the University of Michigan Institutional Animal Care and Use Committee (protocol # PRO00006642) and were in accordance with NIH guidelines for the care and use of laboratory animals.

Auditory Brainstem Response Testing

Auditory Brainstem Response (ABR) testing produces an objective measure of hearing function (Melcher et al., 1996b). These recordings are collected by relatively noninvasive means, making it a credible tool among animals and humans. The signals produced represent an evoked

response from auditory nerve and brainstem auditory pathway activity (Figure 1). Synchronous activity from these groups of nerves is produced by a stimulus, here represented by a click, with activity large enough to shadow any competing background noise from the ABR recording (Melcher et al., 1996a).

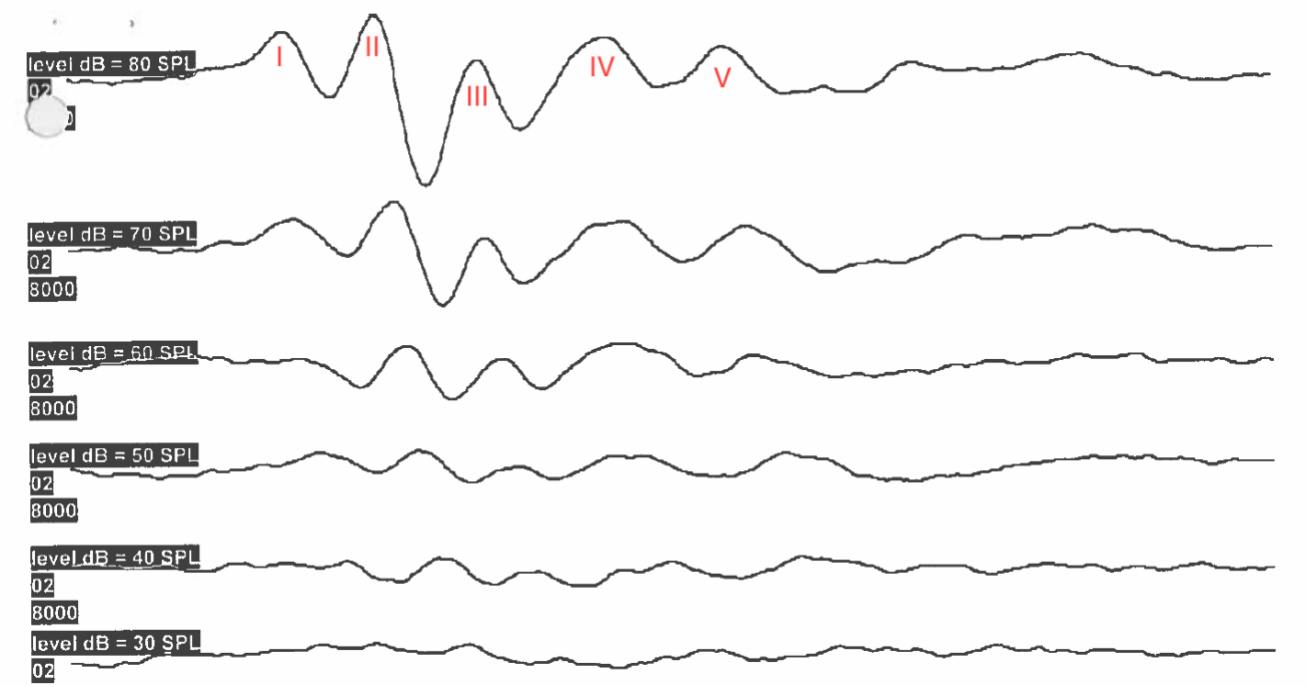

Figure 1. Example of waveforms produced by auditory brain response (ABR) testing. From Treatment 2 mouse recording at 8 kHz. Level dB on the left side represents the decibel level of the tone in the mouse's ear. Roman numerals are used to label waves representing different fiber activity or cellular activity within the auditory system. Wave I: auditory nerve fiber activity. Wave II: neural activity from the cochlear nucleus. Wave III-IV: activity from higher auditory brainstem locations (lateral and medial olivary complexes and lateral lemniscus) resulting from activity in the cochlear nucleus. Wave V: neural activity from the inferior colliculus.

To verify the initial presence of normal auditory functioning and post-surgical unilateral hearing loss, ABR testing was conducted three weeks before surgery and after the 10-day recovery period after surgery. Mice were anesthetized with an initial intraperitoneal injection of ketamine/xylazine cocktail (100 mg/kg ketamine, 10 mg/kg xylazine) and maintained under deep anesthesia via administration of maintenance doses of ketamine (33 mg/kg) as needed throughout the readings.

Further preparations, configurations, and interpretation of output signals aligned with Melcher et al. (1996) studies under the titles "Generators of the brainstem auditory evoked potential in cat. I." and "Generators of the brainstem auditory evoked potential in cat. II." Animals were placed on a heating pad within the sound proof recording booth to maintain body temperature. Three electrodes were inserted under the skin in the following positions: (1) the vertex, (2) the right ear, and (3) the left ear. Further research on electrode placement encouraged us to use the configuration adopted by Shaneen et al. (2015). See Figure 2. Sound was delivered to each ear independently though the use of a calibrated speaker, which administered 4 ms tone pips at a rate of 40/s.

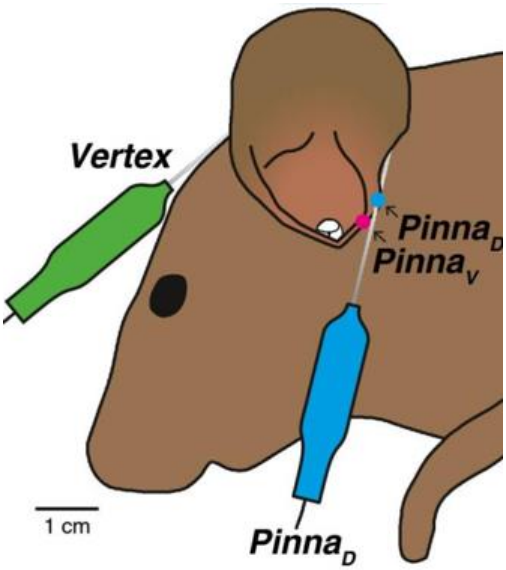

Figure 2. From Shaneen et al. (2010). Schematic of electrode configuration for ABRs. Pinna to pinna configurations used positive electrode placed in identical location on contralateral side (not shown).

A major modification of the technique outlined by Melcher et al. (1996a) included the range of frequencies analyzed for our study. To encompass a wide spectrum of frequencies within the mouse model, we used 8, 16, and 32Hz signals. Based on a collection of literature also cited by Melcher et al. (1996a), we considered 40dB SPL to be the threshold for hearing. Threshold was defined as "the lowest level that could elicit a visually detectable neural response." Of note, signals were subjectively read rather than computationally analyzed considering the nature of our study. Our main goal was to verify the presence or loss of auditory functioning. The limits of our equipment capped our maximum volume at 110dB SPL. An ear was considered successfully deafened when it showed no signal at around 100dB SPL.

Posterior Semicircular Canal Surgery

Our largest barrier to further understanding the impact of SSD on A1 muscarinic receptor density has been the design of a reliable animal model with the ability to exhibit unilateral hearing loss without loss of spiral ganglion neurons during its adult life. This sudden loss beyond the critical stages of development is an important feature of our model as it would reflect the human phenotype.

Suzuki et al. (2017) designed a procedure that successfully delivered adeno-associated virus (AAV) injections into the posterior semicircular canal into an adult mouse without major complication, including threshold shifts, suprathreshold dysfunction, or hair cell damage. A post-auricular incision and blunt dissection of the thin muscle layers overlying the temporal bone allowed them to spot clear landmarks to determine the location of the canal. A 26-gauge hypodermic needle and microcatheter were utilized to administer the injection. This procedure provided us with a supported approach in designing a vector to deliver toxin into the posterior semicircular canal (PSCC).

After verifying animals exhibited normal hearing thresholds, surgical preparations were completed. Sterile drapes were used to cover the workspace. Tools were sterilized via autoclave. A heating pad was placed over the surgery bed and covered with a paper towel to avoid over-heating. Temperature was monitored via rectal probe. A nose cone was also attached to the surgery bed to continually administer isoflurane anesthesia during the procedure. As in Suzuki et al. (2017), a cannula connected to a pressure injector (Nanoject system) was utilized to inject diphtheria toxin (DT) into the posterior canal.

Animals were first anesthetized in a chamber regulated with 1.5% oxygen and 3% isoflurane for 3-5 minutes. After anesthesia induction, animals were placed on surgery bed and within the nose cone, and their eyes protected with artificial tears ointment. Carprofen (5 mg/kg) and buprenorphine (0.1 mg/kg) were injected subcutaneously. Anesthesia was decreased to 1% oxygen and 2% isoflurane. The surgical area (behind the pinna) was shaved and sterilized with three alternating washes of iodine and ethanol. A small incision was made to the skin to reveal the muscles and region of interest. Similar landmarks in the Suzuki et al. (2017) procedure were noted to find the PSCC. Special care was made to prevent damage to the muscles. A #11 scalpel was used to create a ridge on the bone for the 26-gauge needle, which was then rotated to create a substantial hole within the bone for the cannula. The cannula was placed and used to inject approximately 250nl throughout a span of five minutes, delivering a total dose of 1.25ng DT. The cannula was kept in the area for an extra five minutes following injection. A small piece of muscle was placed inside the hole within the bone and surrounding muscles were moved back into place before suturing. Lidocaine was administered over the surgical wound. Mice were then placed in recovery and verified as responsive before return to home cage. In accordance with guidelines from the Institutional Animal Care and Use Committee (IACUC), animals were routinely monitored and fed diet gel throughout the recovery period.

Control mice also underwent anesthetization through the same parameters and were sham-operated. The surgical area was shaved and an incision was made. However, no opening was made in the PSCC. The wound was sutured and lidocaine was administered. Recovery protocol was followed as described above.

Tissue Preparation

After deep anesthetization by isoflurane in an isoflurane drop jar, animals were sacrificed by decapitation and brains were rapidly dissected. Brains were removed and snap frozen in isopentane at 20°C and stored at -80°C until ready for use.

Standard sectioning procedure was completed to prepare slides for autoradiography experiments. Frozen mice brains were thawed from -80°C and sliced at 20μm using Bright Instruments cryostat set to -18°C. Location of A1 was determined through major neurobiological landmarks (Figure 3). Tissue was thaw-mounted on the 1x3 inch poly-L-lysine subbed glass slides until processed for radio-ligand binding.

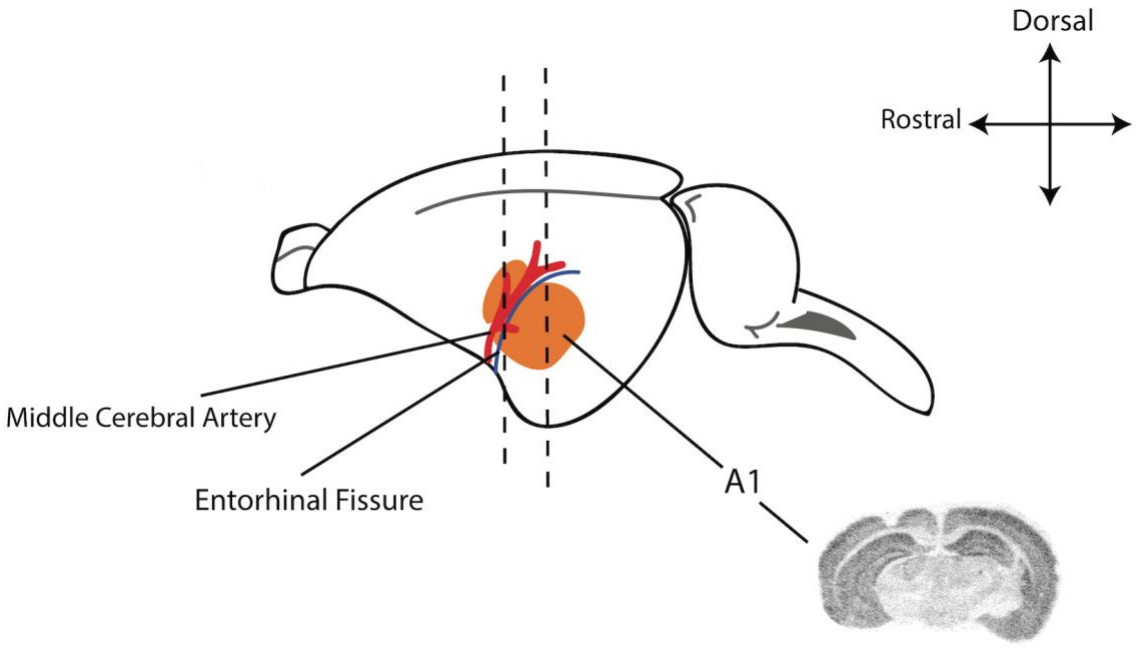

Figure 3. Location of Primary Auditory Cortex (A1) within the mouse brain.

[^{3}H]Scopolamine Receptor Autoradiography

As outlined by Frey and Howland (1992), coronal brain sections containing A1 used for autoradiography experiments were pre-conditioned for 5 min with phosphate buffer saline (PBS) pH 7.4. Sections were transferred to a solution of [^{3}H]scopolamine in PBS pH 7.4 and incubated for 30 min. Subsequently, [^{3}H]scopolamine-labeled tissue sections were washed with PBS pH 7.4 for 2×5 min and rinsed with deionized H_2O for approximately 5 sec. Finally, all slides were dried at room temperature before being exposed to a tritium-sensitive phosphoimager screen (Fuji) for 3 days. The screen was read in a GE Healthcare Typhoon FLA 7000.

Optical Density and Statistical Analysis

Densitometry of the phosphoimager screen was performed with the ImageQuant program. Considering our interest in A1, analysis was focused on this region, as demonstrated by Figure 4. The Allen Mouse Brain Coronal Atlas (2011) was used to determine the location of A1. Receptor density was calculated from co-exposed standards (considering background) and

converted to fmol/μg protein using a standard curve. Once conversions were completed and background non-interference was verified, contralateral (right) A1 densities were compared to ipsilateral (left) A1 densities using a linear mixed model via LME4 library on R Commander. We considered significance to be present at $p \leq 0.1$.

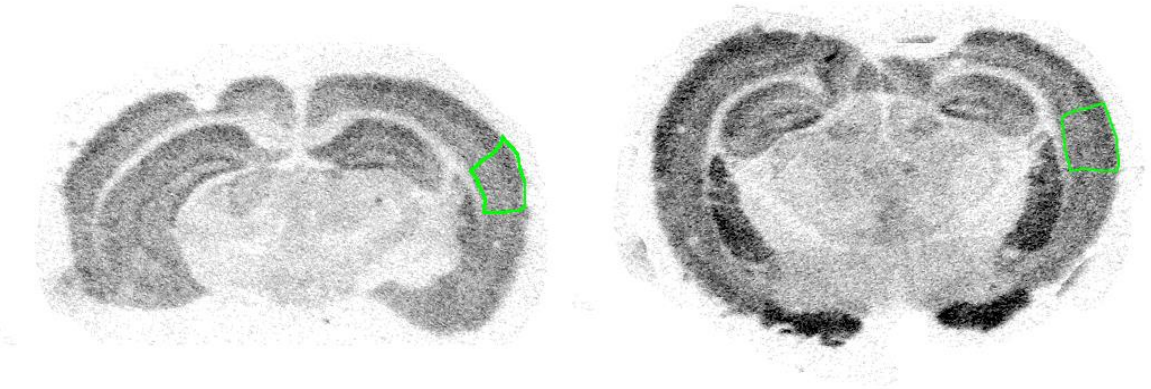

Figure 4. Examples of outlines drawn around primary auditory cortex (A1) in coronal slices of mouse brains to determine optical density in densitometry analysis.

Results

Threshold Shifts Represented by ABR Testing

Before PSCC Surgery. All mice injected with DT (experimental) and control (sham) groups produced normal ABR responses before PSCC surgery. This verified the presence of normal auditory function within the mice prior to experiments.

After PSCC Surgery. After the three-week period of recovery post-surgery, sham mice did not demonstrate an ABR threshold shift and did not show any effects of anesthesia alone on hearing thresholds. Because the exact concentration of DT to achieve SSD is currently not known, experimental mice received different concentrations of DT (Table 1). The experimental mouse exposed to treatment 3 demonstrated a shift in ABR threshold in the surgically injected left ear at 8kHz (30dB SPL to >110dB SPL) and 16kHz (60dB SPL to >110dB SPL). At 32kHz, this mouse presented with thresholds greater than 110dB SPL before and after surgery. No

threshold changes were observed in the right ear (Figure 5). The experimental mouse exposed to treatment 4 demonstrated intact hearing before surgery. However, we could not record ABR responses after surgery as it was difficult to anesthetize this particular animal with our ketamine/xylazine cocktail.

Animal Group	**[DT] (mg/ml)**
Treatment 1	0.01
Treatment 2	0.025
Treatment 3	0.05
Treatment 4	0.1

Table 1. Concentration of diphtheria toxin (DT) used in PSCC surgery on different experimental animal groups.

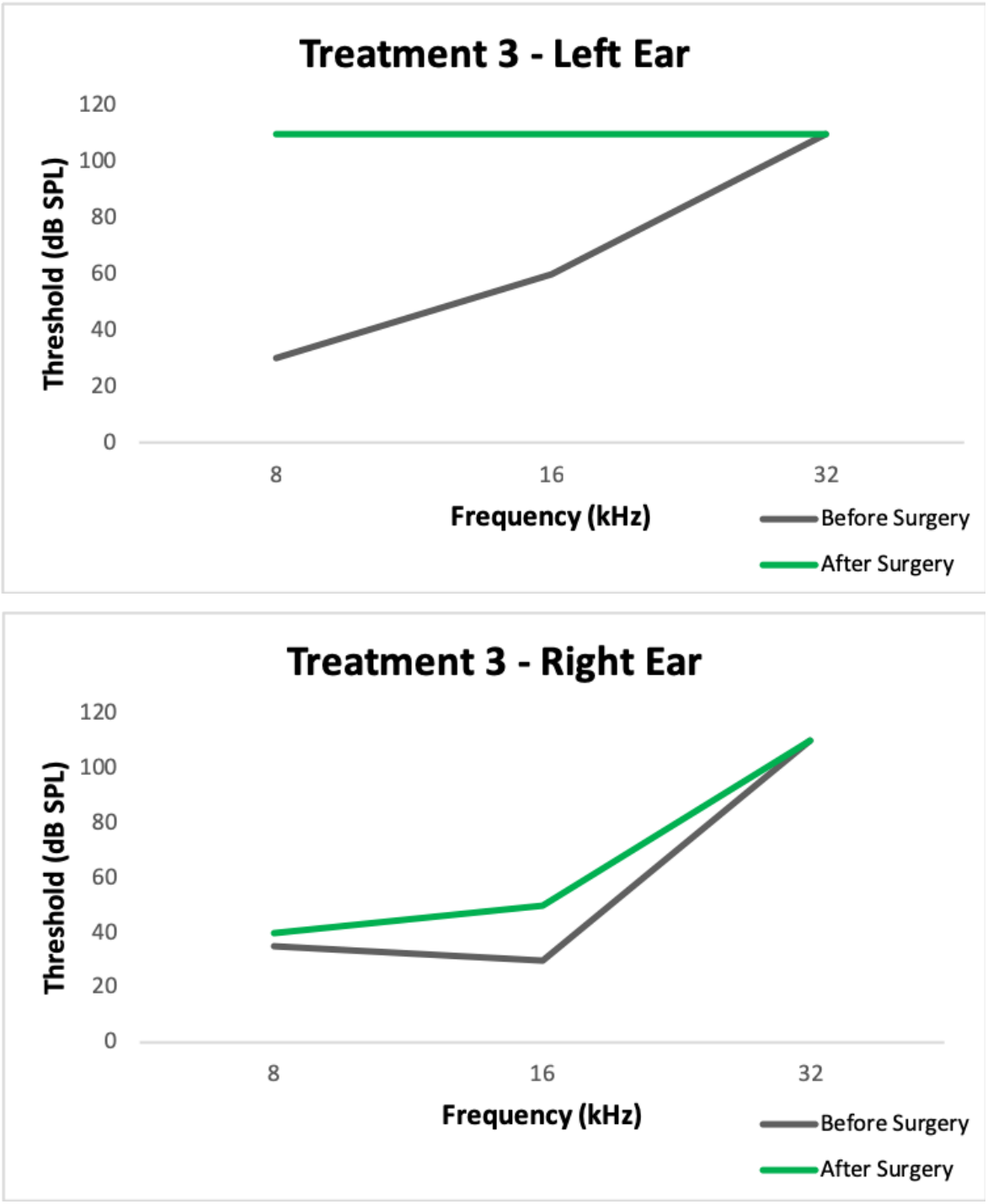

Figure 5. Treatment 3 mouse thresholds of surgically injected (left) ear (above) and non-involved (non-injected) (right) ear (below) before and after surgery recorded via ABR testing.

[^{3}H]Scopolamine Binding in A1

After surgeries and brain harvesting, autoradiography studies of the six (6) sham animal brains demonstrated no significant changes within in A1 optical density measures of [^{3}H]scopolamine binding when comparing the contralateral (right) and ipsilateral (left) hemispheres (Figure 6).

Within the experimental animals, various treatments were classified by the concentration of DT used on the animals (Table 1). Mice within treatments 1 ($p = 0.3028$), treatment 2 ($p = 0.7893$), and treatment 3 ($p = 0.5982$) groups did not demonstrate significant difference in receptor expression between the contralateral and ipsilateral A1. The mouse in treatment 4 did demonstrate a strong trend toward a difference in receptor expression ($p = 0.0865$) between the contralateral hemisphere and ipsilateral hemisphere, with the contralateral A1 demonstrating significantly higher muscarinic receptor expression upon binding with [^{3}H]scopolamine (Figure 7).

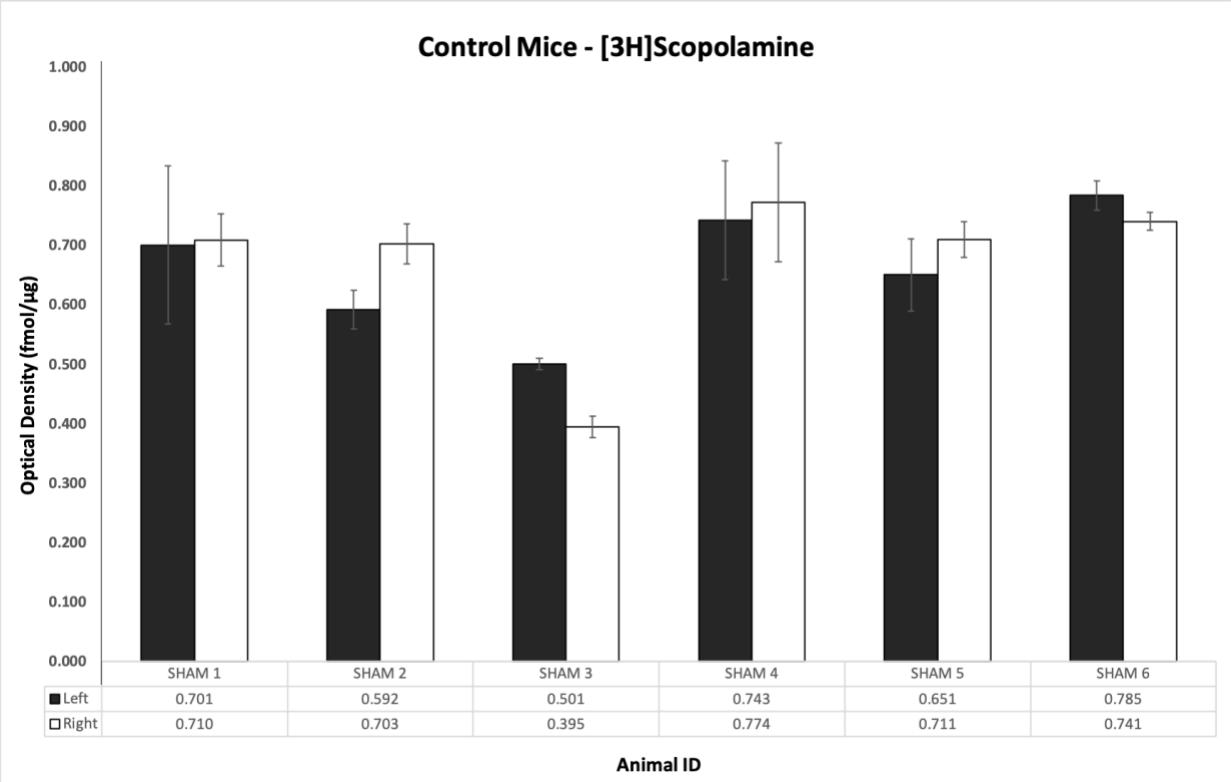

Figure 6. Comparison of optical densities (fmol/µg) of ipsilateral (left) A1 versus contralateral (right) A1 in control mice after exposure to muscarinic acetylcholine receptor antagonist [^{3}H]scopolamine.

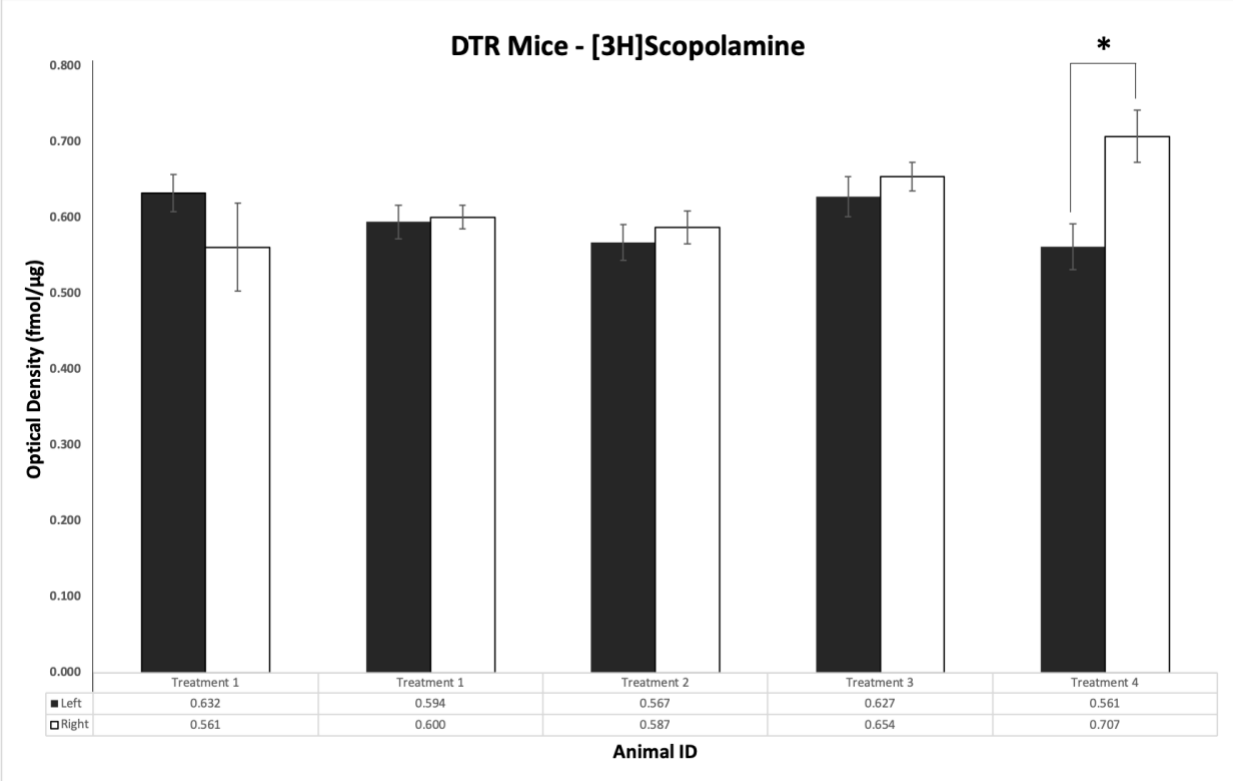

Figure 7. Comparison of optical densities (fmol/µg) of ipsilateral (left) A1 versus contralateral (right) A1 in experimental mice after exposure to muscarinic acetylcholine receptor antagonist [^{3}H]scopolamine ($p < 0.1$).

Discussion

This study attempted to quantify changes in A1 muscarinic receptor density in a possible animal model of SSD utilizing DTR. Our data represents the possibility for the successful development of this novel model for this study and others. It also suggests the likelihood of witnessing changes in muscarinic receptor density after sudden-onset unilateral hearing loss. The development of these methods will allow us to continue to understand patterns of neuroplasticity in A1 after the onset of SSD.

Creating a Model for SSD Using DTR Mice

To understand the impact of SSD on A1, we needed to produce a reliable animal model to represent the phenotype present in humans. Considering current knowledge on the causes of SSD in patients, we determined that targeted cochlear hair cell ablation would produce the most accurate representation. Previous studies have developed models with partial unilateral deafness or SSD by directly impacting the cochlea. Robertson & Irvine (1989), induced the former through direct mechanical lesion of a restricted portion of the Organ of Corti. Lee et al. (2017), induced SSD by disrupting the bony wall of basal turn of the cochlea using a 26-guage needle and followed with irrigation of saline through the perforation to clear the region. Direct trauma to this region is difficult to guarantee. Kim et al. (2018), utilized a more concrete landmark by dissecting the otic bulla to reach the cochlea to avoid impacting the facial nerve. But even when care is taken to avoid impacting other regions, these techniques suggest that SSD is solely induced by trauma to the effected ear. In reality, the causes are much more diverse and complex. Jakob et al. (2015) utilized a similar approach to our study. They injected ototoxic aminoglycoside antibiotic neomycin into the inner ear to destroy cochlear hair cells. While effective, this technique was only demonstrated in mice at postnatal day 10, when the meatus and mesenchyme in the middle ear is still closed and preventing auditory function. Moreover, this technique induced bilateral hearing loss rather than unilateral hearing loss.

A more accurate representation of SSD will allow us to better understand the gaps in our knowledge on SSD. The methods outlined by Golub et al. (2012), proved to be noteworthy as they utilized intramuscular injections of DT to produce bilateral hearing loss of cochlear hair cells. Our study modified their techniques to be the first attempt at unilateral hair cell ablation.

The adult mouse that underwent treatment 3 surgical conditions in our study showed evidence of impacted hearing in the left ear at frequencies of 8kHz and 16kHz and intact hearing in the right, non-surgical ear via ABRs. We were unable to collect recordings from the mouse in treatment 4 due to it being unaffected by our maximum doses of anesthesia. However, it is likely that the increased concentration of DT provided at least the same deafening pattern as in treatment 3. These findings justify future studies that continue to explore the nuances of this technique and its application to our understanding of neuroplasticity throughout the cortex as a result of SSD.

Advantages. This approach allows us to induce SSD rapidly and in adult models to parallel the onset in human patients. In our small sample size, experimental surgeries were completed within an hour and produced mice that had no major issues during the recovery period or behavioral changes that did not already align with unilateral deafness. Therefore, our model is fast, safe, and has the potential to be extremely effective in destroying most hair cells. Further investigation to determine the optimal dose of the DTR required to induced SSD and the timing of SSD-onset after the injection are needed to better characterize the model before it can be implemented widely in future studies.

Disadvantages. As such, the development of this novel mouse model must be critically examined for validity and for the design of future studies. The largest struggle in managing and maintaining this model came from the incorporation of the DTR system. Since its development in 2002, this technique has been used to ablate distinct cell types, especially in the immune system (Ruedl & Jung, 2018). Our model crossed DTR and C57BL/6 in an attempt to mitigate the behavioral abnormalities that characterize homozygous DTR mutant animals. C57BL/6 animals have also been cited to develop severe age-related hearing loss, potentially introducing another variable into our study (Konishi et al., 2017). Even with this precautious measure, we still noted strange behaviors within the experimental mice. Their behaviors were not abnormal in the sense where it impacted recovery, but instead impacted their handling and recordings. Several of the animals were too active to handle and anesthetize, and even within those that were successfully injected, some were difficult to fully anesthetize for the ABR recordings.

Still, many of these reported shortcomings have been cited among limitations with transgenic approaches in general (Ruedl & Jung, 2018). Therefore, efforts should be made to overcome these challenges rather than considering this approach useless.

Auditory Brainstem Response Technique

We utilized ABR testing to verify the presence of intact hearing in our animal population before surgeries and the impact of sham-surgery or PSSC surgery on the sham and experimental animals, respectively. The ABR is an objective measure of hearing function that can be registered relatively noninvasively in animals and humans. The presence of a repetitive tone, noise burst, or click stimulus results in the synchronous firing of cells in the auditory nerve and brainstem. These responses are quantitatively averaged using far-field electrophysiology to avoid the influence of background neural and physiological noise. The resulting waveform contains peaks which represent different fiber or cell activity within the auditory pathway. Studies have been conducted to overlay neural activity with individual peaks (Melcher et al., 1996b).

Damage to the auditory pathway will result in changes within the ABR recordings. Typically, this results in an increase in threshold, as we recognized in treatment 3 and treatment 4 experimental mice who underwent targeted cochlear ablation cell therapy. Other pathologies may impact the latency and amplitude of these waveforms. For example, sensorineural hearing loss (SSNL) can be recognized through a significant change in inter-peak intervals (Hwang et al., 2017). Furthermore, the extent to which a peak or an entire waveform is impacted may depend on the level of damage sustained by the pathway. Therefore, it is possible that a mouse may undergo a change in hair cell number after injection with DT and that the loss is significant enough to be recognized by the animal but not enough to be noted through ABR testing. This may have been a result of the experimental mice in treatment 1 and treatment 2.

Acetylcholine Modulating A1

Neuroplasticity in the adult cortex can occur in response to learning or to peripheral injury. Within A1, neurons have frequency-selective fields that can be modified in response to pairing of an auditory stimulus with a somatosensory stimulus (Banderwoski et al., 2001). Acetylcholine is the neurotransmitter which modulates the reorganization of synapses in response to these events. This has been highlighted in experiments which pair a tone with an iontophoretic injection of acetylcholine and record a larger response as a result compared to unpaired tones. What is defined as significant enough to elicit changes to receptive fields is not fully understood in the auditory system or otherwise.

Evidence suggests acetylcholine to be a critical component in neuroplasticity mechanisms (Bandrowski et al., 2001). Studies have recognized that upon release of acetylcholine into the cortex by stimulation of the nucleus basalis, an increase in excitatory postsynaptic potential (EPSP) amplitude and probability of neuronal discharge is present. Muscarinic acetylcholine receptors in particular have been noted in the process of reducing inhibitory postsynaptic potentials (IPSPs) to contribute in EPSP amplitude elevation. Finally, acetylcholine is classically known to depolarize membranes and increase cellular excitability. This evidence supports the importance of acetylcholine-dependent mechanisms in the modification of A1.

To track neuroplasticity changes associated with acetylcholine and further understand the results of specific stimuli presentation, we used [^{3}H]scopolamine to quantify muscarinic acetylcholine receptor expression in A1. Our results showed no significant changes but do demonstrate a strong trend towards a difference in receptor expression at a high dose of DT, likely destroying most cochlear hair cells out of all of the experimental animals in our study. The effect of this change in phenotype, as the most likely representation of SSD, will be discussed in further detail in this context.

SSD and its Effect on A1

The cerebral cortex can alter synaptic connections within the auditory pathway outside of critical periods of development (Sharma & Glick, 2016). In humans with SSD, changes in auditory experience over time suggest this to be a possible phenomenon as stimuli presentation and any associated learning processes have changed.

Our results in adult mice support the notion that neuroplasticity is an active mechanism throughout the lifespan of an animal. We recognized more drastic changes in muscarinic acetylcholine receptor expression in animals who received a larger dose of DT during PSCC surgery. This technique should destroy a larger quantity of hair cells and more significantly impact auditory perceptions, as we noted in ABR testing in these higher-dose treated animals. That is not to say that no hair cells were ablated in the lower-dose treatments, but that their thresholds did not reach that defined by our study. Therefore, it is possible that auditory function was impacted in these other treatments as well.

Drawing from this hypothesis, we still notice no significant changes in muscarinic acetylcholine receptor density between the contralateral and ipsilateral hemispheres within

treatments. The largest dose of DT withstood by an animal produced a result that demonstrated a strong trend toward a difference in receptor expression, which may suggest that the mechanisms of neuroplasticity are only present upon significant changes in stimuli presentation. Future studies should continue exploring the correlation between changes in stimulus presentation in all senses and neuroplasticity.

It is possible that conditions of mice in future studies will produce significant data representing a down-regulation of muscarinic acetylcholine expression in the ipsilateral hemisphere compared to the contralateral hemisphere, as was alluded to by the treatment 4 mouse in our study. The replication of these results would suggest that muscarinic receptors, and the neurons they inhabit, are susceptible to noise trauma. In particular [^{3}H]scopolamine binds to multiple subtypes but is especially attracted to M_1 and M_2 receptors (Frey & Howland, 1992). Therefore, we can attribute possible down-regulation to these muscarinic receptor types, narrowing our understanding of neuroplasticity in A1. Still, as [^{3}H]scopolamine does bind with lower affinity to other muscarinic acetylcholine subtypes (M_3-M_5), further studies will need to use more specific radioligands to differentiate these subtypes and properly track changes in receptor density (Levey et al., 1991).

Of note, we did notice potential down-regulation in the ipsilateral hemisphere rather than the contralateral hemisphere, as one may anticipate due to the decrease in sensory input. The crossing-over of sensory information to the contralateral hemisphere makes the latter hypothesis more plausible as cochlear damage would disrupt the central auditory pathway (Pickles, 2015). Still, some sensory information can be conserved on the ipsilateral side and may be more dominant in these mechanisms. Further studies should verify that a decrease in ipsilateral receptor density is the definite change in neuroplasticity resulting from the onset of SSD.

Understanding that projections from the inner ear exist in both the contralateral and ipsilateral hemispheres, mechanisms that may result in general down-regulation of muscarinic acetylcholine receptors include neuronal cell death, changes in post-translational processing, and activity-dependent changes mediated by acetylcholine. Neuronal cell death is plausible as the deafening of the left ear involved the death of cochlear hair cells, potentially destroying original pathways downstream. In addition, cellular expression of proteins within the neurons of these pathways may have been impacted by the deafening by damaging messenger RNA (mRNA), which has been shown to impact receptor expression following cerebral cortical damage (Kobori

et al., 2002). Finally, changes in cholinergic inputs following deafness may influence muscarinic acetylcholine expression on pre-synaptic and/or post-synaptic neurons. The accumulation of large amounts of acetylcholine following trauma may result in the down-regulation of these receptors, as has been noted in NMDA glutamate receptor feedback modulating synaptic reorganization following disease and trauma (Scheetz & Constantine-Paton, 1994). Future studies should be aimed at narrowing this list of hypotheses to determine definite causes of changes in A1 muscarinic receptor expression.

Future Directions

We noted a strong trend towards a difference in muscarinic acetylcholine receptor expression within the adult mouse in treatment 4. This mouse, although unrecorded, likely exhibited SSD symptoms within the left ear. The mouse that underwent a less-concentrated dose of DT in treatment 3 also demonstrated signs of unilateral hearing loss in the left ear but no changes in muscarinic receptor density between the contralateral and ipsilateral hemispheres. Mice in treatment 1 and 2 did not demonstrate hearing loss or changes in receptor density. The time limit of this study prevented the administration of other, varying treatments of DT. Nevertheless, the current data suggests these outcomes to be conducted by a dose-dependent mechanism. Future studies should continue exploring other treatments to develop a dose-response curve and to identify an optimal dose for the studying of neuroplasticity in A1.

Limitations

Our study was limited by several variables. As such, additional data is required before we can draw firm conclusions. While we initially intended to maintain an all-male animal population for our studies, DTR animals were both male and female due to the difficulty in breeding adult mice with this strain. We only had the capacity to run a small number of sham (n=6) and experimental (n=5) animals. Furthermore, several doses of DT were used in these animals as we worked to determine an optimal dosage to represent the phenotype of SSD, making the number of animals exposed to each treatment even smaller.

Within our radioligand binding assays, we recognize that [^{3}H]scopolamine binding is limited; it is unable to label all muscarinic receptors despite highly concentrated and long-term exposure to the tissues. Densitometry analysis in this study was focused on A1 but did not

consider the different layers of the cortex and the differences in optical density within them considering the preliminary nature of the study. Our use of [^{3}H]scopolamine on mouse tissue also made this consideration difficult as the picture produced from the phosphoimager screen is not sharp enough to easily distinguish cortex layers. Further studies that utilize this ligand could co-label cell types to mitigate this issue, using techniques such as in-situ hybridization or immunochemistry.

Conclusion

In summary, these data support the DTR mouse as a potential model of SSD witnessed in human patients as it has the ability to replicate characteristics associated with the pathology. The injection of DT into the PSCC of the left ear resulted in the targeted ablation of hair cells to represent the sudden-onset, unilateral nature of SSD. We were successful in inducing unilateral deafness in animals exposed to 0.1 mg/ml and 0.05 mg/ml concentrations of DT during PSCC surgery. By analyzing muscarinic receptor density using radio-ligand binding techniques, we further explored the impact of SSD deafness on central auditory pathways and found a strong trend towards a difference in muscarinic receptor expression in the hemispheres of the brain, specifically thorough a down-regulation in ipsilateral hemisphere receptor density. This was witnessed in the animal treated with the highest concentration of DT in our study (0.1 mg/ml). These data contribute to proving the validity of this model, which will ultimately jumpstart future studies to add to the growing literature on SSD. Our model has the potential to be used to better understand neuroplasticity in A1 resulting from SSD.

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