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SALICYLATE INDUCED TINNITUS IN RATS:
USING A PRE-PULSE INHIBITION MODEL TO ESTIMATE PITCH

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THESIS APPROVAL PAGE

This is to certify that the thesis prepared by Cara Mahoney, entitled Salicylate Induced Tinnitus in Rats: Using a Pre-Pulse Inhibition to Estimate Pitch, has been approved by this committee as satisfactory completion of the requirement for the degree of Doctor of Audiology in the department of Audiology, Speech Language Pathology, and Deaf Studies.

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ABSTRACT

SALICYLATE INDUCED TINNITUS IN RATS:
USING A PRE-PULSE INHIBITION MODEL TO ESTIMATE PITCH

Cara Mahoney

The study of tinnitus in animals has gone through a number of changes with each new animal model of tinnitus that is introduced, and with each change, new information is gained. The advent of the pre-pulse inhibition model has shown that it can be a fast and effective means of measuring the pitch of tinnitus in rats. In this study, the pre-pulse inhibition model was used to determine the pitch of salicylate induced tinnitus in the presence of two levels of background stimuli, 48 and 60 dB SPL. Results showed that a significant difference of PPI was present when comparing baseline and salicylate responses at 16 kHz for the 48 dB SPL background level, indicating the presence of tinnitus with this frequency. Unlike past studies, a significant finding for the 60 dB SPL background level was not found.

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CHAPTER 1

INTRODUCTION

A “phantom auditory perception”, or the perception of the presence of sound without an external sound source, is better known as the phenomenon of tinnitus, which affects up to 15% of the adult population (Hoffman & Reed, 2004; Jastreboff, 1990). While there are many people who experience tinnitus, its’ effects on a person can be very different. In some people with tinnitus, the sound can be ignored, thus rendering it as insignificant ambient sound. However, for those with clinically significant tinnitus, the perceived sound can cause depression, loss of sleep, anxiety, and a decrease in overall well-being (Folmer, Griest, & Martin, 2001). Because clinically significant tinnitus can have such devastating effects on those who experience it, it would be advantageous if the cause of tinnitus were known so that treatments could be developed to aid in the abolishment of the condition. Unfortunately, most of the knowledge regarding the mechanisms of tinnitus is based on theories rather than strict empirical research due to the limitations of human experimentation (Jastreboff, 1990).

Theories of tinnitus generation cover all areas of the auditory pathway ranging from the peripheral system to the central system to a combination of both. It was theorized that if the sound that was heard was caused by a dysfunction in the ear or cochlea, the tinnitus was said to be peripheral in nature. Dysfunctions that can occur in the periphery include abnormal neuronal activity of the hair cells, irregular functioning of the peripheral auditory structures, and morphologic changes in the peripheral auditory

system (Hazel & Jastreboff, 1990; Jastreboff, 1990; Møller, 2003; Torndorf, 1981). In contrast, a central dysfunction occurs in the auditory pathway from the VIII nerve up to the brain (Nelson & Chen, 2004). Although the theories of peripheral and central sound generation anatomically make sense, they do not account for the fact that people with normally functioning auditory systems, deaf people, and people with severed auditory nerves have been documented to have experienced tinnitus (Heller & Bergman, 1953; Møller, 2003; Tucker et al., 2005). Potential explanations are offered by the discordant/dysfunction theory, as well as central theories of tinnitus generation in order to explain why tinnitus does not affect all people in a negative manner. The discordant/dysfunction theory postulates that sound that is perceived as tinnitus is caused by a dysfunction that occurs between the inner and outer hair cells when there is an insult along the basilar membrane and the hair cells are not functioning properly (Jasterboff, 1990). In conjunction with this, the central theory of tinnitus proposes that tinnitus is generated in the central areas of the auditory system and brain. To expand on these two theories, Jastreboff devised the neurophysiological model. The basis of the neurophysiological model proposes that the source of tinnitus related neuronal activity (the tinnitus signal) in a majority of cases is based in the periphery, which is then processed by the central auditory system, and in cases of clinically significant tinnitus, the limbic and autonomic nervous systems as well. When the sound is processed by these latter two systems, which are responsible for regulating memory, attention, emotions, and reaction, negative connotations may be associated with the sound, thus creating clinically significant tinnitus. If negative emotions are not linked with the tinnitus, then it is largely

ignored and deemed as not significant (Jastreboff; Jastreboff & Jastreboff, 2000b; Jastreboff & Jastreboff, 2003a).

Because tinnitus is, by definition, the perception of sound without an external source, the measurement of it as it is experienced by humans is a subjective process. The three core psychoacoustic properties of tinnitus that are usually measured are its pitch match, loudness match, and maskability. In order to match the pitch and loudness of tinnitus, patients are most commonly asked to listen to various simulated sounds of varying pitch and loudness level and to determine which one most closely resembles the sound that they perceive as their tinnitus (Hazel, 1981; Penner, 1993; Vernon & Meikle, 1988; Vernon & Meikle, 2003). In order to determine maskability, white noise or bands of noise are presented at increasing loudness levels until the perception of tinnitus is “masked” (i.e., until it is judged inaudible by the patient) (Vernon & Meikle, 1988). The importance that these psychoacoustic measures play in the role of treatment for tinnitus and expanding the knowledge base of tinnitus is considerable. The only way to measure tinnitus is by subjective accounts, as previously mentioned, as well as simulation experiments. Because of this, great limitations have been placed on the quest to provide the most appropriate treatment for tinnitus patients and to gain knowledge regarding the underlying mechanisms that cause tinnitus to occur.

To address the need for more objective means of measuring tinnitus, researchers developed various animal models in which behavioral changes observed in animals as a result of either salicylate or noise induced tinnitus have indicated the presence of tinnitus. The first of the animal models was developed by Jastreboff, Brennan, and Sasaki (1988). The premise of their model was to induce tinnitus with salicylate injections and observe

changes in trained fear to a period of silence embedded in a constant background noise. It was hypothesized that if the animals, in this case rats, were experiencing tinnitus, there would be decrease in the fear response due to the tinnitus “filling in” the silent period, thus removing silence (Jastreboff et al., 1988). Further works measured pitch and loudness of tinnitus as perceived by rats (Jastreboff & Brennan, 1994). Similar animal models using varying methods of inducing tinnitus and methods of measuring behavioral changes followed the Jastreboff model (Bauer & Brozoski, 2001; Heffner & Harrington, 2002). Through these models, it was objectively revealed that tinnitus experienced by animals has a tonal quality (pitch) and the loudness of the tinnitus is dependant upon the intensity of the noise (or dosage of salicylate) used to induce it, which is consistent with human data.

While these first animal models of tinnitus proved to be useful in the objective determination of the psychoacoustic properties of tinnitus in animals, the employment of their use was cumbersome. In order to carry out experimentation using the early animal models, extensive training of the animals, intricate and complex behavioral manipulations, and lengthy amounts of time (approximately three weeks or more) were needed to complete a full investigation. To address these drawbacks, Turner et al. (2006) devised an animal model that did not require the aforementioned things. Using a pre-pulse inhibition paradigm, the acoustic startle response was measured to determine the presence, as well as the psychoacoustic properties, of tinnitus in rats. The acoustic startle response is a physiological occurrence in which the muscles contract in response to brief intense acoustic stimuli. Studies have shown that when a pre-pulse stimulus (e.g., period of silence) is presented just prior to the intense acoustic stimulus, the acoustic startle

reflex is suppressed (Hoffman & Ison, 1980). If tinnitus were present, it would be seen that this inhibitory effect should be smaller due to the gap of silence (pre-pulse stimulus) being replaced by tinnitus if the tinnitus is smaller than background noise, thus going undetected (Hoffman & Ison). Results found using the pre-pulse inhibition model were in agreement with prior results obtained through the use of earlier animal models, thus validating the usefulness of this model (Bauer & Brozoski, 2001; Turner et al.). Because similar results were obtained with Turner et al.'s model and previous models, and because its use has proven to be a more efficient means of conducting research, the gap detection animal model of tinnitus may give further insight into the mechanisms and psychoacoustic properties of tinnitus in both animals and humans. In this investigation, Turner et al.'s behavioral model of tinnitus will be used to estimate the pitch of salicylate induced tinnitus in rats. Findings from this investigation may lead to knowledge that can be used in future research regarding the use of drugs in the treatment of tinnitus in humans.

CHAPTER 2

LITERATURE REVIEW

Tinnitus

Tinnitus is defined as the perception of sound without the presence of an external sound source, or a “phantom auditory perception” (Jastreboff, 1990). Much of what is known regarding the prevalence of tinnitus in the United States has been obtained through epidemiological studies and surveys. However, due to the lack of standardization of the questionnaires used, greatly varied prevalences have been reported. Across various studies, tinnitus has been reported to affect 4.4 – 15.1% of adults (Hoffman & Reed, 2004). It should be noted that tinnitus itself is not a disease; rather, it is a symptom similar to a headache that can vary from person to person and often appears with conditions such as Meniere’s disease, noise induced hearing loss/acoustic trauma, otosclerosis, ototoxicity, head trauma, and tumors (Nelson & Chen, 2004).

The perception of sound brought upon by tinnitus can vary widely in its loudness, pitch, number of different sounds heard, and sound quality. Tinnitus has been known to range anywhere from a benign ringing that lasts for a few minutes or that occurs intermittently with very few personal or emotion effects to a distressing roar that lasts 24 hours a day and is accompanied by other emotional disturbances and auditory problems (Møller, 2003). Meikle, Johnson, Greist, Press, and Charnell (1995) studied the tinnitus population seen through Oregon Health and Science University, and they found that of the 1625 people surveyed, 53% of people could identify only one sound contributing to

their tinnitus, 26% could identify two sounds, and the remaining 21% heard three or more individual sounds. The same study also revealed that the majority of people described their tinnitus as a “ringing” sound, with “hissing”, “clear tone”, “buzzing” and “high tension wire” descriptions following, regardless of the number of sounds identified in the tinnitus (Meikle et al., 1995).

Mechanisms of Tinnitus

Although studies have shown that 4.4-15.1% (Hoffman & Reed, 2004) of the adult population experiences tinnitus and though it has been studied at length, the exact neural source of tinnitus in humans remains unknown. The general consensus amongst those who specialize in tinnitus is that it is the result of abnormal neuronal activity along the auditory pathway which is incorrectly interpreted as sound input by the auditory processing centers in the brain; however, after this general consensus, the various theorists cease agreement (Jastreboff, 1990). Until the advent of the animal model of tinnitus (Jastreboff, Brennan, & Sasaki, 1988), much of the understanding regarding the mechanisms of tinnitus was based upon theory rather than strict clinical research due to the limitations of human experimentation (Jastreboff, 1990).

Most theories regarding tinnitus generation can be classified into two general categories, peripheral and central, depending on the site of dysfunction. If the dysfunction was concentrated in the ear and cochlea, the tinnitus was said to be peripheral in nature. Conversely, if the dysfunction was located in the auditory pathway from the VIII nerve up to the brain, it was a central dysfunction (Nelson & Chen, 2004).

Peripheral. There are many theories of sound generation that lead to the phenomenon of tinnitus. While some of the theories are rooted in abnormal neuronal

activity of the hair cells, others speculate that the sound is generated by irregular functioning or morphologic changes in the peripheral auditory system (Møller, 2003). One theory of sound generation involving abnormal neuronal activity focuses on the damaged cochlea that arises when one has a hearing loss, or the “edge-effect” (Gerken, 1996; Jastreboff, 1990). The outer hair cells (OHC) in a normal hearing cochlea are arranged along the basilar membrane in a tonotopic fashion, with hair cells that are tuned to high frequency sounds appearing at the base and low frequencies at the apex. When a hearing loss is present, the OHCs along the area of the basilar membrane that correlate with the effected frequency range no longer function to their full potential. When this area is damaged, the OHCs become less inhibited in their firing patterns in response to sound in an attempt to enhance incoming sound input, resulting in an increase in gain applied to the incoming signal, as well as in the neural activity of these neurons (Gerken, 1996). The dysinhibitory action may spread to the surrounding healthy OHCs and cause them to “overamplify” the ambient noise present in the cochlea at those frequencies. This overamplification would then be perceived as a sound signal louder than the environmental noise at those specific frequencies (Hazel & Jastreboff, 1990).

A second theory of peripheral sound generation had been proposed based upon Harris’s (1968) findings that free-flowing stereocillia from the OHCs produce an ambient noise level of up to 30 dB. In a healthy cochlea, the largest stereocillia of the OHCs are embedded in the tectorial membrane, and the shearing action of the stereocillia allows neural signals to be sent to central auditory processing centers when an external sound input is present. Torndorf (1981) hypothesized that if the coupling force between the OHCs and the tectorial membrane is broken or loosened, then the ambient noise produced

from the free-flowing stereocillia could be processed as sound input without there being an external sound source present (i.e., tinnitus). Dependant upon where the detachment has occurred along the basilar membrane and how wide of an area is affected, the resulting perceived sound could vary in its pitch as well as its frequency specificity (Jastreboff, 1990).

A third theory of sound generation in the periphery was proposed by Møller (1984). This theory involved the loss of the myelin sheath covering individual axons in auditory cells and auditory nerve. In a normal functioning auditory system, hair cells and/or auditory nerve fibers are covered in a myelinated sheath, which both insulates the neurons and aids in neural transmission, and each auditory nerve fiber fires in a random manner when in silence. When this myelin sheath is broken down, the individual nerve fibers can “communicate” with each other and their random firing patterns can become synchronized when no sound source is present. This communication has been described as “cross-talk” and results in an increased phase correlation of adjacent fibers, which, in turn, leads to an increased number of fibers firing at once. When these fibers become phase-locked or synchronized with each other, the auditory processing centers no longer see this firing as random, but as the detection of a sound signal (Jastreboff, 1990; Møller, 1984). Measurements of neuronal activity from people with normal peripheral systems argue against this postulate, as no enhanced correlation was found.

A final, and most accepted, theory of tinnitus sound causation is known as the discordant/dysfunction theory. By this theory, Jastreboff (1990) proposed that sound perceived as tinnitus is caused by a dysfunction that occurs between the inner and outer hair cells when there is an insult along the basilar membrane and the hair cells are not

functioning properly. When there is damage to the basilar membrane, the basal (high-frequency) area is affected first, with dysfunction occurring in the OHCs (afferent Type II innervation) prior to the inner hair cells (IHC) (afferent Type I innervation). When this occurs, there may be areas along the basilar membrane with properly functioning IHCs and improperly functioning OHCs, leading to mechanical disturbances of the organ of Corti, as well as abnormal afferent fiber activity (Jastreboff, 1990; Jastreboff, 1995).

Mechanical disturbances to the Organ of Corti occur when damaged OHC stereocilia can no longer maintain the mechanical coupling between the basilar and tectorial membranes. When this occurs, abnormal movement of the basilar membrane or total collapse of the tectorial membrane may occur, resulting in a decrease in the space normally seen between the cilia of the IHCs and the tectorial membrane. The decrease in area between the intact IHCs and the tectorial membrane can cause tonic depolarization of the IHCs, resulting in the appearance of irregular afferent fiber activity stemming from the Type I IHC fibers. The increase in afferent activity leads to a decrease in efferent fiber activity due to the lack of input from the damaged OHCs. If the OHCs are not functioning properly (i.e., they are damaged or changed) along a given section of the basilar membrane, then a decrease in OHC Type II fibers occurs, resulting in inhibition and higher levels of IHC Type I fiber activity. This dysfunctional increase in IHC Type I afferent activity along the damaged area of the basilar membrane is also thought to be seen in normal functioning adjacent areas, which is then processed as sound (tinnitus) in the brain (Jastreboff, 1990; Jastreboff, 1995).

The theories of the peripheral causes of tinnitus may aid in the determination of where sound is generated. However, sound generation is only one part of the puzzle. In

order for a sound to be detected as tinnitus, it must be processed and perceived in higher cortical areas (Jastreboff, 1990). Research has shown that tinnitus does not affect all people with hair cell damage, so factors other than peripheral damage to the auditory system must be necessary for tinnitus to occur (Jastreboff, Brennan, & Sasaki, 1988; Møller, 2003).

Central. Although it has been theorized that the source of tinnitus is generated in the peripheral auditory system, there are several indications that there is the potential for other systems to assist in its development. One such indication was highlighted by Heller and Bergman (1953). In their study, they wanted to see what, if any, effects low level ambient noise would have on people with normal hearing. In other words, would they develop tinnitus, and if so, how would it be characterized? The results of their study indicated that after spending five minutes in a quiet environment, 94% of normally hearing subjects experienced tinnitus. A second study by Tucker et al. (2005) expanded on Heller and Bergman's methodology by increasing the amount of time spent in quiet. The incidence of tinnitus in normal hearing subjects found by Tucker et al. was 64%, which was lower than what was found by Heller and Bergman. If an abnormal peripheral system were the only determinant for experiencing tinnitus, it would be expected that individuals with a normal peripheral system would not be able to perceive this type of sound. It has also been shown that deaf people can experience tinnitus, as can individuals in whom the auditory nerve has been cut (Møller, 2003). For these reasons, it is thought that tinnitus is not simply a peripheral or central dysfunction, but rather a combination of both (Jastreboff, 1990).

Neurophysiological model. The neurophysiological model came about as a way to explain the interaction between the peripheral and central systems in the development of tinnitus, as well as why some people with tinnitus are impacted in a negative manner and why others are not. Those who are affected in a negative manner (stress, loss of sleep, negative feelings, etc.) by the presence of “phantom sounds” are labeled as having clinically significant tinnitus. Conversely, people who experience the “phantom sounds” who are not impacted negatively are thought to have tinnitus that is not clinically significant (Jastreboff & Jastreboff, 2000b). The neurophysiological model of tinnitus, which was proposed by Jastreboff in the 1980s (as cited in Jastreboff, 1990), postulates that the auditory system is not the primary contributor to tinnitus induced problems. Rather, the focus of the neurophysiological model states that in clinically significant tinnitus, various parts of the nervous system plays a role in the detection, perception and evaluation of the sound generated in the periphery (Jastreboff, 1990; Jastreboff & Jastreboff, 2003a).

In this theory, structures in the central nervous system, specifically the limbic and autonomic nervous systems, play crucial roles in the formation of clinically significant tinnitus. The theory of nervous system involvement arose from observations seen in previously conducted research in the field (Jastreboff & Jastreboff, 2003a). The first research observations that lead to this theory were that of all of the people who experience tinnitus, only a reported 25% suffer from clinically significant tinnitus. Moreover, there were no correlations found between the perceived severity of the tinnitus (i.e., loudness and pitch ratings and maskability) and the level of distress that was reported (McFadden, 1982). If there were correlations between the two, then it would be

expected that the louder the tinnitus perception, the more distress it would cause.

Secondly, it was observed that people who report similar psychoacoustical perceptions of tinnitus do not suffer equally, nor do they react in a similar fashion to treatment methods (Jastreboff, Hazell, & Graham, 1994). The fact that the psychoacoustical perception of tinnitus does not have a direct correlation with the amount of distress that is caused leads to the notion that there must be some other factors in play besides the generation of the sound in the periphery.

After the phantom sound is generated by abnormal activity in the cochlea or auditory nerve, it travels to the central nervous system to be processed. When the tinnitus-related neuronal activity is processed in the central nervous system, it triggers not only the auditory processing centers which process the sound stimulus, but also other cortical areas that are responsible for memory, attention, emotions, and reaction, specifically the limbic and autonomic nervous systems (Jastreboff & Jastreboff, 2003a). The limbic association areas, which include the hippocampus, amygdala, and the hypothalamus, are involved with emotions and memory storage/recall. The limbic system is also responsible for the activation of the autonomic nervous system, one of the two main divisions of the nervous system. Further, the autonomic nervous system can be divided into two subsystems, the parasympathetic and the sympathetic systems, with the latter playing a dominant role in the formation of clinically significant tinnitus (Jastreboff & Jastreboff, 2000b; Seikel, King, & Drumright, 2000). It has been shown that the sympathetic nervous system has many connections with the hair cells in the cochlea. (Henry & Møller, 2000). This system also plays a part in the “fight or flight” reaction that one feels when in danger or in situations of great stress (Seikel, King, & Drumright).

For clinically significant tinnitus to be present, four steps must occur: a sound is generated in the periphery, the auditory association areas process it, the sound is evaluated by the auditory area and other sub-cortical areas, and the activation of the limbic and autonomic nervous systems is sustained (Jastreboff & Jastreboff, 2000b). When the limbic system is activated, a memory or emotion is linked to the incoming stimulus. In some cases, the emotion can be positive; however, in the case of clinically significant tinnitus, the associated emotion is negative. The negative associations formed with tinnitus enhance the attention that is applied to it, therefore strengthening its detection and perception, regardless of the sound's psychoacoustical properties. Without the sustained activation of the limbic and autonomic nervous systems and negative connotations, the tinnitus is not deemed as a negative experience, thus rendering it not clinically significant. Hence, once the negative associations incurred with the sounds are removed by treating the neurological aspect, the distressing effects of the tinnitus are diminished, even if the perception of the psychoacoustical characteristics of the tinnitus remains the same (Jastreboff, 1990). Because tinnitus has been shown to be alleviated with this kind of treatment, the neurophysiological theory that the auditory system plays a secondary role in the formation of clinically significant tinnitus is supported (Jastreboff, Gray, & Gold, 1996).

Hyperacusis

Reorganization of the normal functioning of the central nervous system can result in tinnitus as well as other auditory abnormalities. One such abnormality that may accompany tinnitus is decreased sound tolerance or oversensitivity to sound (Jastreboff & Jastreboff, 2003a). There are two elements to decreased sound tolerance: hyperacusis and

misophonia (Jastreboff & Jastreboff, 2000b). Decreased sound tolerance often times will accompany tinnitus and has been referred to as a pre-tinnitus state (Jastreboff, 1990).

Hyperacusis is one of the elements of decreased sound tolerance and can be defined as “abnormally strong reactions to sound occurring within the auditory pathways” (Jastreboff & Jastreboff, 2003a). It is characterized by a decrease in Loudness Discomfort Levels (LDLs) to sounds that would otherwise not be deemed as uncomfortable by normal listeners (Formby & Gold, 2002) along with physical reactions, such as pain or physical discomfort, that would not be apparent in the average population in response to sounds of similar intensity and/or frequency. It has been postulated that people who suffer from hyperacusis exhibit similar neuronal patterns in the presence of soft inputs to those of normal listeners in the presence of substantially louder inputs (Jastreboff & Jastreboff, 2003b). While the exact cause of hyperacusis is unknown, the generation of sound may be caused by either peripheral or central mechanisms affecting the auditory pathway, which results in an increase in the gain of the auditory system (Jastreboff, 1990; Jastreboff & Jastreboff, 2000b). Peripherally, within the cochlea, the OHCs are responsible for providing amplification of approximately 60 dB to low intensity sounds (generally less than 60 dBHL). If the OHCs act abnormally and provide this same amount of amplification to greater intensity inputs or provide much more initial amplification to the low intensity sounds, then this increase in gain is processed in the central auditory system as being much louder than it would be for someone with a normal acting system (Jastreboff & Jastreboff, 2000b). On the other hand, the central manifestation of hyperacusis can result from oversensitive neurons in the auditory pathway. Through the work of Boettcher and Salvi (1993) and Gerken (1993) it was

shown that when there is a decrease in the intensity of input into the auditory system, the response thresholds of a considerable number of neurons in the auditory pathway are altered. When the input is decreased, these neurons exhibit an abnormally high sensitivity to input, thus increasing the amount of gain present in the auditory system. This abnormally high gain may lead to the detection of small fluctuations in the spontaneous firing patterns of neurons or other irregular activity in the auditory system, therefore leading to its detection. Because this activity would not normally be processed, hyperacusis and tinnitus may have similar mechanisms of neuronal manifestation (Jastreboff & Jastreboff, 2000b).

The second element of decreased sound tolerance is misophonia or the dislike of sound. Hyperacusis and misophonia often coexist and frequently occur with tinnitus. According to various studies, it has been estimated that 40-86% of those with tinnitus also exhibit decreased sound tolerance (Jastreboff & Jastreboff, 2000b). Misophonia, in a slightly similar fashion to clinically significant tinnitus, is a result of the overactivation of the limbic and autonomic nervous systems without abnormally high activation of the auditory system. Many people who initially experience hyperacusis and misophonia may go on to develop phonophobia (fear of sound) due to the negative reactions that accompany the perception of soft or moderate inputs as being “too loud”. With phonophobia, the extent of the reaction to sound is dependant mainly upon past experiences, the context of the occurrence of the sound, and the person’s psychological profile and not the acoustic properties of the sound (Jastreboff & Jastreboff, 2003b)

Problems Associated with Tinnitus

As the neurophysiological model of tinnitus theorizes, systems other than the auditory system have a great impact upon the formation of clinically significant tinnitus. As stated previously, in order for tinnitus to be classified as clinically significant, the limbic system and autonomic nervous systems must remain active, and sustained activation of these two systems can lead to emotional problems such as anxiety and depression, as well as problems with sleep (Folmer, Griest, & Martin, 2001; Jastreboff & Jastreboff, 2000b).

Problems with sleep and tinnitus can be linked to the sustained activation of the autonomic nervous system. When this system is alert and active, the body is kept in a state ready for physical action, thus inhibiting the body's ability to rest (Coles, 1996). To illustrate the effects of tinnitus and sleep, Folmer and Griest (2000) studied 147 people with severe tinnitus over a four-year period. On the initial questionnaire, tinnitus was rated subjectively on the parameters of perceived severity and loudness and how it affected their ability to sleep. Initially, the subjects received counseling and education to learn how to manage their tinnitus. On the follow-up questionnaire, subjects were once again asked to rate the severity and loudness of the tinnitus and sleep problems experienced. While a number of people reported that they no longer encountered sleep problems to as great a degree, a significant number of people indicated that as the severity of their tinnitus increased, so did the problems with sleep. Loss of sleep is a problem that usually coexists with other disorders or problems. With the loss of sleep, or insomnia, emotional issues such as anxiety and depression often occur. Folmer, Griest, and Martin (2001) studied the affect that sleep interference, caused by tinnitus, had on

self-ratings of anxiety and depression. They found that the more tinnitus interfered with sleep, the higher the ratings on both an anxiety and a depression inventory were and these problems compromised a “vicious circle of symptoms”.

The negative emotions that are linked to tinnitus are reinforced by the activation of the limbic system. Once a negative connotation is attached to an auditory signal, each reoccurrence of the signal reinforces those negative feelings until the tinnitus is deemed clinically significant (Jastreboff & Jastreboff, 2000b). Folmer, Griest, Meikle, and Martin (1999) showed that an association between tinnitus severity and depression does exist, and those people who suffer from tinnitus and depression concurrently exhibit negative emotional markers such as irritability, nervousness, anxiety in social situations, and an overall decreased enjoyment of life.

Psychoacoustic Properties of Tinnitus in Humans

Some tests of auditory function, such as otoacoustic emissions, have an objective means of measurement. In measuring otoacoustic emissions, a probe is placed in the ear and the echo produced by the OHCs in reaction to a sound stimulus is recorded and measured in an objective manner. In other words, the measurement of this event is not dependant upon patient input; rather, it is purely a measurement of a physiological event. This is not the case for measuring tinnitus in humans. In order to measure the psychoacoustic properties of tinnitus in humans, clinicians must rely on the patient’s subjective accounts of the tinnitus as well as simulation experiments such as pitch and loudness matching and maskability (Hazell, 1981). Mitchell, Vernon, and Credon (1993) stated that the main objective in measuring the psychoacoustic characteristics of tinnitus is to monitor its’ course over time in order to provide the most appropriate forms of

treatment or therapy possible. Other objectives in the measurement of tinnitus are to provide reassurance to patients that what they are experiencing is real, as well as to demonstrate to family members what the patient is experiencing (Tyler, 1992).

Pitch matching. Pitch is defined as the psychological correlate to acoustic frequency (Henry & Meikle, 2000). Pitch matching is often accomplished by presenting two tones or narrowbands of noise to a patient and asking them to make a two-alternative forced choice between the two based upon which sound most resembles the pitch of their tinnitus. This forced-choice method is continued until the most precise match is found (Vernon & Meikle, 2003). While this process sounds simple enough, several obstacles can prevent the patient from making an accurate determination of pitch. The first of the confounding factors that may impede the accurate determination of pitch is that nearly 50% of people who experience tinnitus report that their tinnitus is not entirely tonal; rather, it is composed of several pitches (Meikle et al., 1995). Penner (1993) found that when tinnitus patients were asked to match the pitch of their tinnitus to pure-tones (comprised of one sine wave) and then tones comprised of several sine waves, patients reported that the tones comprised of several sine waves more closely matched the tinnitus that they perceived. Further, it was revealed that as additional sine wave components were added to the imitation tinnitus, the perceived level of similarity to the patients' tinnitus also increased. Secondly, pitch matching can be highly variable due to the fluctuating nature of pitch in tinnitus. Studies have shown that pitch can vary from day to day or within the timeframe of one day, as well as over a long period of time (Meikle & Greist, 1991). Another phenomenon that may confound results is "octave-confusion", which has been shown to exist in approximately 60% of the tinnitus population (Meikle

& Greist). Octave-confusion occurs when a patient indicates that a presented pitch matches their tinnitus, when, in fact, it is an octave below the true pitch match (Vernon, 1988). Lastly, tinnitus that is only audible at low sensations levels may be susceptible to masking by tones presented via audiometric equipment intended for comparison purposes, thus decreasing the reliability of pitch masking procedures (Tyler, 2000).

Studies examining the trends of pitch matching have attempted to make an association between how a person matches the pitch of their tinnitus to their type of hearing loss or auditory pathology. Meniere's disease, which often produces a low frequency fluctuating hearing loss and roaring tinnitus, has been consistently shown to have pitch matching results in the low frequency range, most notably, under 1000 Hz (Douek & Reid, 1968; Nodar & Graham, 1965). On the other hand, a noise induced hearing loss is most noted for a high frequency loss beginning at approximately 4000 Hz and has produced far less consistent pitch matching results. Douek and Reid found that persons with noised induced hearing loss matched the pitch of their tinnitus in the range of 2000 to 8000 Hz. Several studies have shown that pitch matching most often occurs in the frequency region where hearing loss is greatest or where a region of normal hearing meets a frequency region of hearing loss (Fowler, 1942). If this were the case, noise induced hearing loss patients would notably match their tinnitus in the region of 4000 Hz or above.

To look at the trends of how people with tinnitus subjectively rate the pitch of tinnitus, Meikle et al. (1995) studied 1514 people through the Oregon Hearing Research Center. The protocol for the pitch matching of tinnitus called for the use of the two-alternative forced-choice method with the test ear being contralateral to the ear in which

the tinnitus was predominant. Prior to each tone presentation, the intensity of the tone was adjusted so that it was equivalent to the perceived loudness of the tinnitus. The order of tone presentations was also randomized so that the lower frequency tone was not always the first presented in the pair. Once a pitch match was indicated, an octave-confusion test was performed to ensure an accurate match. Results from their study indicated that most people (83%) experience a high-pitched tinnitus over 3000 Hz and that the median value for pitch is 6000 Hz (testing only conducted to 8000 Hz). In a similar study by Meikle and Walsh (1984), it was revealed that 54% of people matched their tinnitus to a tone over 3000 Hz, which was lower than the percentages reported by Meikle et al.

Because results vary greatly from study to study, and the number of possible confounding factors that may lead to less reliable results, it is very important that proper pitch matching procedures are followed and that several replications of the testing are conducted to provide the appropriate treatment course for an individual (Tyler, 2000). Properly matched tinnitus may have a highly desirable effect on treatment. One example of this can be seen when tinnitus patients report that there are several pitches present in their tinnitus. When even one of the pitches (usually the highest) is able to be eliminated through the use of treatment methods, such as Tinnitus Retraining Therapy (TRT) which employs the use of sound generators, up to 84% of patients reported that their tinnitus was more manageable and report this as a positive treatment effect (McKinney, Hazell, & Graham, 1999 as cited in Jastreboff & Jastreboff, 2000a; Vernon & Meikle, 2003).

Loudness matching. Just as pitch is the psychological correlate to acoustic frequency, loudness is the correlate of acoustic intensity. Loudness matching is

performed by presenting a tone or noise at various intensities across a number of frequencies (often at the pitch match frequency and a frequency in which hearing sensitivity is within normal limits) and having the patient indicate when the intensity of the generated noise matches that of their tinnitus (Goodwin & Johnson, 1980; Tyler, 2000). In order to determine an accurate loudness match, the presentation of the tone is presented at an intensity level below threshold and increased in 2 dB steps until threshold is reached. Once threshold is reached, the intensity is increased in 1 dB steps until the intensity of the tone matches the perceived loudness of the tinnitus (Vernon & Meikle, 1988). The loudness of the matches have been described using a variety of psychoacoustic units including sones and “personal loudness units” (PLUs), but they are most often expressed in decibel Sensation Level re: hearing threshold (dBSL) (Henry & Meikle, 2000; Jastreboff & Jastreboff, 2003a; Vernon & Meikle, 2003).

In clinically significant tinnitus, patients assess their tinnitus as being so loud and bothersome that they experience many side effects such as depression, loss of sleep and a decrease in overall well-being (Folmer, Greist, Meikle, & Martin, 1999). However, studies have shown that when tinnitus patients match the loudness of their tinnitus to an outside sound source, results often show that the intensity of their matched loudness is just slightly greater than their measured threshold of hearing. As such, the sensation levels of the perceived loudness are significantly lower than what would be expected from the subjective rating scales of tinnitus disturbance and complaints put forth by tinnitus patients (Henry & Meikle, 2000). It would be thought based upon the complaints from those with clinically significant tinnitus that the loudness matches would be equal to

a loudness level that would be classified as disturbing by those who do not experience tinnitus.

In the second part of the aforementioned study by Meikle et al. (1995), 1422 subjects were asked to match the loudness of their tinnitus at both the pitch match frequency and at 1000 Hz. Similar to the pitch matching protocol, the contralateral ear was used as the test ear. Once auditory threshold was determined for each frequency, tones were presented to the subject below threshold and increased in 1 dB steps until it was noted that the intensity of the external sound source matched the perceived loudness of the tinnitus. This level was recorded in sensation level (dB SL) above threshold. The results indicated that 68.9% of people matched their loudness within 6 dB SL and 83.8% matched within 9 dB SL of threshold at the pitch matched frequency of their tinnitus. These findings go against what normal listeners would classify as being a “loud sound” (Meikle et al.). To illustrate this, Meikle et al. also had their subjects match the loudness of their tinnitus at 1000 Hz, a normal hearing frequency. They found that these results were much different, and only 37.9% of loudness matches were within 9 dB of hearing threshold compared to 83.8% at the pitch match frequency. Similar findings were demonstrated by Meikle and Walsh (1984). Out of 502 subjects tested, only 20% matched the loudness of their tinnitus as greater than 6 dB SL over threshold at the frequency in which the pitch was matched. Goodwin and Johnson (1980) found that when loudness was matched at the pitch frequency and a normal hearing frequency, the difference between the mean loudness matches was 17 dB (24 dB SL at frequencies at which the audiometric threshold was 20 dB HL or less and 7 dB SL at the pitch match frequency).

The differences seen in the loudness matching results at the pitch match frequency and a normal hearing frequency suggest that there may be something interacting with the perceived loudness of tinnitus. Goodwin and Johnson (1980) suggested that this anomaly may be due to the presence of recruitment (abnormally rapid loudness growth) in tinnitus patients. Because of the presence of recruitment in some tinnitus patients, it is important to measure loudness matches at both the pitch match frequency and at a normal hearing frequency. While differences in loudness matching are seen between frequencies, it should be noted that, unlike pitch matching, which can be highly variable, loudness matching is usually consistent. Patients who match their tinnitus on a regular basis often do so within a few decibels (Vernon & Meikle, 1988).

Masking. Unlike pitch and loudness, which are both one-dimensional, masking is a phenomenon in which the perceived sound is “covered up”. In masking techniques, the minimal masking level, or level in which the tinnitus becomes inaudible, is determined by presenting a band of noise (3,000-12,000 Hz) and slowly increasing the intensity until the patient indicates that they are no longer able to hear their tinnitus. Masking is presented to the ear in which the tinnitus is most prominent, and in cases of bilateral tinnitus, each ear is tested separately (Hazell, 1981; Vernon & Meikle, 1988). When the tinnitus becomes completely inaudible, then “complete masking” has taken place; when tinnitus is still heard, yet the loudness is reduced, then “partial making” has occurred (Vernon & Meikle, 2003). Complete masking has been shown to be attainable in approximately 90% of patients. On the other hand, partial masking is achieved in 5%, while the remaining 5% cannot be masked at all (Vernon & Meikle, 1988). Similar to loudness matching results, minimum masking levels are found close to auditory

threshold. Meikle et al. (1995) found that 42% of subjects established minimum masking levels at or below 6 dB SL and 58% were established at or below 9 dB SL.

The groundwork for modern masking techniques was laid by Feldman (1971). In his studies, he examined the masking characteristics of 200 tinnitus patients and found that tinnitus could be masked at similar intensities by both noise and pure tones. These results contradicted previously used psychoacoustical masking tests and indicated that tinnitus was processed in a different manner than externally applied sounds (Hazell, 1981). From Feldman's studies, five masking pattern categories emerged: congruence, distance, persistence, convergence, and divergence. A congruence masking pattern showed that tinnitus could be masked at low sensation levels throughout the frequency range. In these cases, tinnitus was tonal in quality, which leads to the threshold curve and masking curve almost overlapping. Conversely, distance patterns were masked at high sensation level throughout the frequency range. To mask the tinnitus for these patients, the masking sound had an intensity well above the subjective threshold. If masking was not attainable, then that person exhibited a persistence pattern. For people with precipitous high-frequency hearing loss, tinnitus was masked with a convergence pattern, in this case, at high sensation levels in low frequencies and low sensation levels in high frequencies. For these patients, the masking threshold and subjective threshold meet at the pitch match frequency and coincide at the higher frequencies. Lastly, a divergence masking pattern was indicated by low sensation levels in the low frequencies and high sensation levels in the high frequencies. People with a divergent masking pattern most often had mild-to-moderate hearing loss. From these findings of the various patterns of maskability, Feldman concluded that the hair cells which were responsible for the tinnitus

activity were not identical with the pattern of hair cells that are activated by an external sound source. He also goes on to say that this phenomena cannot be “masking” in the truest sense since the two areas simulated (tinnitus generation site and external sound source site) are different based upon the physical interaction along the basilar membrane. Rather, this interaction may be thought of as neural inhibition (Feldman). Although Feldman describes these masking patterns, which may be attributable to These masking patterns, as well as the establishment of pitch matching, loudness matching, and minimal masking levels in patients, can provide a framework for the clinical use of ear-level treatment devices to ease the effects of tinnitus (Vernon & Meikle, 1998).

Animal Models of Tinnitus

Qualitization and quantization of the psychoacoustical properties of tinnitus by humans is wholly subjective in nature. The reasoning for the subjective nature of the psychoacoustical measurement of tinnitus is that tinnitus is generated by internal sound sources, which are then processed based on personal experiences. In other words, there is no “tangible” sound stimulus to be measured objectively. Because of the lack of external sound sources which would lead to more objective measurements, past investigations into the characteristics of tinnitus using human subjects have lead to a mostly superficial, rather than empirical, understanding of the nature of tinnitus (Bauer, 2003; Jastreboff, Brennan, & Sasaki, 1988). Along with the subjective nature of tinnitus, several factors have left researchers with little room to gather the empirical data needed to get a true understanding of tinnitus such as the heterogeneity. Some of these factors include the heterogeneity of the tinnitus population, the reactive nature of tinnitus, and the ethical limits of human investigation (Bauer). In order to get a better understanding of tinnitus,

researchers have devised animal models in which both the underlying causes as well as the psychoacoustic characteristics of tinnitus can be studied. Because animals are not able to verbally communicate their experiences, animal models of tinnitus have been based upon behavioral paradigms in which the behaviors and performances of animals in reaction to external stimuli have been used.

Jastreboff procedure. The first animal model of tinnitus was developed by Jastreboff, Brennan, and Sasaki (1988) to investigate the hypotheses of sound generation in the auditory pathway, as well as to explore the psychoacoustic characteristics of tinnitus in rats. The premise behind their model was based upon Pavlovian conditioning techniques in order to examine whether or not pigmented rats developed tinnitus after being injected with an equivalent dosage of sodium salicylate, which has been shown to induce tinnitus in human subjects. Pavlovian techniques condition subjects to perform a desired behavior in the presence of some type of stimuli, which is referred to as a conditioned stimulus. In order for the desired behavior to take place, the conditioned stimulus is paired with some kind of consequence, known as the unconditioned stimulus. In the Jastreboff procedure, the rats were conditioned to respond to or fear a period of silence (conditioned stimulus) by associating it with an undesirable foot shock (unconditioned stimulus) (Jastreboff, Brennan, & Sasaki).

During the conditioning period, the rats were trained to fear a one minute period of silence in the presence of constant background noise by imposing a low-level foot shock at the end of the period when the background noise was “shut off”. The level of fear was measured by the well established method of monitoring the licking behavior of the rats that had previously been water deprived. A decrease in licking behavior was a

signal that the rats were fearful, thus, they detected the silence. Once the rats were sufficiently conditioned, sodium salicylate injections were given to a portion of the rats, while others remained as a control. Results showed that the rats who received the sodium salicylate injection had a lower level of fear of the silence period than that of the control group as measured by their licking behavior (experimental subjects did not significantly decrease the rate of licking during the periods of silence). Presumably, the lack of fear of the silence of the experimental rats was due to the absence of the silent period to which the rats were conditioned. In other words, the perceived noise caused by tinnitus filled in the periods of silence (Jastreboff et al., 1988). This method was also expanded to show similar results to noise induced tinnitus (Jastreboff, Jastreboff, Kwon, Shi, & Hu). Although this model was successful in showing a change in behavior in reaction to a tinnitus phenomenon being present, it took approximately three weeks to complete, which hinders its applicability.

Other animal models. Following the success seen by Jastreboff et al.'s (1988) behavioral model, several other researchers attempted to create additional animal models of tinnitus in order to build upon and expand the knowledge that had been found. These later models used similar conditioning techniques that were utilized by Jastreboff et al.; however, in these later models, tinnitus was induced via high intensity noise exposure due to its' relevance to clinical practice. The pitch and loudness of tinnitus, as experienced by rats, were measured as well (Bauer & Brozoski, 2001; Heffner & Harrington, 2002)

Bauer and Brozoski (2001) introduced a psychophysical animal model which allowed them to study rats over a 17 month period in which they were able to test the use

of GABAergic drugs as an effective means of treating tinnitus. The Bauer and Brozoski model was designed after the same basic principles as the Jastreboff procedure. During the conditioning period, rats were conditioned to fear a period of silence, just as in the previous procedure; however, in this animal model, the behavior that signaled fear was decreased lever-pushing for a food pellet rather than the suppression of licking. It was hypothesized that rats that had tinnitus due to exposure to high intensity noise would show a lower suppression level of lever-pushing in relation to their control group peers. Extensive testing utilizing this model allowed researchers to detect and qualify chronic tinnitus (over a 17-month period), screen for potential therapeutic treatment for tinnitus, and examine the qualitative characteristics of tinnitus (Bauer & Brozoski). However, like the Jastreboff procedure, the initial experimentation was cumbersome in the training, conditioning, and testing of the animals, thus also being quite time-consuming.

Following the Bauer and Brozoski model, Heffner and Harrington (2002) established another procedure in a similar fashion to examine the effects of noise exposure on the formation of tinnitus. Likewise to the Jastreboff procedure, Heffner and Harrington trained hamsters to lick water in the presence of a sound and suppress licking during a silent period through conditioning them to fear silence. Also in a similar fashion to the preceding models, Heffner and Harrington were able to measure some psychoacoustic measures of tinnitus; however, this was accomplished over a lengthy period of time. Although the research endeavors of Jastreboff et al. (1988), Bauer and Brozoski (2001), and Heffner and Harrington (2002) successfully added to the working knowledge base of tinnitus, they were far from an ideal method. Due to the extensive training of the animals, intricate and complex behavioral manipulations, and time needed

to complete a full investigation, these methods served as a cumbersome means of conducting research (Turner et al., 2006).

Pre-pulse inhibition. To address the inefficiency of previously contrived animal models, Turner et al. (2006) devised a new model, which utilized a pre-pulse inhibition design in order to measure the psychoacoustic properties of tinnitus. In comparison to the older models, the pre-pulse inhibition model does not require that animals go through water/food deprivation, nor is there any conditioning period needed. Because these two items are not required for this model, experimentation can take place in as little as one 40-minute session following tinnitus induction, which allows researchers to conduct more trials, as well as test many more subjects in a shorter period of time (Turner et al.).

The premise underlying the pre-pulse inhibition model is the acoustic startle reflex (ASR). The ASR has been shown to be apparent in many mammalian species, including humans and rats, and is characterized by the rapid contraction of muscles following a brief, intense acoustic stimulus (Koch & Schnitzler, 1997; Pilz, Schnitzler, & Menne, 1987). Davis, Gendelman, Tishler, and Gendelman (1982) described the ASR pathway in rats based upon their studies of various lesion sites within the auditory system. They concluded that the main auditory areas responsible for the ASR pathway consist of the auditory nerve, ventral cochlear nucleus (receives the primary auditory input), lateral lemniscus (receives direct input from the ventral cochlear nucleus), pontis caudalis (gives rise to muscle tracts), spinal interneurons, lower motor neurons, and muscles. The minimum intensity needed to elicit an ASR in rats was explored by Pilz et al. (1987) and was found to be dependant upon the frequency of the stimulus used. Although the intensity of the stimulus needed to elicit a response changed as a function of

frequency, the lowest intensity stimulus required was approximately 80 dB SL. It has also been found that the magnitude of the ASR is dependant on a variety outside factors, one of which is a pre-startle stimulus or pre-pulse inhibition. When a less intense/different stimulus is presented to the rat just prior to the intense startle stimulus, ASR has been shown to be reduced in magnitude. Specifically, ASR magnitude is most affected when the pre-startle stimulus is more prominent or closer to a subject's threshold, such as a gap of silence (Hoffman & Ison, 1980).

Using this knowledge of the ASR and pre-pulse inhibition, it was hypothesized that when the background noise in which the gap of silence (pre-pulse stimulus) was qualitatively similar to the tinnitus experienced by the rat, then poorer detection of the silence period would occur. In other words, the gap of silence would be replaced by the tinnitus if the tinnitus experienced by the rat was similar to that of the background noise. If this was the case, and the silence period was not successfully detected, then it would be seen that the acoustic startle reflex would be greater in magnitude when compared to control subjects (Turner et al., 2006). The ASR would be higher in the test subjects due to their inability to successfully suppress the ASR without the presence of the pre-pulse stimulus. To test this hypothesis, rats were placed in specially designed chambers which emitted a constant level background noise with gaps of silence placed (pre-pulse stimulus) at intervals just prior to the introduction of the startle stimulus. The ASR magnitudes of the rats were then recorded automatically within the chambers. Results from the Turner et al. (2006) study supported the proposed hypothesis. It was revealed that rats that had noise induced tinnitus showed higher ASR magnitudes/smaller suppression of ASR in comparison to their control group counterparts. Results found

using the pre-pulse inhibition models were also in agreement with prior results obtained by Bauer and Brozoski (2001), thus validating the usefulness of this model.

Psychoacoustic Properties of Tinnitus in Animals

Using the aforementioned animal models of tinnitus, researchers have been able to study the psychoacoustic properties of tinnitus (pitch and loudness) in a manner not previously available due to the limitations of human experimentation. Assessing the presence and qualitative aspects of tinnitus using these animal models can have significant bearings on clinical implications for treatment of tinnitus in humans, as well as expanding the breadth of knowledge regarding the tinnitus phenomenon.

Pitch. In order to understand tinnitus as it occurs in animals, it must be evaluated qualitatively. In other words, to get a full understanding of tinnitus, the psychoacoustic properties, as they appear in animals, should be investigated. One of the first animal models of tinnitus developed by Bauer and Brozoski (2001) attempted to explore the qualitative properties of noise induced tinnitus in male Long-Evans rats. In order to induce tinnitus via exposure to noise, the rats were subjected to a narrowband of noise with a center frequency of 16 kHz at an intensity level of 105 dB. The rats were then trained and conditioned to behave in certain ways to the presence and absence of sounds (as discussed earlier). The rats were then placed in cages with narrowband background noise at center frequencies ranging from 10-30 kHz. Results from their study indicated that rats exposed to a 16 kHz intense noise for one hour showed the greatest amount of suppressed level-pressing when the background noise had a center frequency of 20 kHz (Bauer & Brozoski). These findings support a theory which states that tinnitus is tonal in nature.

The tonal nature of tinnitus was supported in a study conducted by Heffner and Harrington (2002) that also examined the psychoacoustic properties of tinnitus in hamsters as a result of noise exposure. Unlike the Bauer and Brozoski (2001) study, Heffner and Harrington exposed Syrian golden hamsters to a 10 kHz tone rather than a narrowband of noise like in previous studies and further in depth examined what affect the length of time of noise exposure had on tinnitus formation. Results indicated that hamsters exposed to a 10 kHz tonal noise for either two or four hours, rather than for shorter time periods, suppressed licking behavior most when experimental conditions included periods of silence embedded in a 10 kHz background noise. In other words, tinnitus sensation was most prominent in these hamsters, and the tinnitus demonstrated a tonal quality based upon the suppression of licking to a tonal background noise (Heffner & Harrington). The tonal nature of tinnitus was demonstrated, once again in this study, and the frequency of the tinnitus was been shown to be dependant on the noise in which caused the tinnitus to appear.

Loudness. Unlike pitch, the exploration of tinnitus loudness in animals has had fewer endeavors. The success seen by Jastreboff et al.'s (1988) behavioral animal model of tinnitus aided in gaining knowledge regarding general mechanisms of tinnitus, yet knowledge of the psychoacoustic properties of tinnitus had gone largely unexplored. To address this, Jastreboff and Brennan (1994) devised a study to evaluate the loudness of salicylate induced tinnitus in rats and the related changes seen with experimental manipulations of the dosages given. It was hypothesized that, as the dosage of salicylate increased, the loudness of tinnitus would also increase, as has been shown in human subjects (Day et al., 1989; Jastreboff & Brennan). Jastreboff and Brennan also intended to

prove that the rats demonstrated marked behavior changes as a result of the variations in the perceived loudness of the tinnitus by comparing the rats' behavioral patterns of the salicylate induced tinnitus to tinnitus induced by noise exposure.

In the first part of Jastreboff and Brennan's experiment, rats were given various dosages of salicylate ranging from 75-300 mg/kg. Following training and conditioning, the rats were placed in cages with a constant background noise level of 62 dB SPL. Results showed that those that received the highest dosage of salicylate, 300 mg/kg, failed to suppress licking behavior, with the lower dosage levels each showing successively higher suppression behavior as the dosage amount decreased. In other words, the rats injected with the highest amount of salicylate failed to detect periods of silence embedded in the 62 dB SPL background noise more than any other group, thus not suppressing licking behavior. To further expand the knowledge from experimentation and to investigate how loud the tinnitus produced by the salicylate injections was, it was necessary to create a "frame of reference" for the tinnitus by indicting what intensity external sound is needed to elicit similar behaviors from the rats as seen in the first experiment.

To facilitate finding the perceived loudness of tinnitus, Jastreboff and Brennan (1994) exposed the rats to a constant 10 kHz signal at varying intensity levels (32, 42, 52, 62, 72, and 82 dB SPL) instead of the various dosages of salicylate in experiment one. In a similar fashion to the previous experiment, the suppression behaviors of the rats were monitored to see which group failed to detect silence gaps in the presence of the perspective background noise. Findings from experiment two showed that rats that were exposed to a 10 kHz tone at an intensity level of 62 dB SPL showed the lowest level of

suppressive behavior, indicating that the intensity of the exposing noise inducing tinnitus is similar to the perceived loudness of the tinnitus (Jastreboff & Brennan).

Salicylate to Induce Tinnitus

In the aforementioned behavioral models of tinnitus, there have been several methods employed to induce tinnitus in the experimental subjects, one of which is through the introduction of high doses of salicylate into the systems of the subjects being used. Salicylates, in humans, are one of the most commonly used types of drugs, with the most common form being acetylsalicylic acid, better known by its' common name of aspirin (Jastreboff, Issing, Brennan, & Sasaki, 1988). Because there are very few side effects when used in relatively small doses in humans, it is often prescribed as an anti-inflammatory or pain-reducer. However, when used in larger doses, it has been shown to induce a temporary tinnitus sensation in humans, a sign of acute systemic salicylate toxicity (Chyka, Erdman, Christianson, Wax, Booze, & Monoguerria, et al., 2007). Through the numerous animal models of tinnitus that have been discussed thus far, it has been shown that there are similar findings when it comes to high doses of salicylate given to animals.

In order to discover why high doses of salicylate induces the sensation of tinnitus in rats, Guitton, Caston, Ruel, Johnson, Pujol, and Peul (2003) studied what affects could be seen in the auditory system when salicylate was given and the rats were conditioned to perform a behavioral task. To study this question, Guitton et al (2003) also introduced mefenamate to the rats as a comparison, which is known to be a potent cyclooxygenase inhibitor. Cyclooxygenase works by performing the first step in the creation of prostaglandins. By adding oxygen molecules to arachidonic acid, a set of reactions occur

to create a host of unusual molecules. Salicylate (or aspirin) blocks the binding of arachidonic acid in the cyclooxygenase active site. When the arachidonic acid is blocked, the normal messages are stopped from being delivered, and pain is not felt as much (Goodsell, 2001). Further, it has been shown that arachidonic acid potentiates NMDA receptor currents. Results from Guitton et al.'s study showed that both the salicylate and the mefenamate increased the number of false positive results seen during the behavioral testing; indicating that salicylate induced tinnitus may be due to the inhibition of cyclooxygenase. Further introduction of NMDA antagonists into the cochlea blocked the increase of behavioral responses seen. These findings suggest that salicylate induced tinnitus is a result of activation of cochlear NMDA receptors. Additionally, studies have also shown that through electrophysiological measures, salicylate increases the spontaneous activity within the auditory nerve, which may lead to a perception of tinnitus (Evans & Borerwe, as cited on Guitton et al.).

Because it is known that salicylate induces a tinnitus reaction in rats, it must also be known the time course that should be used to produce the desired effect. To investigate this, Jastreboff, Issing, Brennan, and Sasaki (1988) studied the time needed for the uptake of salicylate in pigmented rats. During experimentation, 21 pigmented rats were given either 300 mg/kg or 400 mg/kg of salicylate acid injected intraperitoneally, and were then anesthetized. Blood samples were then taken from the tails of the rat at different time intervals to determine when the greatest concentration of salicylate was seen. Additionally, samples of CSF were taken from the exposed cisterna magna, and samples of perilymph were drawn from the cochlea to test their uptake properties as well. Results from Jastreboff, Issing, Brennan, and Sasaki showed that the peak levels of

salicylate in the serum of the rats were seen after 90 minutes, while peak levels in the CSF and perilymph occurred at approximately two hours after injection. Additional findings from this investigation showed that when salicylate injections were given over a period of several days, the salicylate did not accumulate in the serum of the rats.

Statement of Purpose

The exploration of the psychoacoustic properties of tinnitus in animals is an under explored research endeavor. If this field is researched more thoroughly, new knowledge may be gained regarding the various aspects of tinnitus, such as the source of its generation, how various pathologies affect the pitch and loudness of tinnitus, and methods of treating and alleviating the affects of its presence. Further, it may be of particular importance to monitor changes in tinnitus source caused by medical interventions such as medications and electrical or magnetic stimulation. Up until the recent advent of the pre-pulse inhibition model developed by Turner et al. (2006), previous animal models of tinnitus were cumbersome in their procedures and were not easily carried out due to the vast amount of training and time needed to complete research. The Turner et al. model allows for much faster detection and characterization of tinnitus, as there is no need for animals' training. The purpose of this investigation was to determine the pitch of salicylate induced tinnitus in rats utilizing Turner et al.'s pre-pulse inhibition model of tinnitus. Findings from this research may lead to knowledge that can potentially aid in future investigations regarding delineating the effects of new drug treatments for the alleviation of tinnitus in humans.

CHAPTER 3

METHODS AND MATERIALS

Subjects

Sixteen male Long-Evans pigmented rats, aged approximately 3 months at the onset, were used during experimentation. The rats were housed at the Towson University Hearing Research Lab located in the Psychology building on the main campus of the university. Each rat was housed individually in a room that was maintained between 70 – 72 degrees Fahrenheit with a twelve-hour light/twelve-hour darkness light schedule. The experimental protocol for this investigation was approved by the Towson University Institutional Animal Care and Use Committee (IACUC). The approval letter is included as Appendix A.

Pre-Pulse Inhibition (PPI)

Pre-pulse inhibition (PPI) testing was conducted using eight Hamilton-Kinder startle reflex cages (Hamilton-Kinder Behavioral Testing Systems, Poway, CA) and software that was designed for this investigation by Dr. Pawel J. Jastreboff (Emory University, Atlanta, GA). In order to measure the magnitude of the acoustic startle reflex (ASR), short bursts (20 ms) of white noise were presented at a level of 115 dB SPL into the chambers through the use of Pioneer speakers Model A1365 located 15 ms above the rats' heads, which were mounted in the ceiling of the test chamber. The magnitude of the ASR was measured through the use of a calibrated piezo transducer affixed to the floor, which, when force was exerted upon it, provided an amplitude of force in Newtons. The

walls of the restraining cage, along with an adjustable-height roof allowed enough room for the rats to turn around and move, while at the same time, preventing the rats from rearing up. This arrangement prevented stress that would be induced if the animals were immobilized.

For PPI measurements, the rats were exposed to background noise that consisted of 1/6 octave band-passed (slope of 48 dB/octave, Butterworth filter) filtered noise with center frequencies spaced every 1/3 octave covering frequencies from 5 to 32 kHz (i.e. 4.00, 5.03, 6.35, 8.00, 10.08, 12.70, 16.00, 20.16, 25.40, and 32.00 kHz) in the testing chambers. The presentation levels of the background noise were 48 and 60 dB SPL. In order to decrease the variability of PPI, each trial was preceded by a pure startle response (SR) condition in which the background noise was not interrupted. For the pure SR trials, the narrow band background noise was presented continuously before the sound was presented that induced the startle response, without any type of gap or silence preceding it, which may have reduced the startle response. For each experimental condition, e.g., the ten different center frequencies, four pairs of SR and PPI were run, which was repeated four times during each session, for a total experimental running time of approximately 120 minutes. Intertrial intervals were changed in a random manner from 5 to 10 seconds, with a mean of 7 seconds. The frequencies of the background noise were changed in a pseudorandom manner so that they were not presented in a sequential manner. In between the running of each condition, the rats remained in separate housing chambers. The levels of the environmental and background noises were measured and calibrated utilizing a Brüel & Kjaer sound level meter model 2231 with 1/3 octave filter model 1625. The sounds were controlled by the computer program to assure that for all

frequencies used, the maximal level of the spectrum was 60 dB SPL. In order to control for consistency amongst the various conditions, all testing was conducted under the same circumstances, while only changing the center frequency of the background narrowband noise. All testing sessions began with a one-minute acclimation period and two pure SR responses. This reflects the observation that the first two SR conditions were typically larger, with subsequent responses stabilized. A schematic of the PPI protocol is exhibited in Figure 1.

Auditory Brainstem Response

Auditory Brainstem Response (ABR) testing was conducted to monitor the hearing thresholds of the rats before salicylate administration was started, as well as with salicylate administration (following PPI testing). ABR testing consisted of tone bursts from 4 to 20 kHz, in 1/3 octave steps (i.e. 4.0, 5.0, 6.3, 10.0, 12.5, 16.0, and 20.0 kHz). Hearing thresholds measured prior to testing served as a baseline, while those measured after testing gave information regarding the hearing status of the rats after exposure to salicylate. ABR testing was performed in IAC sound treated testing chambers, and ABR measurements were obtained via BioSig Cambridge Electrical Design software. Prior to conducting ABR testing, the rats were anesthetized by giving them a dosage of Nembutal that was equal to 50 mg/kg. In order to maintain an anesthetized state, an additional dosage equal to 1/3 of the original dosage was given if needed as judged by paw withdrawal reflex. Testing protocol called for the use of three electrodes; ground was set in the rat's right arm, vertex at the midline of the head, and reference at the level of the mastoid on the left side. Insert earphones were placed in both the right and left ears.

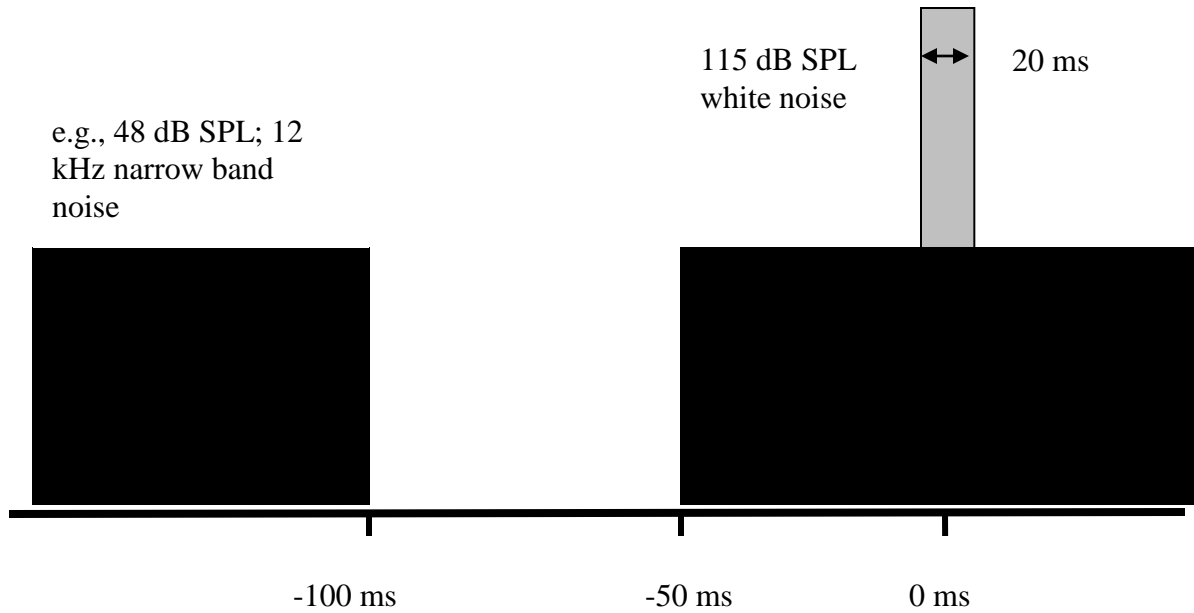


Figure 1. Schematic of PPI presentation protocol. A constant background sound of a certain dB SPL level and frequency level is presented. 100 ms prior to the presentation of the startle stimulus (115 dB SPL white noise), a period of silence is introduced into the background sound, which lasts for 50 ms. 50 ms after the gap of silence is over, the startle stimulus is presented.

Distortion Product Otoacoustic Emissions

Distortion Product Otoacoustic Emission (DPOAE) testing was conducted to monitor the functional status of the outer hair cells in the rats before and during salicylate administration. DPOAE testing was conducted in IAC sound treated chambers, and measurements were obtained using the Starkey Lab DP2000 Measurement System. Like ABR testing, DPOAEs measured under Nembutal anesthesia prior to the presentation of any experimental conditions served as a baseline measurement at the same time as ABR testing. Testing protocol for DPOAEs consisted of obtaining measurements for the frequency range of f_2 from 4 to 20 kHz, 6 points per octave, with primary levels of 65 and 55 dB SPL, and f_1/f_2 ratio of 1.2. All DPOAE measurements were acquired using a modified insert earphone to the left ear.

Procedure

Prior to beginning experimental conditions in which the rats were given salicylate to induce tinnitus, the subjects were measured for baseline levels. Baseline measurements of ABR and DPOAE testing were conducted over a two day period. Following ABR and DPOAE testing, two days of rest were given to allow the Nembutal to be completely expelled from the body. Two days were then needed to measure baseline PPI levels in all conditions. These baseline measurements served as controls for the experiment. After obtaining baseline control measurements, the rats were each injected with a 275 mg dosage of salicylate (321 mg/kg of sodium salicylate) in order to induce tinnitus. To achieve a plateau of salicylate in the serum, experimental measurements were not started until two days following the initial injection. Additional dosages of salicylate were administered each day (one per day) during the period of experimentation. Each day in

which experimentation took place, measurements were performed two hours after salicylate administration to allow for salicylate to reach plateau in the perilymph (Jastreboff, Issing, Brennan, & Sasaki, 1988). A total of two days of PPI testing were needed to carry out the multiple sessions of data collection, in which one session was conducted per day. In order to assess the extent of the salicylate effect on hearing and OHC function, ABR and DPOAE testing was carried out again at the conclusion of all testing sessions, as described above. Prior to conducting the final two sessions of PPI testing to determine if ASR returned to baseline, two additional days of rest were given to the rats, as previously described, to allow time for both the Nembutal and salicylate to be expelled from the rats' systems. Two testing runs were completed, so that testing on rats 1 through 8 was conducted first, with testing conducted on rats 9 through 16 following. Each set of rats took 16 days, and for all testing to be completed, a total of 32 days were needed (Table 1).

Measuring Startle and Calculating PPI

During the course of experimentation, startle was measured in two different ways. One way was to measure the maximum force in Newtons, while the second way was to measure the average force, or "area under the curve". Additionally, in order to calculate PPI, two different techniques were used, an "individualized" PPI and an "averaged" PPI. By either technique, PPI is calculated by taking the ratio of startle with a gap to startle alone (gap/alone). If there was no reduction in the startle response when a pre-startle stimulus (gap in continuous sound background) was present, then the ratio would be equal to one, and PPI would not be seen. Alternatively, if there is inhibition in the

Table 1. Number of days needed to complete testing on one group of rats.

Day	Action
1	ABR and DPOAE testing
2	ABR and DPOAE testing
3	Rest
4	Rest
5	Baseline PPI testing
6	Baseline PPI testing
7	Salicylate administration
8	Salicylate administration
9	Behavioral PPI testing with salicylate administration
10	Behavioral PPI testing with salicylate administration
11	ABR and DPOAE testing with salicylate administration
12	ABR and DPOAE testing with salicylate administration
13	Rest
14	Rest
15	After salicylate PPI testing
16	After salicylate PPI testing

response, then the PPI ratio would be less than one; the farther away from one, the more inhibition or stronger PPI present.

CHAPTER 4

RESULTS

A total of 16 Long-Evans pigmented rats were used during experimentation with the aim of determining the pitch of salicylate induced tinnitus using the pre-pulse inhibition animal model of tinnitus (Turner et al., 2006). Although 16 rats were used at the onset, two rats were eliminated from experimentation due to technical difficulties experienced with the equipment during the time course of the investigation. The rats were tested in two groups of eight, with the second group undergoing manipulation once all experiments were conducted on the first set in its entirety. This was done due to a limitation in the number of testing chambers available. Unlike Turner et al.'s protocol, the methodology used during this experimentation consisted of not only the use of a 60 dB SPL background stimulus, but also the use of a 48 dB SPL background stimulus.

Behavioral Results

In order to measure behavioral responses and calculate PPI, both the startle alone and startle with gap inserted 100 ms before the startle stimulus were collected during the various experimental conditions. The first experimental condition consisted of measuring baseline behavioral responses for two days. Following baseline measurement, the rats were given daily injections of salicylate equal to a dosage of 275 mg (321 mg/kg sodium salicylate) over a period of two days to induce tinnitus. Next, behavioral responses were measured again over a period of two days, continuing daily injections of salicylate to document any change that the salicylate may have had. After the ceasing of salicylate and

after allowing for a recovery period of at least two days, a final set of behavioral responses were measured to see if responses returned to baseline. All measurements were taken in the presence of two levels of background narrow band noise, 48 dB SPL and 60 dB SPL, with test frequencies spaced every 1/3 octave covering frequencies from 4 to 32 kHz.

As mentioned in the methodology, two methods of calculating PPI were employed, “individualized” and “averaged”. Because PPI is dependant upon the change in the startle reaction when a gap is inserted, it should be seen that the startle alone response is not affected by the interaction of condition, day, block and/or frequency. ANOVA with repetitions were run to test the significance of these interactions for the 48 and 60 dB SPL background levels. When all data was analyzed for the 48 dB SPL background level (Table 2), significant findings were seen for Frequency ($F(9,63) = 14.066, p <.000$), Day ($F(1,7) = 14.082, p <.007$), and Block ($F(3,21) = 46.637, p <.000$), as well as the interactions of Condition x Day ($F(2,14) = 5.766, p <.015$), Condition x Frequency x Day ($F(18,126) = 1.858, p <.025$), Frequency x Block ($F(27,189) = 2.699, p <.000$), and Day x Block ($F(3,21) = 4.749, p <.011$). All other interactions were found to be not significant for this background level. For the 60 dB SPL background level (Table 3), significant findings were seen for Condition ($F(2,16) = 9.129, p <.002$), Frequency ($F(9,72) = 40.546, p <.000$), Day ($F(1,8) = 20.278, p <.002$), and Block ($F(3,24) = 30.756, p <.000$), as well as for the interactions of Condition x Frequency ($F(18,144) = 3.007, p <.000$), Condition x Day ($F(2,16) = 6.299, p <.010$), Condition x Block ($F(6,48) = 3.213, p <.010$), Frequency x Block ($F(27,216) = 3.136, p <.000$), Condition x Frequency x block ($F(54,432) = 2.200, p <.000$), Condition x Day x Block ($F(6,48) =$

Table 2. ANOVA with repetitions for startle alone in the 48 dB SPL background narrow band noise centered on range of frequencies.

Variables	F Sat.	df	p value
Condition	3.197	2/14	.630
Day	14.802	1/7	.007
Frequency*	14.066	9/63	.000
Block*	46.637	3/21	.000
CxD	5.766	2/14	.015
CxF	1.145	18/126	.318
DxF	.690	9/63	.715
CxDxF	1.858	18/126	.025
CxB	1.685	6/42	.149
FxB	2.699	27/189	.000
CxDxB*	1.008	6/42	.433
DxB*	4.749	3/21	.011
CxFxB*	1.233	54/378	.137
DxFxB	1.351	27/189	.127
CxDxFxB	1.235	54/378	.135

Values of statistical significance are marked with an asterisk in this and subsequent tables. Variables: Condition (baseline/salicylate/after salicylate), Day, Frequency, Block, x (interaction between variable(s)).

Table 3. ANOVA with repetitions for startle alone in the 60 dB SPL background narrow band noise centered on range of frequencies.

Variables	F Sat.	df	p value
Condition*	9.129	2/16	.002
Day*	20.278	1/8	.002
Frequency*	40.546	9/72	.000
Block*	30.756	3/24	.000
CxD*	6.299	2/16	.010
CxF*	3.007	18/144	.000
DxF	1.627	9/72	.124
CxDxF	1.360	18/144	.161
CxB*	3.213	6/48	.010
FxB*	3.136	27/216	.000
CxDxB*	3.567	6/48	.005
DxB	1.066	3/24	.382
CxFxB*	2.200	54/432	.000
DxFxB	1.059	27/216	.392
CxDxFxB*	1.605	54/432	.006

Variables: Condition (baseline/salicylate/after salicylate), Day, Frequency, Block, x (interaction between variable(s)).

3.567, $p < .005$), and Condition x Frequency x Day x Block ($F(54,432) = 1.605$, $p < .006$). Because these findings show that difference trends were seen, data was collapsed in order to investigate the overall interactions of frequency and condition (Tables 4 and 5). There was no significant findings for this interaction for either 48 dB SPL ($F(18,144) = 1.207$, $p > .05$) or 60 dB SPL ($F(18,144) = 1.207$, $p > .05$), indicating that the startle response was stable in all conditions.

To calculate PPI using the first method, “individualized”, PPI was calculated for each pair of startle alone and startle with gap responses separately over the four testing repetitions in each testing block and then the average of these four PPIs was taken to get an average for the given experimental condition within one block. The second, typically used, approach was to calculate PPI by using the averaged gap responses from the four repetitions within a given block divided by the averaged startle alone responses averaged over the four repetitions in the same block. This resulted in an “average” PPI for a given experimental condition with one block. In theory, it is possible to expect that one or the other approach should provide smaller variability of PPI. Specifically, if the animal’s behavior is correlated in short time (seconds) then “individualized” PPI should be superior. If, however, responses of the rats exhibit large variability within a short time, then “averaged” method should be superior as it decreases variability caused by random, low response to startle.

Over the course of the investigation, the rats showed varied responses during experimentation. In order to demonstrate this variable nature, two rats were chosen for use in the following set of figures, rats 1 and 2. Presented in Figures 2a and 2b and 3a and 3b are examples of the raw data, startle alone and startle with a gap, obtained from these

Table 4. ANOVA with repetitions for startle alone in the 48 dB SPL background narrow band noise centered on range of frequencies – data collapsed.

Variables	F stat.	df	p value
Frequency*	13.451	9/72	.000
Condition*	4.649	2/16	.026
FxC	1.027	18/144	.434

Variables: Frequency, Condition (baseline/salicylate/after salicylate), x (interaction between variable(s)).

Table 5. ANOVA with repetitions for startle alone in the 60 dB SPL background stimulus
– data collapsed.

Variables	F stat.	df	p value
Frequency*	13.451	9/72	.000
Condition*	4.649	2/16	.026
FxC	1.027	18/144	.434

Variables: Frequency, Condition (baseline/salicylate/after salicylate), x (interaction between variable(s)).

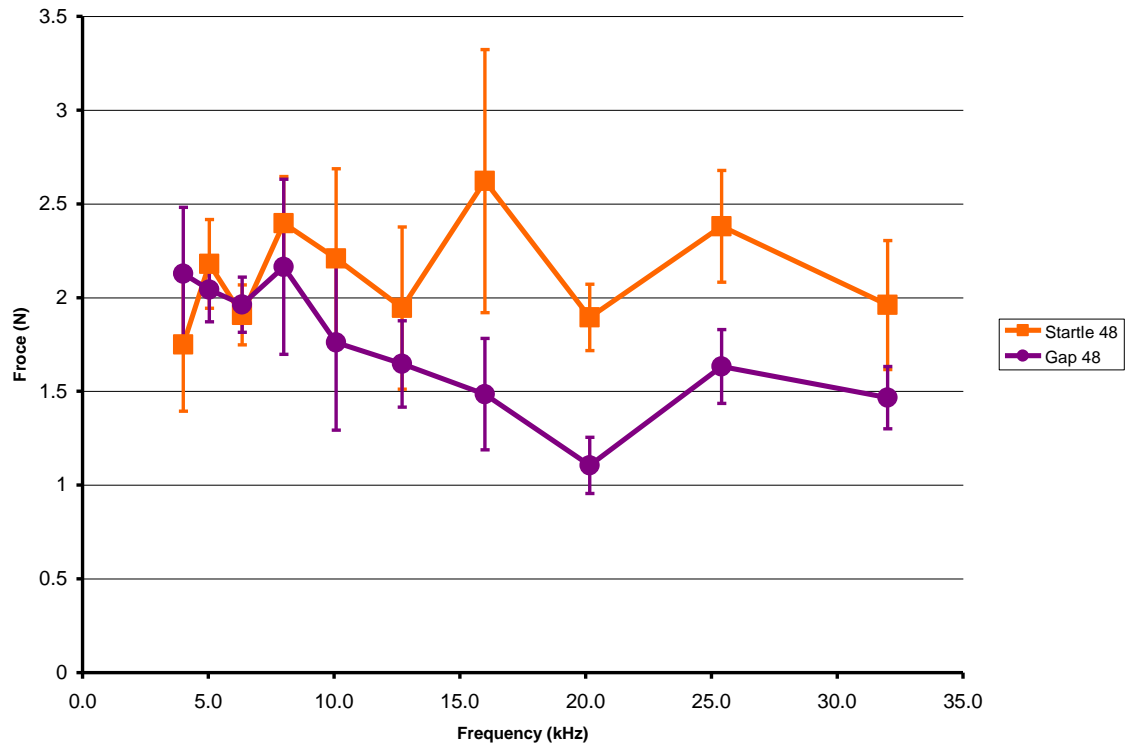


Figure 2a. Startle alone (square) and startle with gap (circle) responses of Rat 1 in the presence of a 48 dB SPL background stimulus expressed as maximum force. Response measured as a maximum force in Newtons within a 250 msec time window from the startle stimulus in this and subsequent figures. Note that the responses with a gap are consistently smaller than without a gap for frequencies above 6 kHz, indicating the presence of PPI. Vertical bars represent the standard error of mean (SEM) in this and subsequent figures.

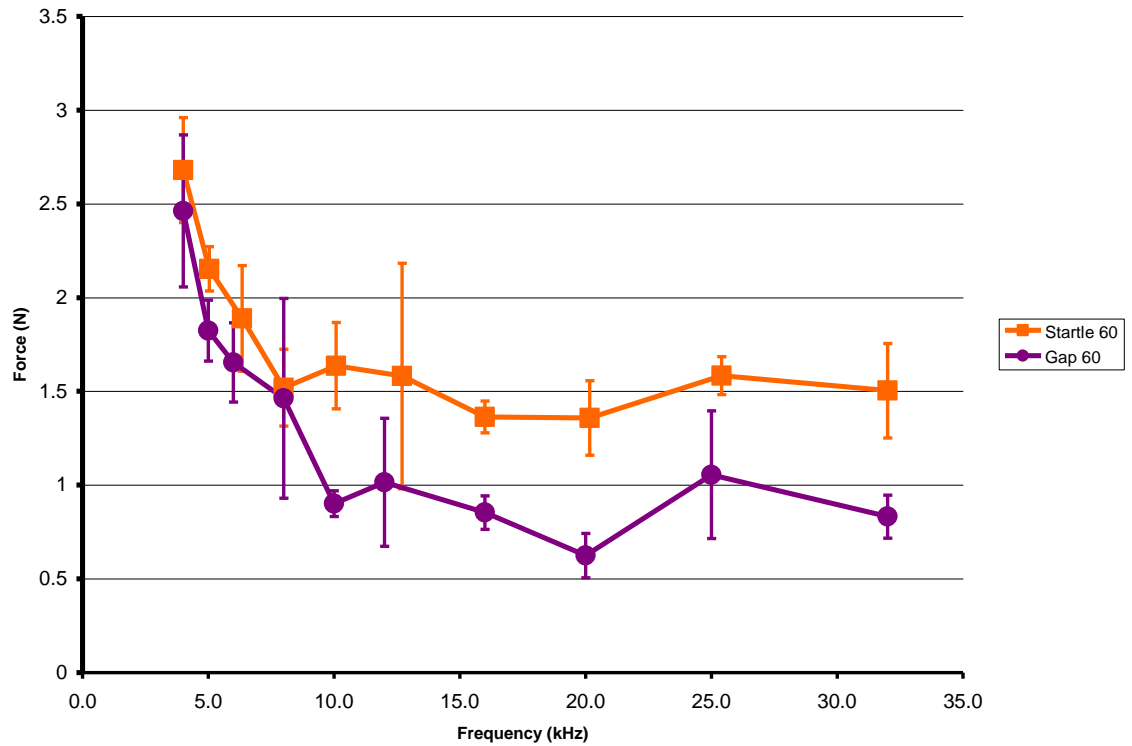


Figure 2b. Startle alone (square) and startle with gap (circle) responses of Rat 1 in the presence of a 60 dB SPL background stimulus expressed as maximum force. Note that as for the 48 dB SPL background, responses with a gap are consistently smaller than without a gap, indicating the presence of PPI.

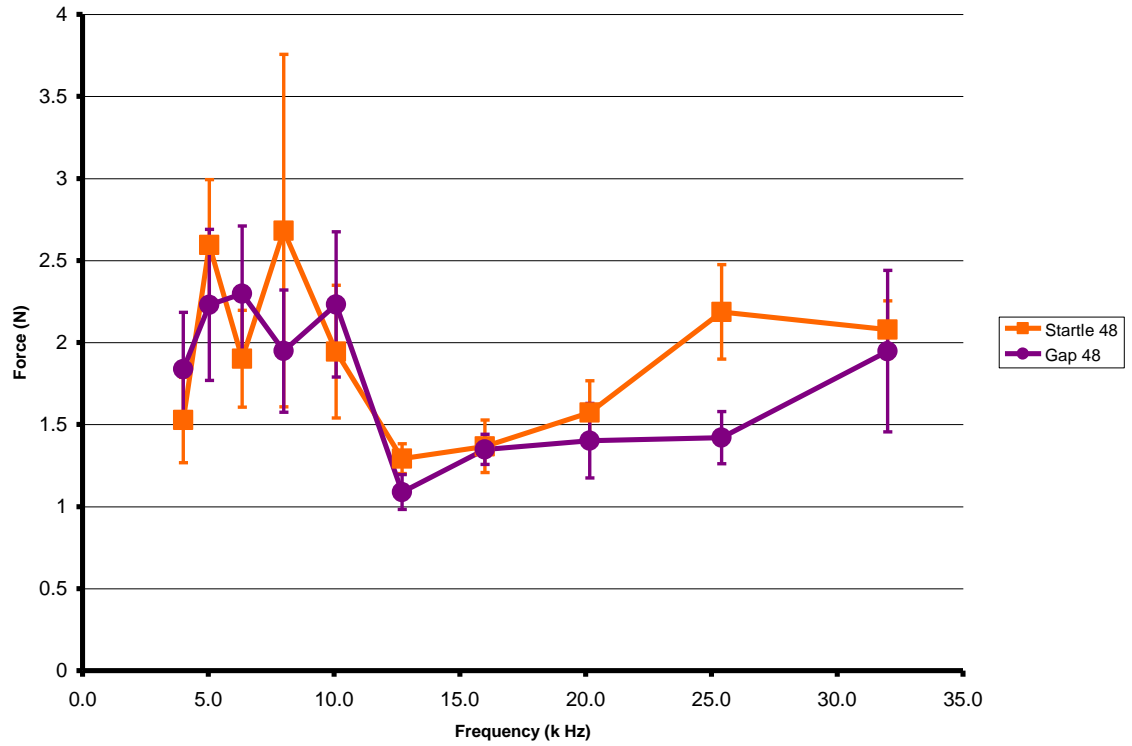


Figure 3a. Startle alone (square) and startle with gap (circle) responses of Rat 2 in the presence of a 48 dB SPL background stimulus expressed as maximum force. Responses show lack of clear indication of PPI presence, except for 25 kHz.

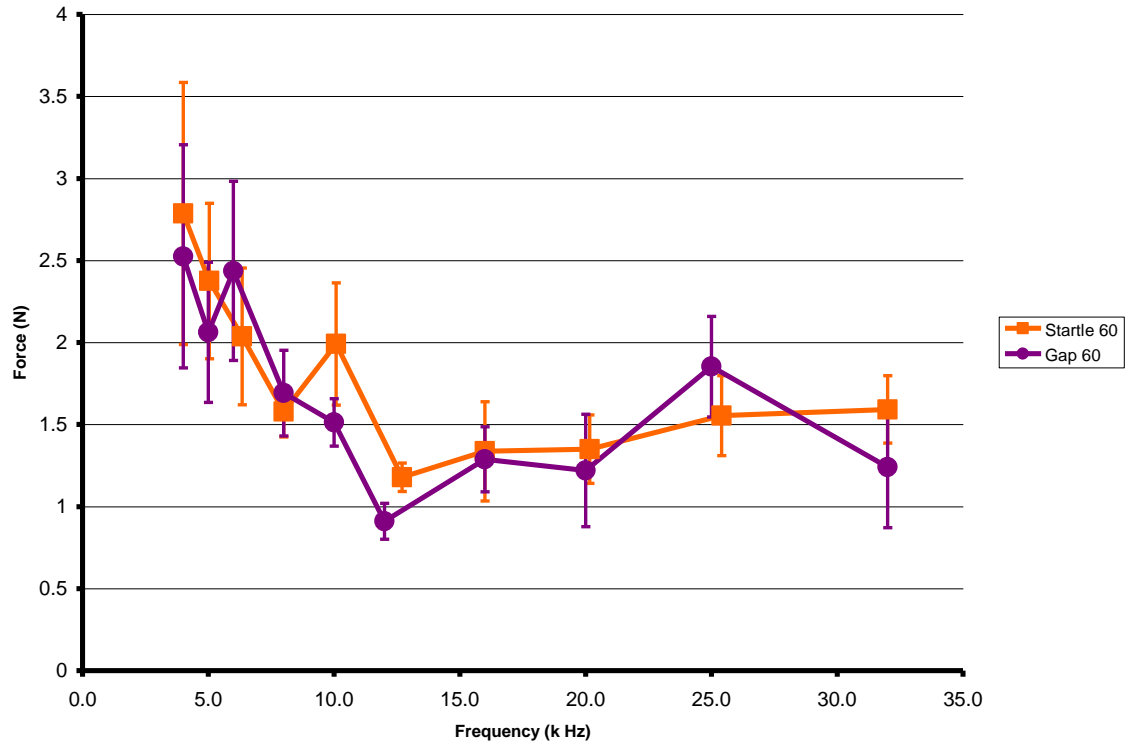


Figure 3b. Startle alone (square) and startle with gap (circle) responses of Rat 2 in the presence of a 60 dB SPL background stimulus expressed as maximum force. Responses show a clear lack of PPI for Rat 2.

two rats in the presence of the two levels of background stimulus seen within a 250 msec time window from the presentation of the startle stimulus. These sets of data were collected on the first day of baseline measurement. Behavioral responses were measured as a maximum level of force (in Newtons) that was applied to the piezo transducer as the rats were startled in the test chambers. As can be seen by examination of these four figures, especially when looking at differences between rats 1 and 2, there was a lot of variability between the rats as well as for a given rat between the two levels of background stimuli. In spite of variability, it can be seen that the responses with gap (circle) tend to be smaller than startle alone (square), therefore indicating the presence of PPI. It can also be noted that the responses for both startle alone and startle with the gap are frequency dependent, as they change with each frequency; however, the change seen from one frequency to another is unpredictable. Although there is a considerable amount of variability in the responses displayed by the individual rats, when examined as a group (Figures 4a and 4b) there are far fewer fluctuations observed with a fairly flat frequency response seen for both levels of background noise. From these two figures, a systematic decrease in the response for both startle alone and startle with a gap can be seen for the two background test conditions, with the 60 dB SPL condition having a greater dependence on the frequency response. The responses seen with the 60 dB SPL background stimuli appear to be lower (in Newtons) than what is seen for 48 dB SPL. Despite the differences seen, both background conditions show that when a gap is inserted prior to the startle stimulus, the overall force exerted is less, indicating inhibition of the startle response. This is most evident for the frequencies above 10 kHz.

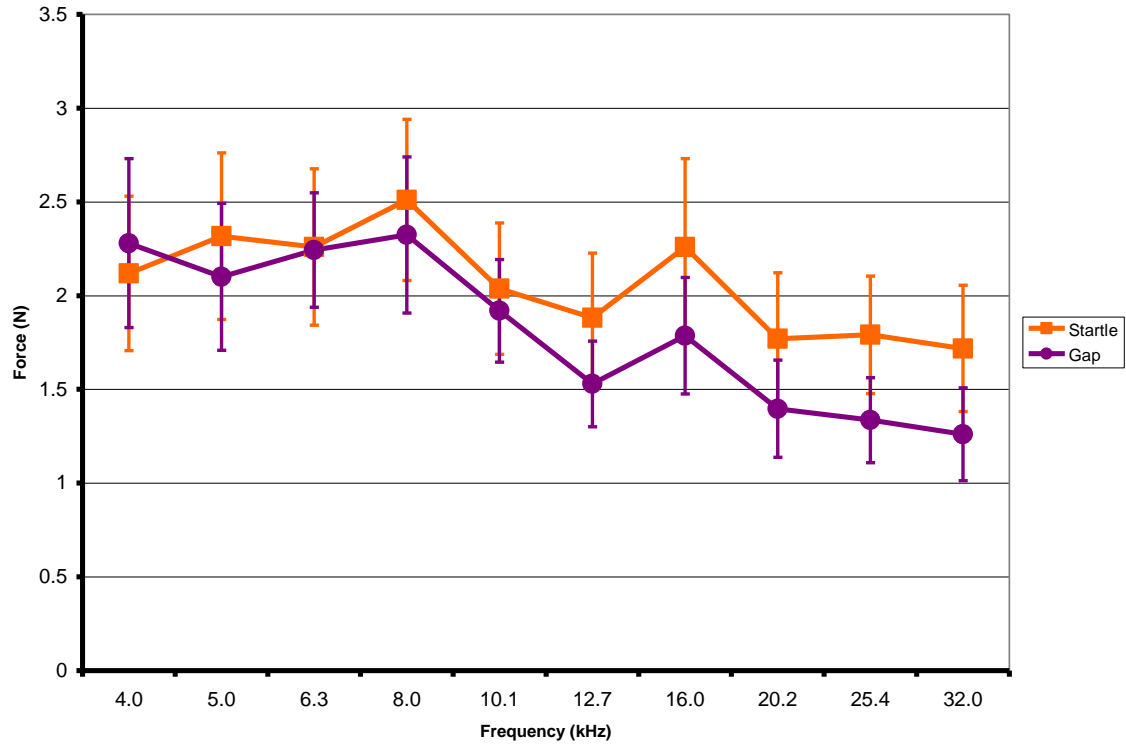


Figure 4a. Startle alone (square) and startle with gap (circle) responses of all rats in the presence of a 48 dB SPL background stimulus expressed as maximum force. Note that the response with a gap is smaller than without a gap, indicating the presence of PPI, especially for frequencies over 10 kHz.

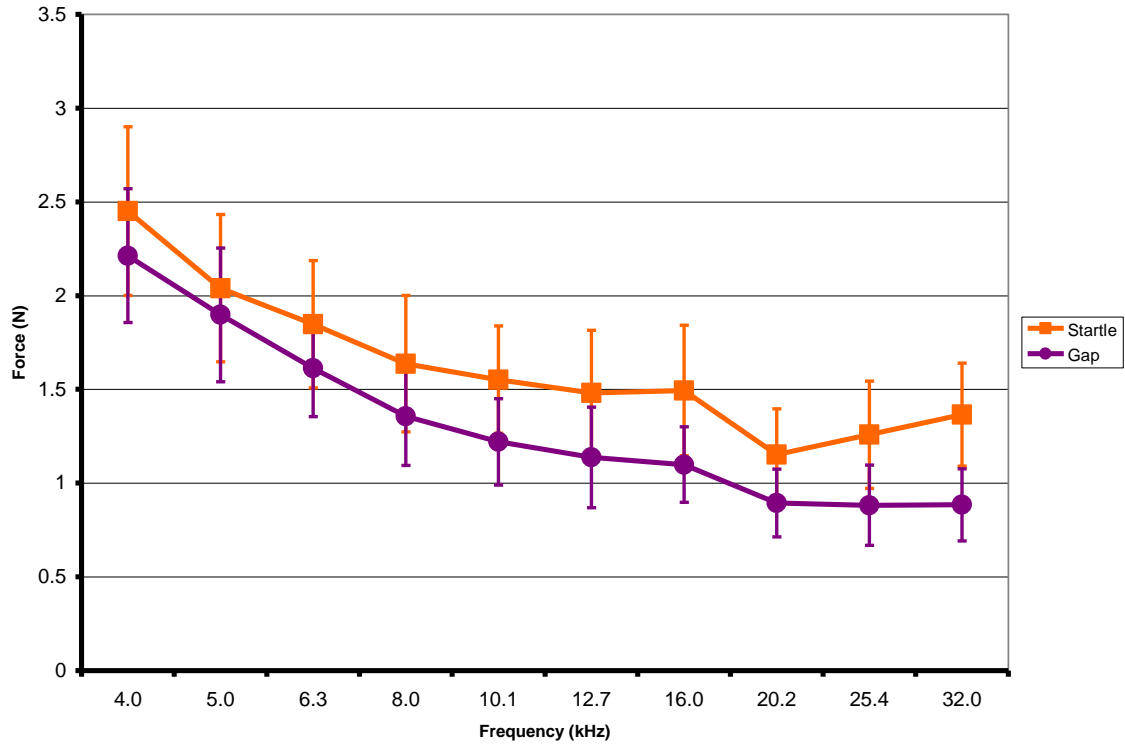


Figure 4b. Startle alone (square) and startle with gap (circle) responses of all rats in the presence of a 60 dB SPL background stimulus expressed as maximum force. For the entire group of rats, there is a clear indication of PPI present at all frequencies, with greater PPI in the higher frequencies.

When measuring behavioral responses, there are two ways of showing the value of the force exerted by the rats. The first method, as shown in the aforementioned figures, uses the maximum level of force. A second way of showing this data is by measuring the area under the curve, which represents an average force during the 250 msec time window. For this experimentation, both methods were used to evaluate the startle responses of the rats. Figures 5a, 5b, 6a, and 6b show the startle alone and startle with a gap response for rats 1 and 2 in the presence of both background conditions as measured by the area under the curve representing the average force exerted.

The responses expressed as the average force are presented in Figures 7a and 7b for all experimental rats used. Note the similarity between Figures 2 and 5, 3 and 6, and 4 and 7. There is little difference when comparing the responses expressed as average and maximum force. Evaluation of results gathered by these two methods revealed that they are highly correlated as seen upon graphical inspection and statistical evaluation ($R^2=0.9426$, $df= 5998$, $p< .001$) (Figure 8). It is possible, however, to expect a different variability of responses when max force versus average force is used. As there is no clear indication of an advantage to using the average force approach and as current literature (Turner et al., 2006) is currently using maximum force, subsequent analysis will focus solely on the maximal force method.

As previously mentioned, there are two methods of calculating PPI, the “individualized” method, and the “averaged” method. Figures 9a and 9b, as well as 10a and 10b show the PPI measurements for rats 1 and 2 utilizing these two approaches of calculating PPI for both background levels (“averaged” – diamonds, “individualized” – circles).

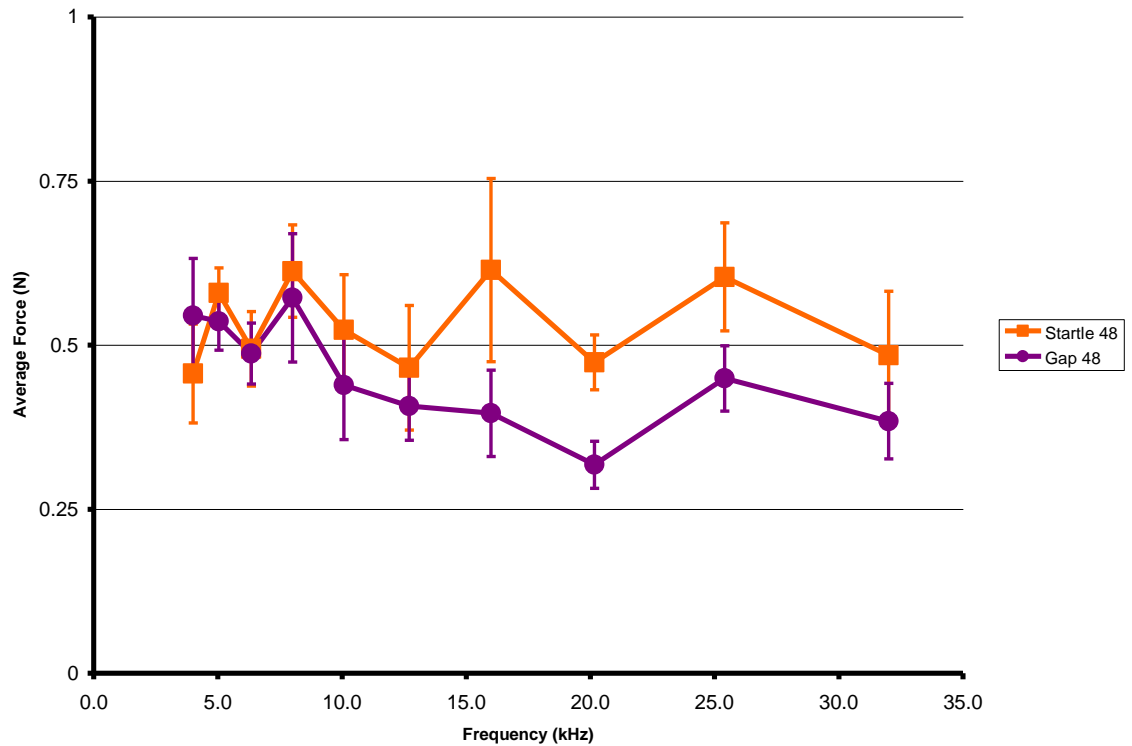


Figure 5a. Startle alone (squares) and startle with gap (circle) responses of Rat 1 in the presence of a 48 dB SPL background stimulus expressed as average force. Response measured as the area under the curve, which represents the average force exerted in Newtons within a 250 msec time window from the startle stimulus in this and subsequent figures. Note the similarity between this figure and Figure 1a.

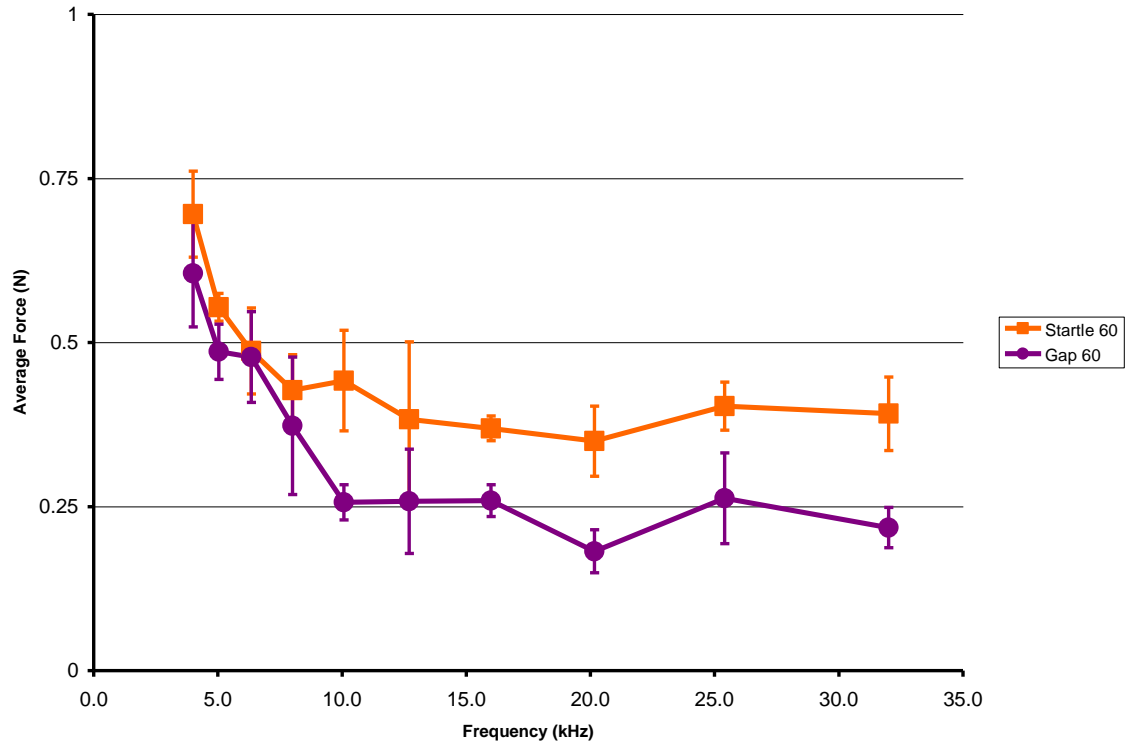


Figure 5b. Startle alone (square) and startle with gap (circle) responses of Rat 1 in the presence of a 60 dB SPL background stimulus expressed as average force. PPI is present for all frequencies above 10 kHz. Note the similarity between this figure and Figure 1b.

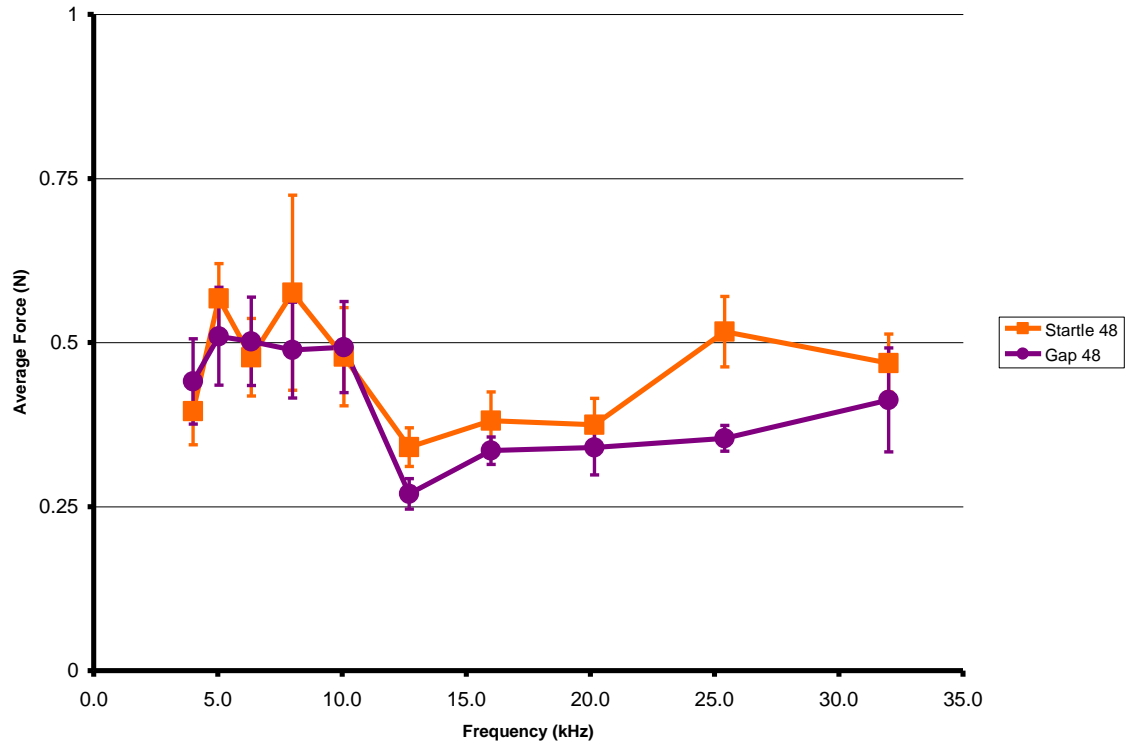


Figure 6a. Startle alone (square) and startle with gap (circle) responses of Rat 2 in the presence of a 48 dB SPL background stimulus expressed as average force. The only indication of PPI present is at 25 kHz. Note the similarity between this figure and Figure 2a.

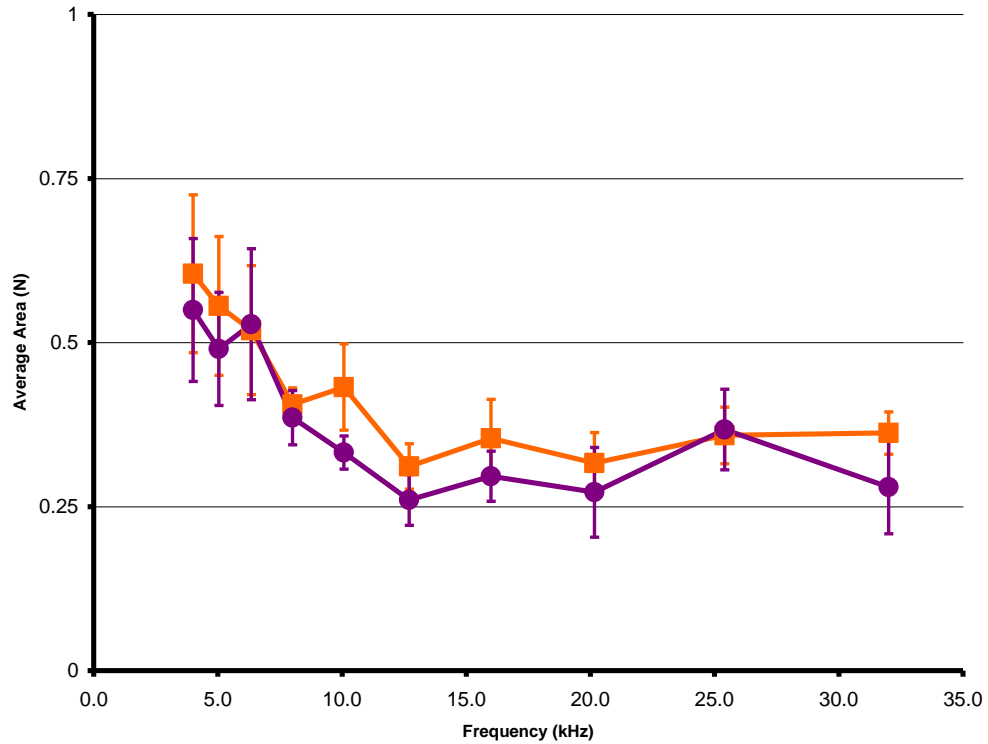


Figure 6b. Startle alone (square) and startle with gap (circle) responses of Rat 2 in the presence of a 60 dB SPL background stimulus expressed as average force. There is no clear indication of PPI at any frequencies as seen by the closeness of the startle alone and startle with gap responses. Note the similarity between this figure and Figure 2b.

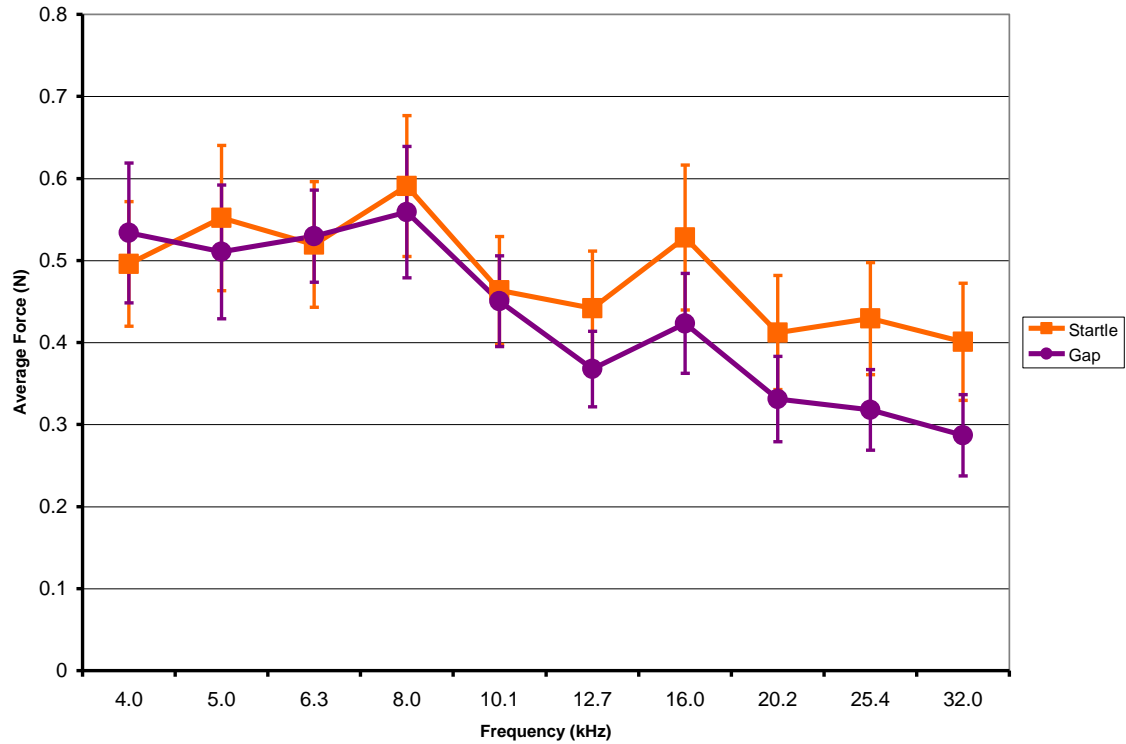


Figure 7a. Startle alone (square) and startle with gap (circle) responses of all rats in the presence of a 48 dB SPL background stimulus expressed as average force. PPI is present in the higher frequencies, starting at 12.7 kHz. Note the similarity between this figure and Figure 3a.

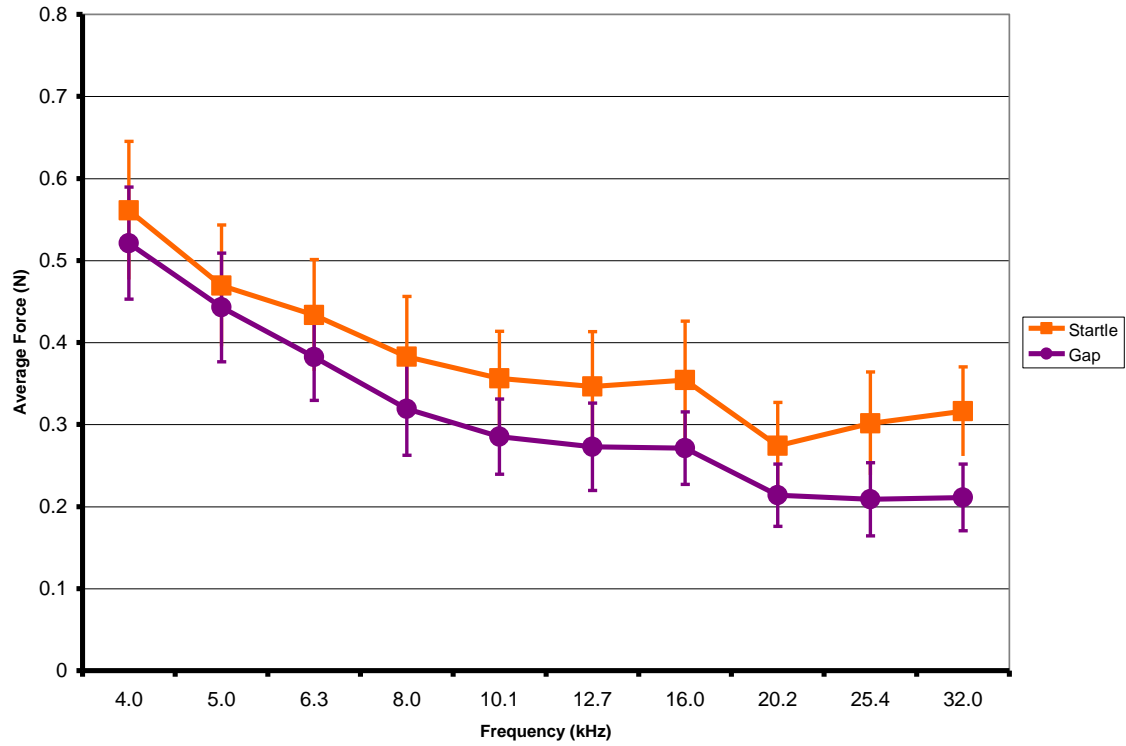


Figure 7b. Startle alone (square) and startle with gap (circle) responses of all rats in the presence of a 60 dB SPL background stimulus expressed as average force. There is a clear indication of PPI at most frequencies. Note the similarity between this figure and Figure 3b.

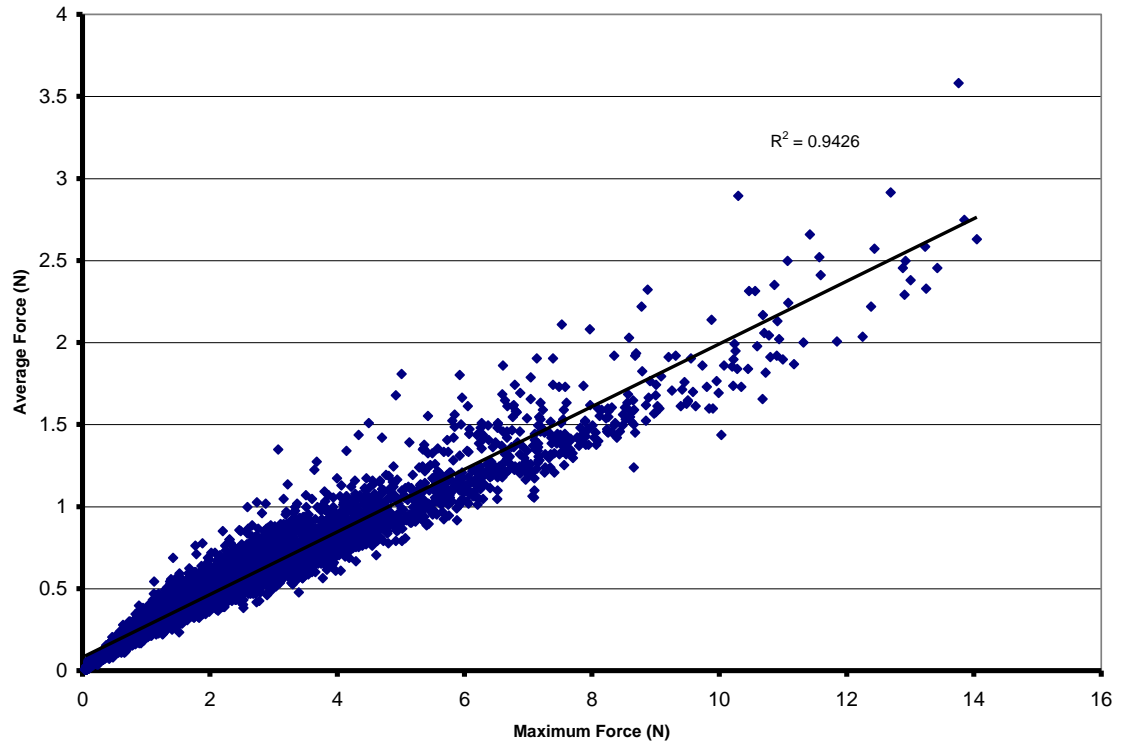


Figure 8. Correlation seen between measuring the startle response using the maximum force and the average under the curve. Statistical analysis reveals a high level of correlation between the two measures ($R^2=0.9426$, $df= 5998$, $p< .001$). Because of this statistical correlation and because the maximum measurement is more widely used, this will be used in all subsequent graphs and analyses.

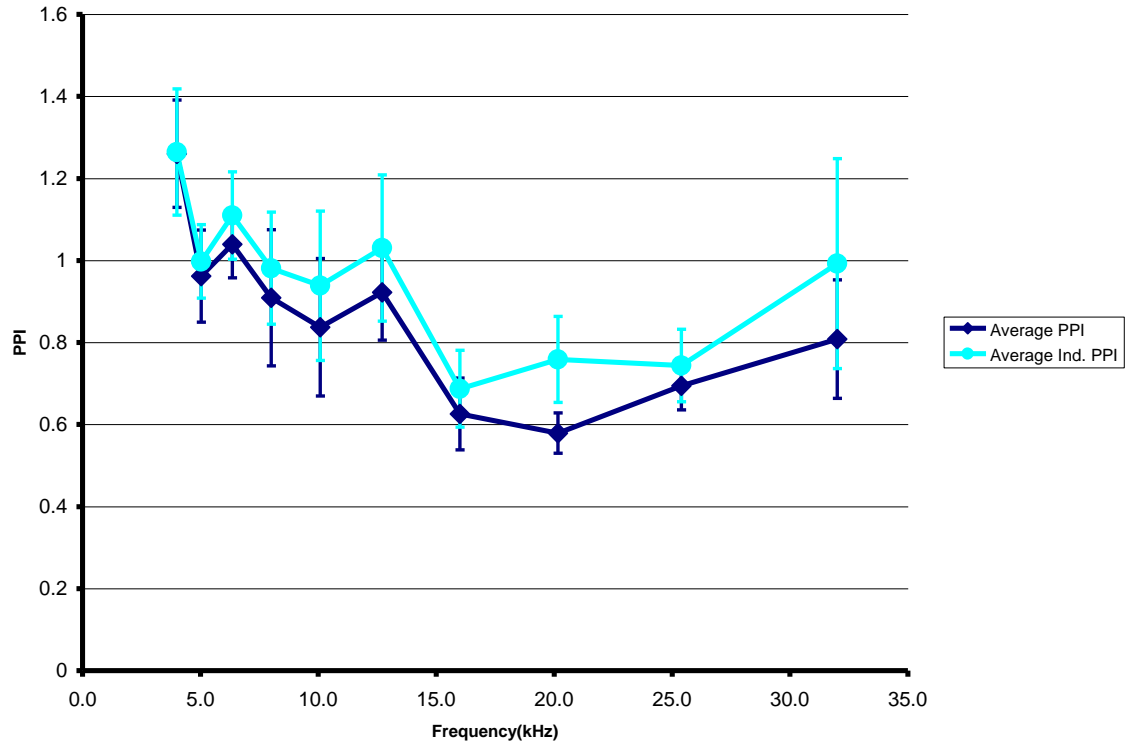


Figure 9a. PPI responses of Rat 1 in the presence of a 48 dB SPL background stimulus conducted using an “averaged” method (circle) and an “individualized averaged” method (diamond). The individualized method of calculating PPI shows a smaller value of PPI when compared to the averaged method as indicated by the individualized tracing being closer to a value of 1.

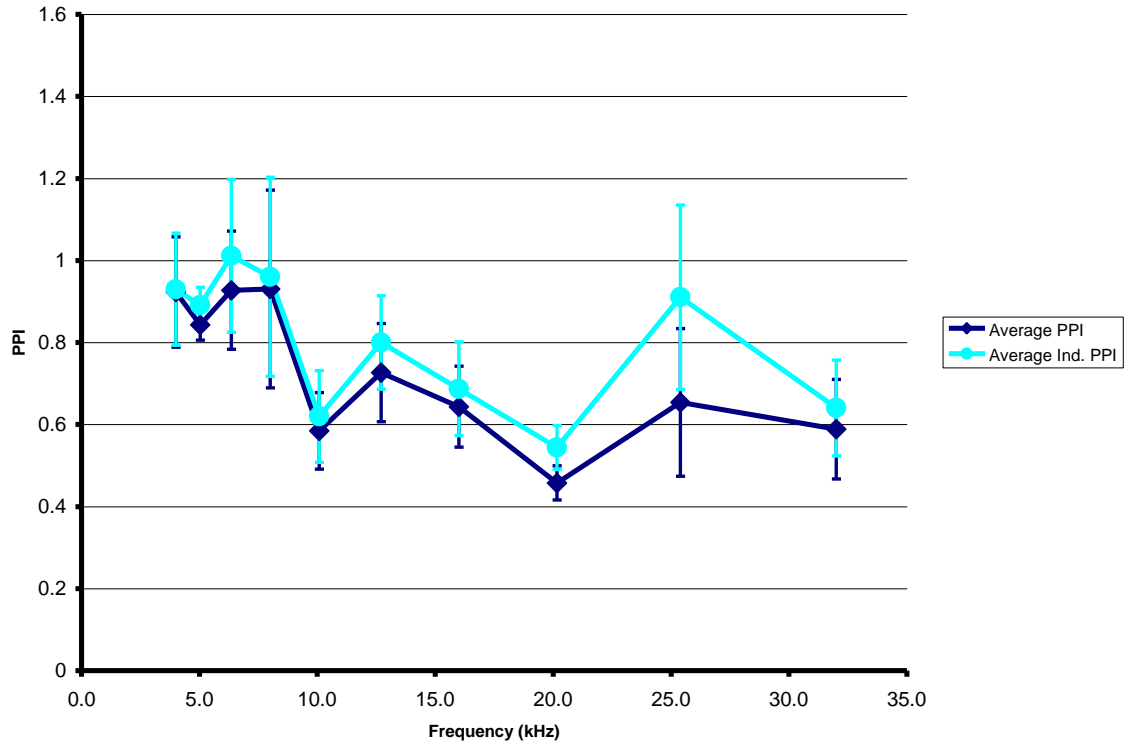


Figure 9b. PPI responses of Rat 1 in the presence of a 60 dB SPL background stimulus calculated using an “averaged” method (diamond) and an “individualized averaged” method (circle). The individualized method shows a smaller value of PPI, especially at 25 kHz.

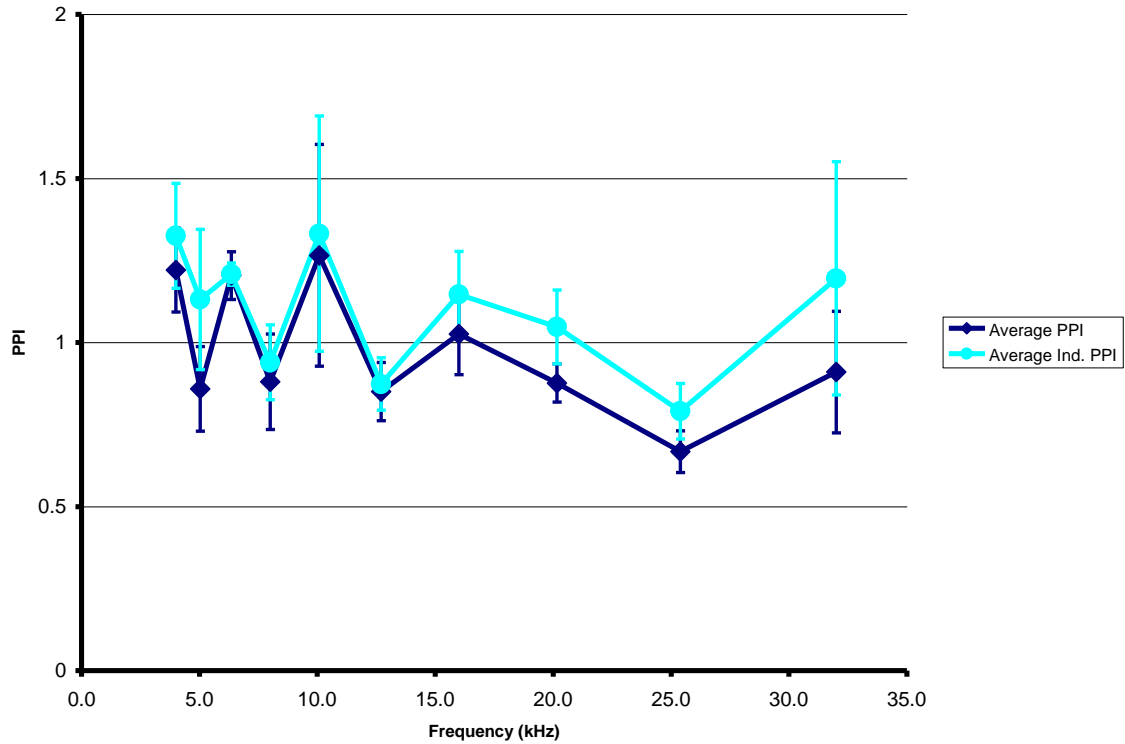


Figure 10a. PPI responses of Rat 2 in the presence of a 48 dB SPL background stimulus calculated using an “averaged” method (diamond) and an “individualized averaged” method (circle). There is no evidence of PPI for in the individualized methods, except at 25 kHz. The averaged method shows PPI at 20 kHz and above.

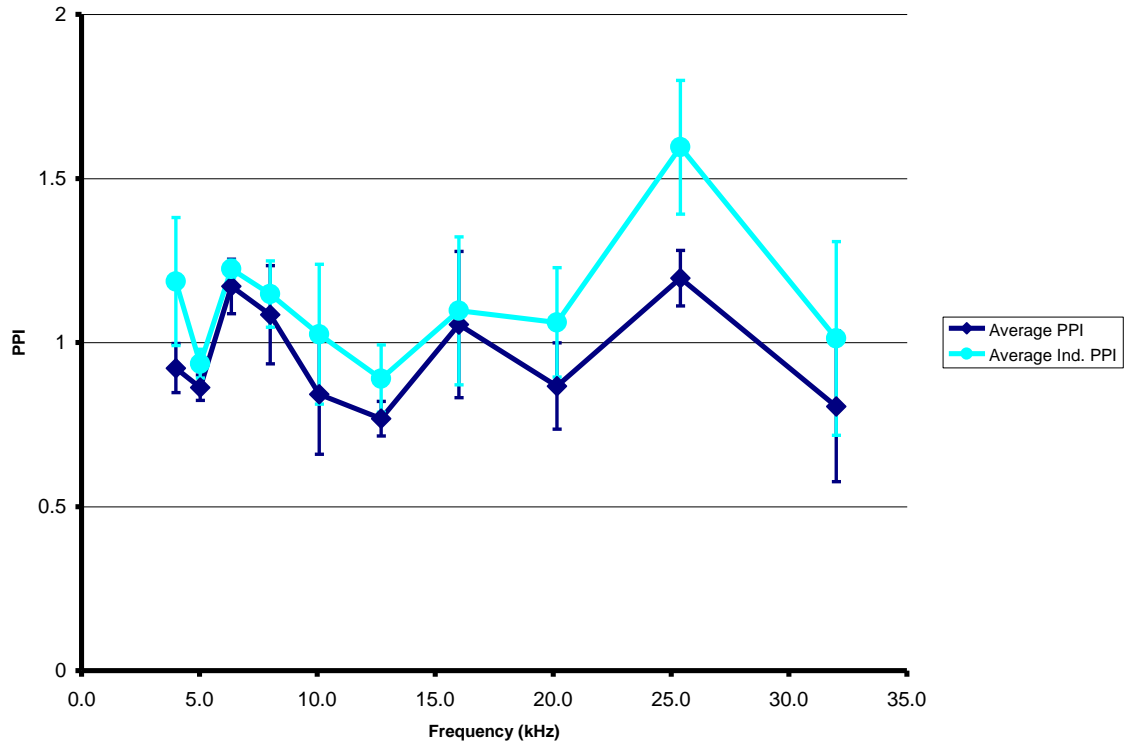


Figure 10b. PPI of Rat 2 in the presence of a 60 dB SPL background stimulus calculated using an “averaged” method (diamond) and an “individualized averaged” method (circle). There is no clear evidence of PPI for the individualized method at any frequency.

The individually calculated PPI shows a systematically smaller value of PPI along with a greater amount of variability for the two rats. Moreover, for inspection of all data (Figures 11a and 11b), it was seen that in a number of pairs of startle alone versus startle with gap, there was a very small response to startle alone, which lead to biased data using this method (e.g., see PPI in Figure 11b, 20.2 kHz). Thus, this method can greatly skew the results. Due to these factors, subsequent analysis will focus on the typically used “averaged” method of calculating PPI, which is much less susceptible to this type of bias.

Thus far, all figures have shown the behavior of rats on the first experimental day (baseline day 1). However, the goal of this investigation is to determine what, if any, systematic affects salicylate may have on the PPI measurements over time and experimental conditions which may be related to a presence of tinnitus. Figures 12a and 12b show the averaged startle results for the entire group of rats as obtained over all six experimental days and three experimental conditions (pre-salicylate, salicylate, and post-salicylate). For both the 48 dB SPL and 60 dB SPL background stimuli, changes can be seen from day to day, although not in a systematic manner. For example, a decrease in the startle response for the 48 dB SPL condition is seen from baseline day 1 (diamonds) to baseline day 2 (squares), but then increases for the two salicylate days (circles and Xs). These unsystematic changes are seen for both 48 dB SPL and 60 dB SPL conditions. Examination of the startle responses for both dB SPL levels show the trend that 60 dB SPL is more frequency dependant, with the tendency to see the highest responses on day 1.

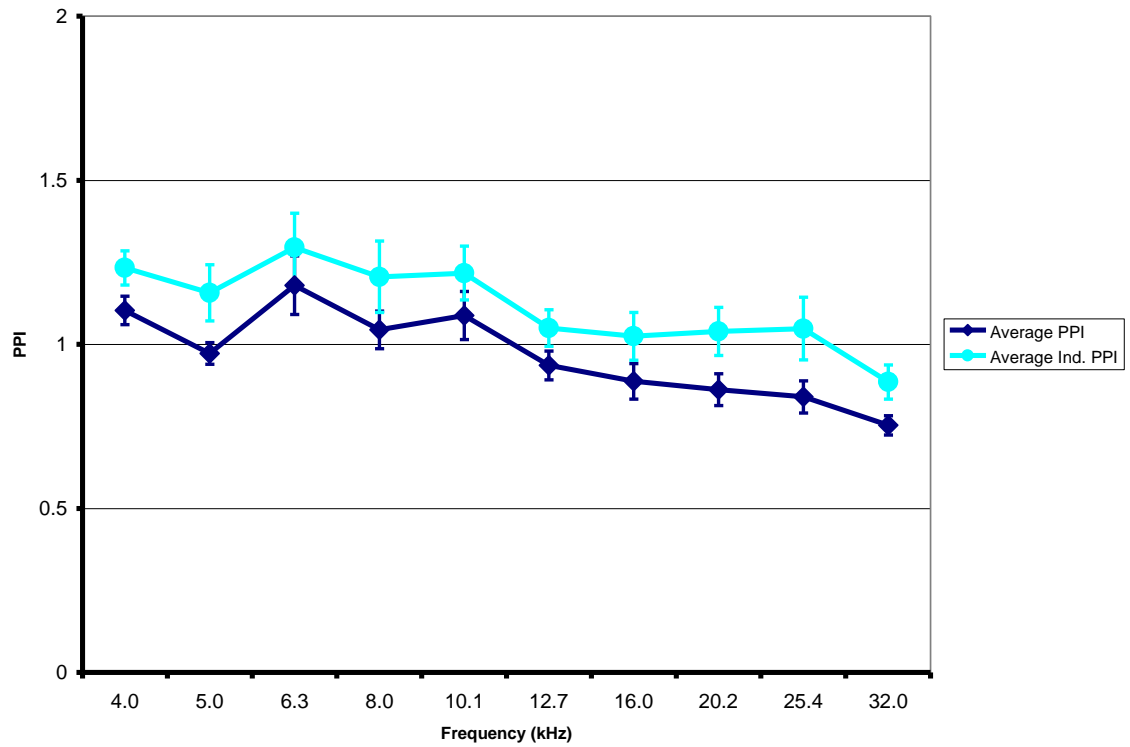


Figure 11a. PPI responses of all rats in the presence of a 48 dB SPL background stimulus calculated using an “averaged” method (diamond) and an “individualized averaged” method (circle). There is no clear evidence of PPI for the individualized method at any frequency, whereas the averaged method shows PPI at 16 kHz and higher.

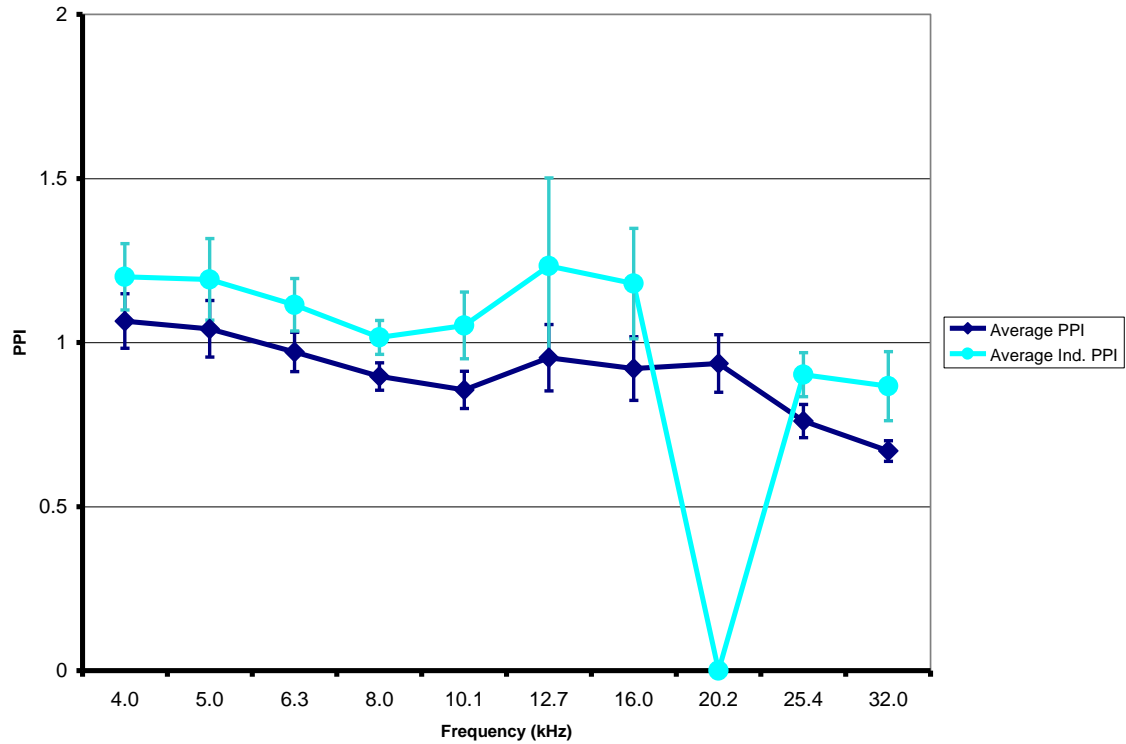


Figure 11b. PPI responses of all rats in the presence of a 60 dB SPL background stimulus calculated using an “averaged” method (diamond) and an “individualized averaged” method (circle). Note the response for the individualized method at 20.2 kHz, which was caused by a small response to startle alone. This type of response leads to bias in the data using this method.

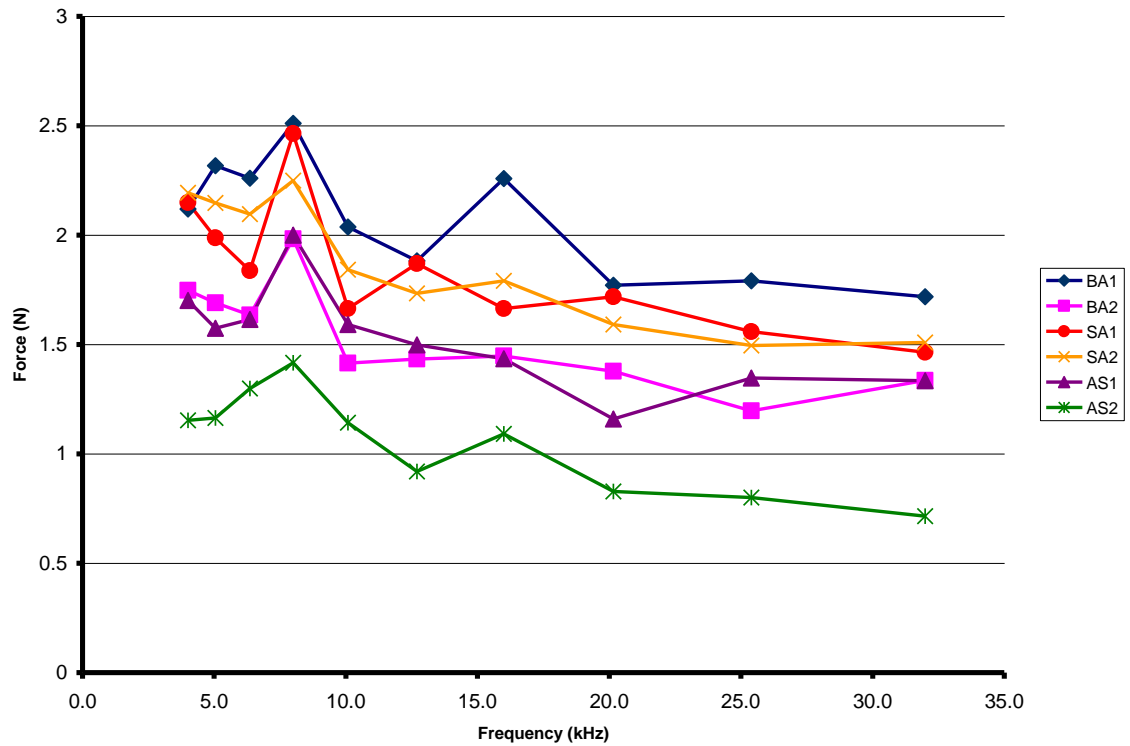


Figure 12a. Average startle responses for all rats tested seen over the course of all testing days in the presence of a 48 dB SPL background stimulus. The first pair of days represent the baseline condition (BA – diamond and square), the second pair days represent the salicylate condition (SA – circle and X), and the third pair of days represent the after salicylate condition (AS – triangle and asterisk). Changes in the startle response are seen from day to day, although not in a systematic manner (e.g., see a decrease in startle from BA1 [diamond] to BA2 [square] and then a subsequent increase for SA1 [circle] and SA2 [x]).

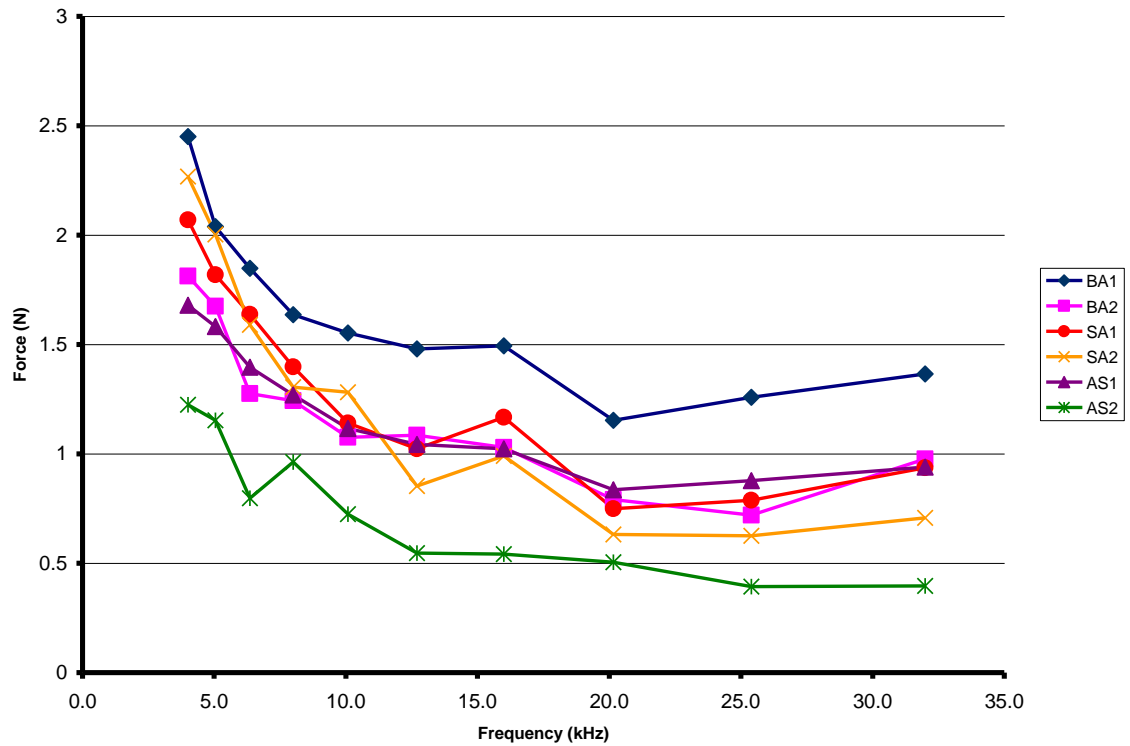


Figure 12b. Average startle responses for all rats tested seen over the course of all testing days in the presence of a 60 dB SPL background stimulus. The first pair of days represent the baseline condition (BA – diamond and square), the second pair days represent the salicylate condition (SA – circle and X), and the third pair of days represent the after salicylate condition (AS – triangle and asterisk). Changes in the response behavior are unsystematic; however, changes appear more frequency dependant than for 48 dB (Figure 11a).

Further examination into the initial baseline PPI measurements was made. In order to detect changes in PPI for a given rat, that rat needs to display the presence of clear PPI on the initial day. It has been shown in the past that salicylate can affect the PPI response in a systematic manner (i.e., salicylate decreased PPI for some frequencies of background) (Turner et al., 2006). If no PPI is present at the onset, then its' decrease cannot be detected.

Figures 13a and 13b show the PPI measurements obtained during baseline day 1, as well as the differences seen when responses from successive days are subtracted from baseline day 1 for rat 1 in the presence of the two background levels. A clear indication of PPI at 8 kHz and above for the 48 dB SPL condition and for all frequencies for the 60 dB SPL condition can be seen, with more prominent PPI in the higher frequencies. When examining the difference lines, the closer the line is to 0, the less PPI difference or change is seen. For example, when looking at the after salicylate day 2/baseline day 1 difference line (X) at 16 kHz in Figure 12a, the value is very close to 0. Hence, when the baseline measurement was subtracted from the salicylate measurement, a difference of close to 0 was found, indicating little change in the response. On the other hand, when looking at the salicylate day 1/baseline day 1 line (circle), a difference of a difference of close to 0.5 is seen, indicating a large change in the response. Although many fluctuations are observed over the period of time, there is a tendency of the two salicylate days to show a decrease of PPI as indicated by the positive differences for some frequencies.

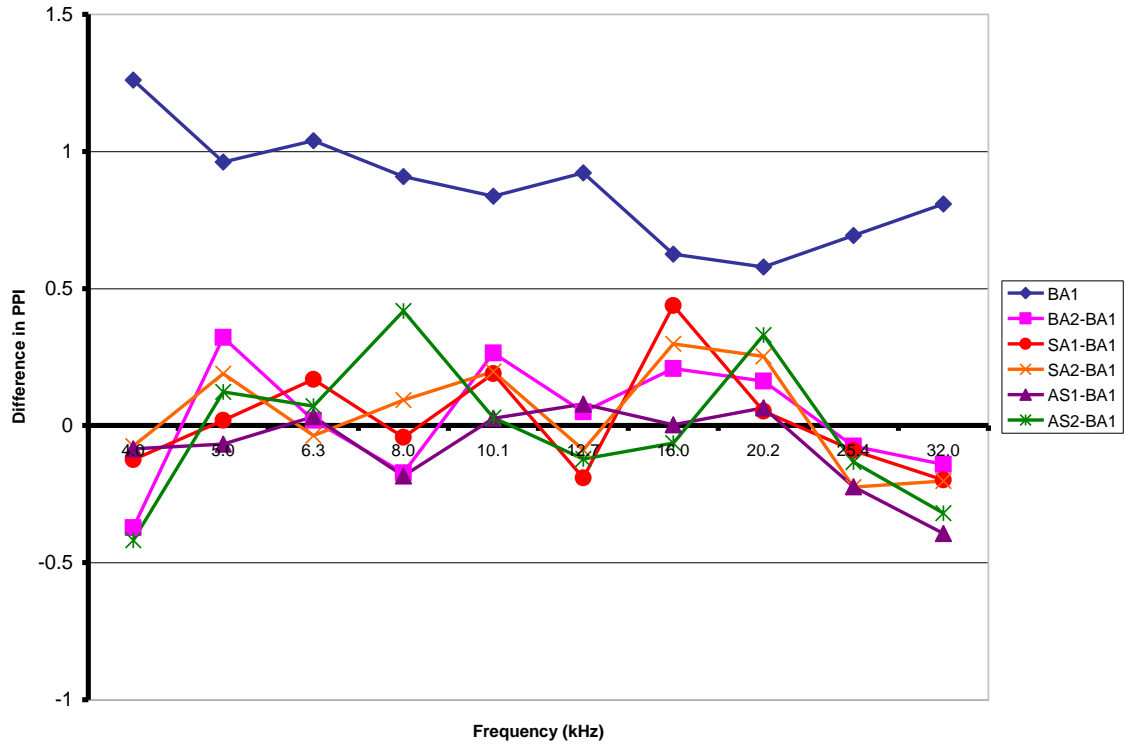


Figure 13a. Differences in PPI measurements for Rat 1 in the presence of a 48 dB SPL background stimulus seen when each subsequent day after Baseline day 1 is subtracted from baseline. Differences of zero indicate no change, whereas positive differences indicate a decrease in PPI. Rat 1 demonstrated good baseline PPI, especially for frequencies of 16 kHz and higher. The difference lines show the largest decrease in PPI for Salicylate day 1 (circle).

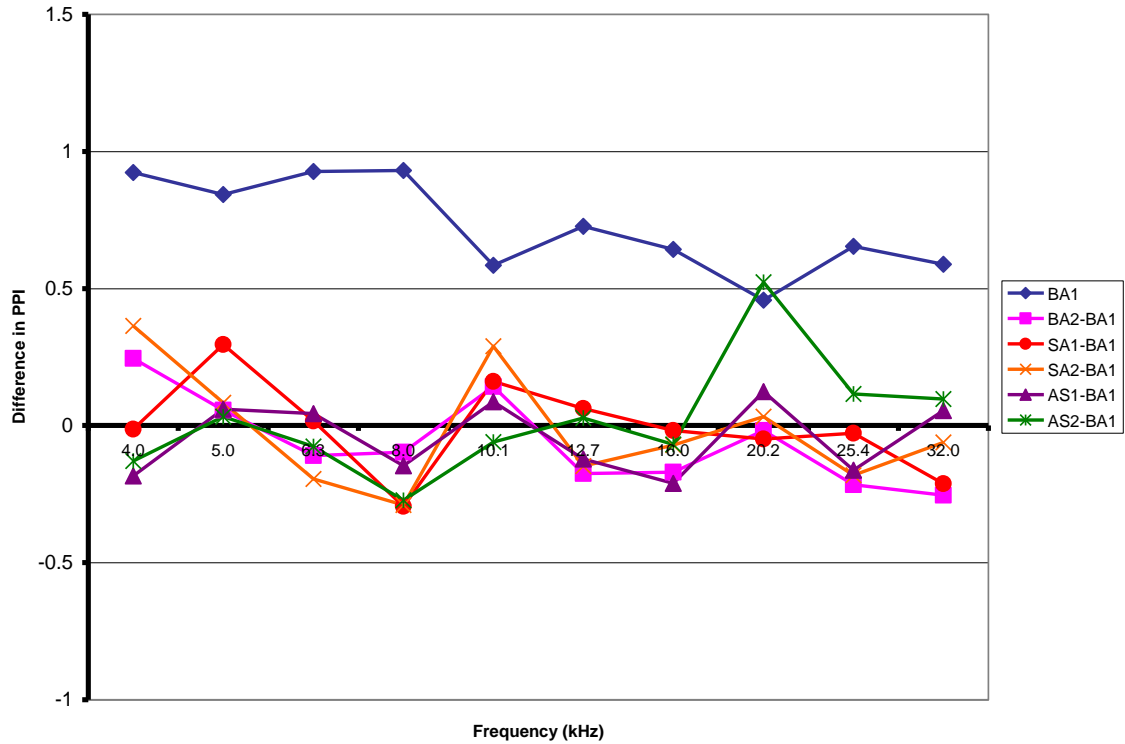


Figure 13b. Differences in PPI measurements for Rat 1 in the presence of a 60 dB SPL background stimulus seen when each subsequent day after Baseline day 1 is subtracted from baseline. Differences of zero indicate no change, whereas positive differences indicate a decrease in PPI. There is very little change in PPI for any of the days, as indicated by difference values around 0, with the exception of After Salicylate day 2 (asterisk).

In contrast to rat 1, Figures 14a and 14b show little or no baseline PPI for rat 2, with the exception of the very highest frequencies for 48 dB SPL and in the mid frequencies for 60 dB SPL. Based upon the initial baseline PPI measurements (individual day and averaged data from the two baseline days), rats which did not show the presence of clear PPI during baseline days were omitted from statistical analysis and final presentation.

Figures 15a and 15b, as well as 16a and 16b show the averaged responses over two days of a condition for rats 1 and 2 for each of the three experimental conditions and the differences seen between those conditions. As expected from the individual days data, rat 1 showed good baseline PPI in response to both background stimuli levels, and was therefore included in the statistical analysis. Conversely, rat 2 showed poor baseline PPI for 48 dB SPL and was omitted from statistical analysis. Rat 2, however, was included in the analysis for 60 dB SPL due to good baseline PPI for that background level.

Once all rats that did not show clear baseline PPI were omitted, the remaining rats were grouped together for statistical analysis. Figure 16a and 16b show the averaged PPI responses for all rats used in the statistical analysis for the various background levels as a function of frequency. There is a clear difference between the PPI measurement obtained from the baseline condition versus when salicylate was present in the rats' systems at 16 kHz for the 48 dB SPL background intensity (Fig. 17a). Additionally, PPI measurements for the after salicylate condition return to near baseline levels following the ceasing of salicylate administration.

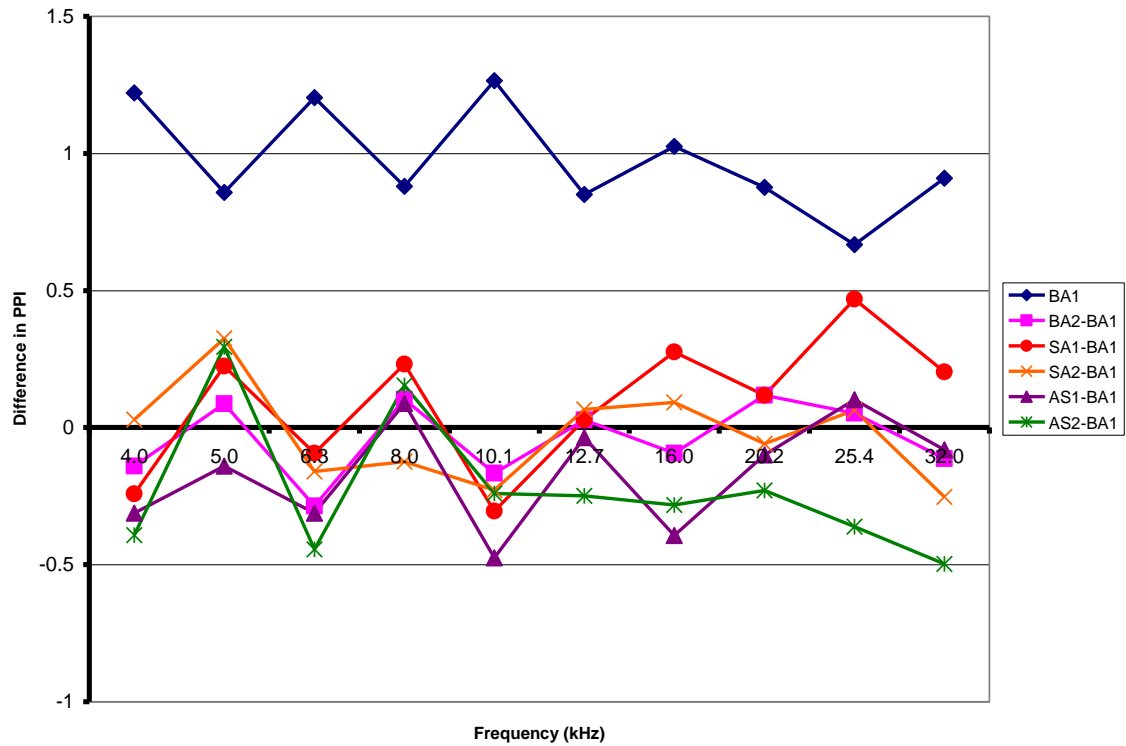


Figure 14a. Differences in PPI measurements for Rat 2 in the presence of a 48 dB SPL background stimulus seen when each subsequent day after Baseline day 1 is subtracted from baseline. Differences of zero indicate no change, whereas positive differences indicate a decrease in PPI and negative differences indicate an increase in PPI. An increase in PPI is seen for Salicylate day 1 (circle) for all frequencies of 16 kHz and higher, and for Salicylate day 2 (X) at 16 kHz.

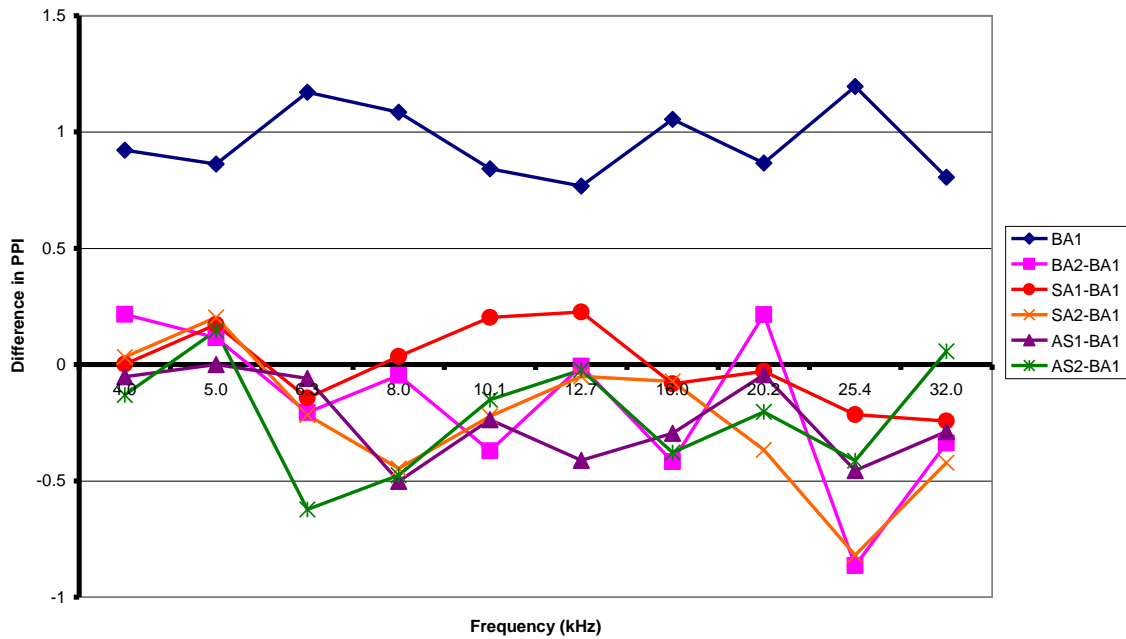


Figure 14b. Differences in PPI measurements for Rat 2 in the presence of a 60 dB SPL background stimulus seen when each subsequent day after Baseline day 1 is subtracted from baseline. Differences of zero indicate no change, whereas positive differences indicate a decrease in PPI and negative differences indicate an increase in PPI. The only clear affect of salicylate is seen for Salicylate day 1 (circle) at 10 and 12 kHz, however, a clear indication of baseline PPI (diamond) is not evident.

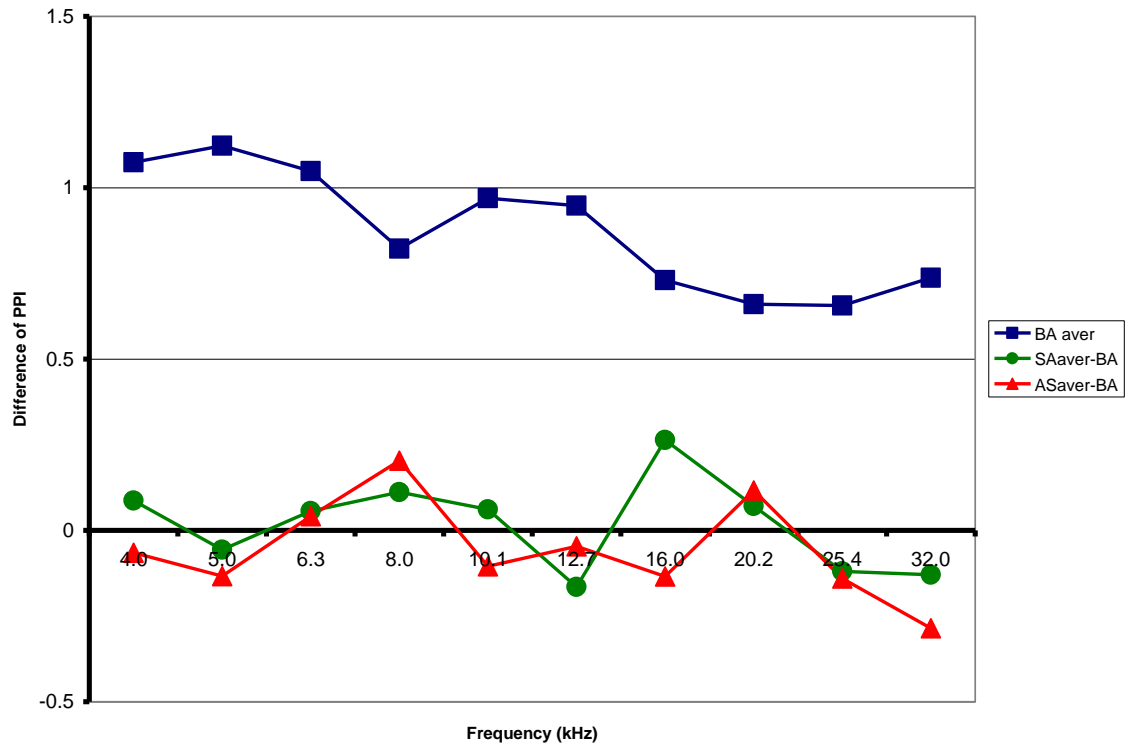


Figure 15a. Differences in averaged PPI measurements for Rat 1 in the presence of a 48 dB SPL background stimulus seen when each subsequent condition following baseline is subtracted from baseline. Differences of zero indicate no change, whereas positive differences (above zero) indicate a decrease in PPI and negative differences (below zero) indicate an increase in PPI. There is a clear indication of baseline PPI (square) at 16 kHz and above, therefore this rat was included in statistical analysis for 48 dB. Additionally, there is a strong indication of a salicylate affect (circle) at 16 kHz.

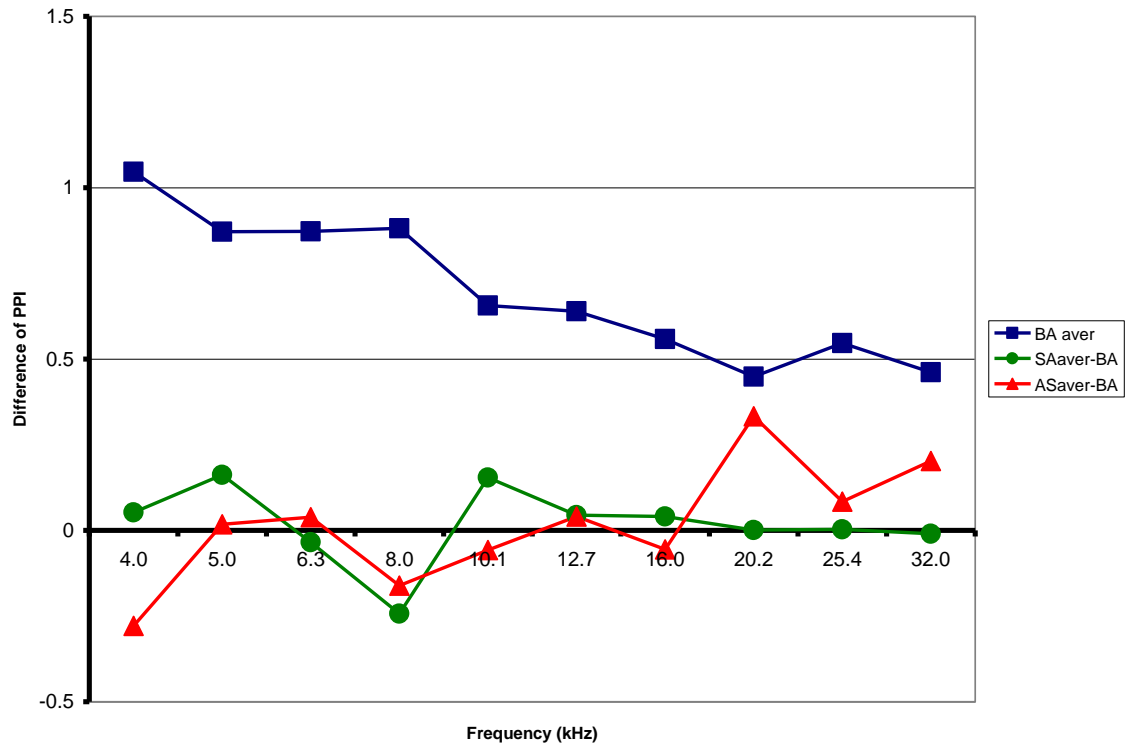


Figure 15b. Differences in averaged PPI measurements for Rat 1 in the presence of a 60 dB SPL background stimulus seen when each subsequent condition following baseline is subtracted from baseline. Differences of zero indicate no change, whereas positive differences (above zero) indicate a decrease in PPI and negative differences (below zero) indicate an increase in PPI. Baseline PPI (square) is very strong across all frequencies except 4 kHz. This baseline PPI qualifies this rat for inclusion in statistical analysis; however, salicylate (circle) affects are negligible.

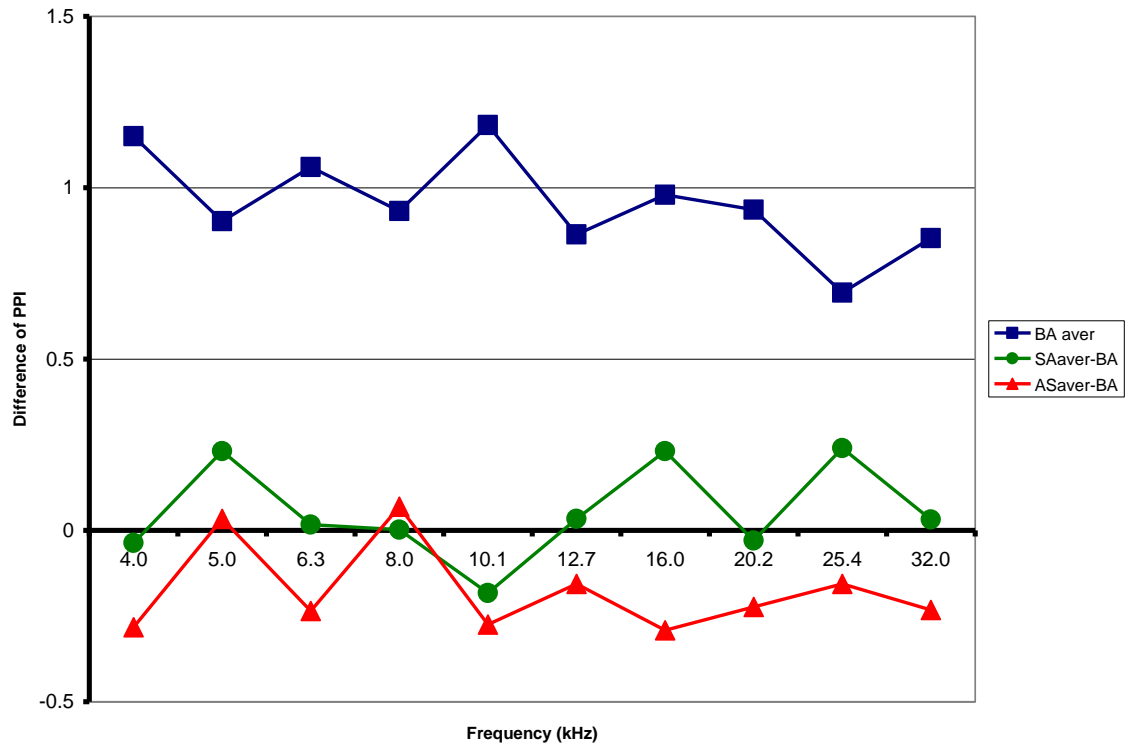


Figure 16a. Differences in averaged PPI measurements for Rat 2 in the presence of a 48 dB SPL background stimulus seen when each subsequent condition following baseline is subtracted from baseline. Differences of zero indicate no change, whereas positive differences (above zero) indicate a decrease in PPI and negative differences (below zero) indicate an increase in PPI. Baseline (square) PPI is minor, therefore this rat was omitted from subsequent analysis for 48 dB.

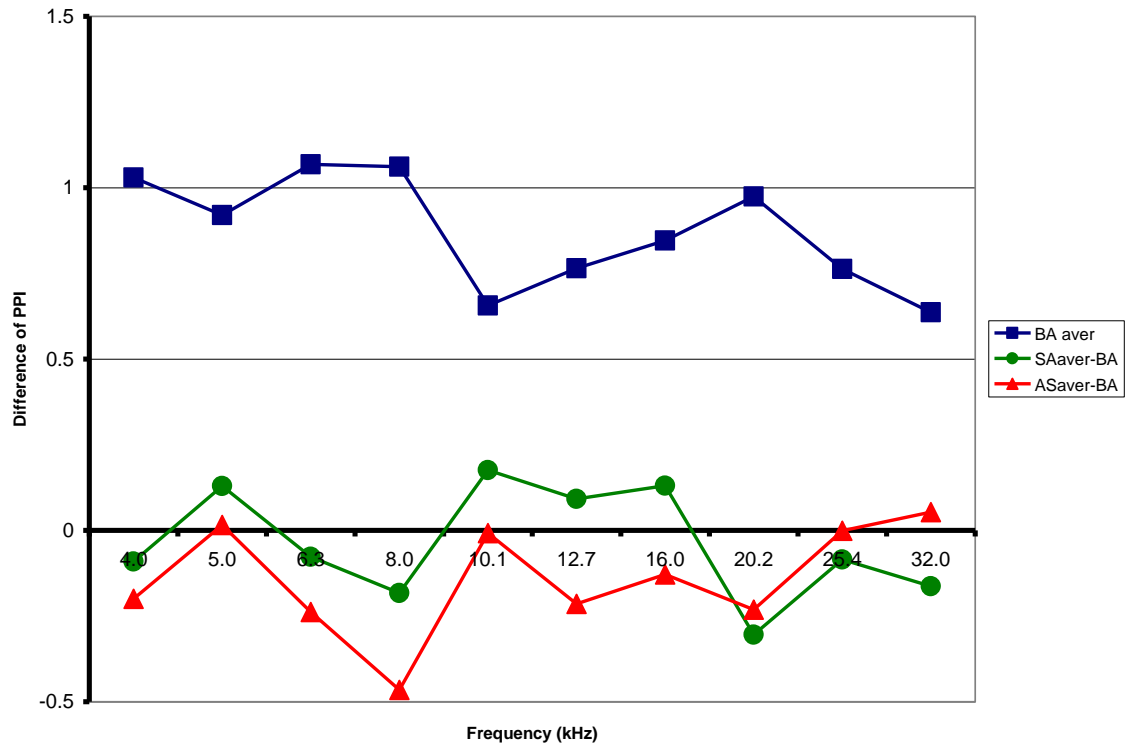


Figure 16b. Differences in averaged PPI measurements for Rat 2 in the presence of a 60 dB SPL background stimulus seen when each subsequent condition following baseline is subtracted from baseline. Differences of zero indicate no change, whereas positive differences (above zero) indicate a decrease in PPI and negative differences (below zero) indicate an increase in PPI. PPI baseline (square) presence is strong over 10 kHz (with the exception of 20.2 kHz), hence, this rat was included in subsequent analysis for 60 dB.

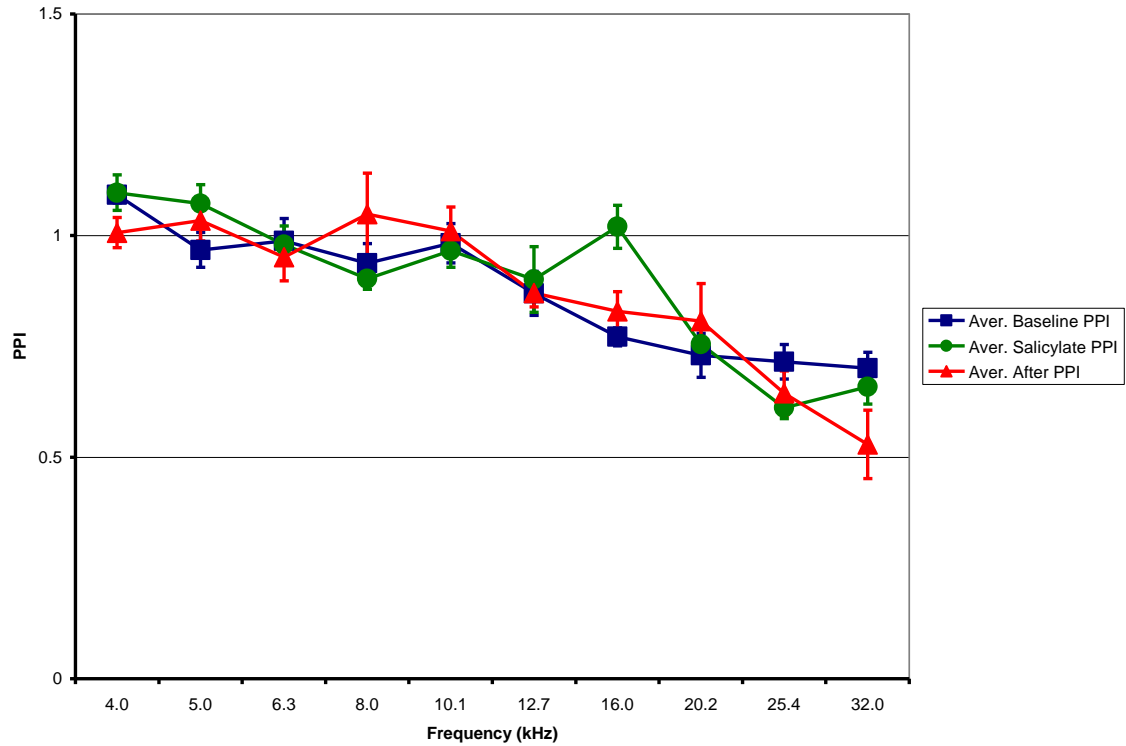


Figure 17a. Average PPI responses seen over the course of the three testing conditions (Baseline [square], Salicylate [circle], After Salicylate [triangle]) in the presence of a 48 dB SPL background stimulus for rats selected to be included in final data analysis. Baseline PPI is strong for 12.7 kHz and above. Additionally, there is a very clear indication of a positive salicylate affect at 16 kHz.

When all data was examined using a repeated measure ANOVA for the 48 dB SPL background level, there were significant differences in PPI seen for Frequency ($F(9,63) = 33.579, p < .000$) and Block ($F(3,21) = 4.225, p < .017$), as well as for the interactions of Condition x Day x Block ($F(6,42) = 2.778, p < .023$), Frequency x Block ($F(27,189) = 1.963, p < .005$), and Condition x Frequency x Block ($F(54,378) = 2.045, p < .000$). All other variables and interactions were not significant (Table 6). When data was collapsed to examine overall trends (Table 7), a repeated measure ANOVA was calculated to examine the effects of Condition (baseline, salicylate, after salicylate) and Frequency (4-32 kHz) on the behavioral response of PPI. The main effect for frequency was highly significant ($F(9,72) = 36.253, p < .000$), while the main effect of condition was found not significant ($F(2,16) = .244, p > .05$). A significant Frequency x Condition interaction was, however, present ($F(18,144) = 2.098, p < .009$) indicating various effects of conditions for different frequencies. Post-hoc paired T-Student tests were conducted and revealed that the administration of salicylate had a significant affect over baseline measurements at 16 kHz with a decrease of PPI (i.e., values closer to 1) ($t = -4.175, df = 8, p < .003$). Significant findings were also present at 25 kHz ($t = 2.672, df = 8, p < .05$), however, upon inspection of Figure 17a, it is seen that this significant finding is due to an increase in PPI (i.e., values farther from 1) rather than a decrease in PPI as should be seen with the administration of salicylate. All post-hoc T- Student test results are presented in Table 8 for the interaction of the baseline condition and the salicylate condition.

Table 6. ANOVA with repetitions for PPI in the 48 dB SPL background narrow band noise centered on range of frequencies.

Variables	F Sat.	df	p value
Condition	.085	2/14	.919
Day	1.043	1/7	.314
Frequency*	33.579	9/63	.000
Block*	4.225	3/21	.017
CxD	3.325	2/14	.060
CxF	1.549	18/126	.084
DxF	1.001	9/63	.449
CxDxF	.556	18/126	.924
CxB	2.815	6/42	.063
DxB	.585	3/21	.632
CxDxB*	2.778	6/42	.023
FxB*	1.963	27/189	.005
CxFxB*	2.045	54/378	.000
DxFxB	.840	27/189	.695
CxDxFxB	.850	54/378	.764

Variables: Condition (baseline/salicylate/after salicylate), Day, Frequency, Block, x (interaction between variable(s)).

Table 7. ANOVA with repetitions for PPI evoked by a gap in presence of the 48 dB SPL background stimulus – data collapsed.

Variables	F stat.	df	p value
Frequency*	36.253	9/72	.000
Condition	.244	2/16	.786
FxC*	2.098	18/144	.009

Variables: Frequency, Condition (baseline/salicylate/after salicylate), x (interaction between variable(s)).

Table 8. Post-hoc paired T-student tests for PPI in the presence of a 48 dB SPL background sound: comparisons between baseline condition (B) versus salicylate condition (S).

Variables	T stat.	df	p value
B4-S4	-.134	8	.896
B5-S5	-1.455	8	.184
B6-S6	.216	8	.834
B8-S8	.862	8	.414
B10-S10	.240	8	.816
B12-S12	-.355	8	.732
B16-S16*	-4.175	8	.003
B20-S20	-.340	8	.742
B25-S25*	2.672	8	.028
B32-S32	1.017	8	.339

Each frequency tested is indicated by a number after the letter denoting the condition. T statistics and degrees of freedom (df) are marked with an asterisk in this and subsequent tables.

Significant findings for 16 kHz were also seen between the salicylate condition and the after salicylate condition ($t = 3.140$, $df = 8$, $p < .014$). All results for this analysis are presented in Table 9. Additional post-hoc analysis showed that the difference between baseline PPI and after salicylate PPI was not significant for any of the frequencies (Table 10), indicating that PPI measurements returned to near baseline levels following the experimental manipulation.

In contrast to Figure 17a, Figure 17b, which shows the same set results but for the 60 dB SPL intensity, indicates no decrease of PPI for the salicylate versus baseline conditions. Alternatively, PPI appears to increase at almost all of the frequencies. Repeated ANOVA for all original data, as shown in Table 11, for the 60 dB SPL background level shows a high level of significance for Condition ($F(2,18) = 4.602$, $p < .024$), and Frequency ($F(9,81) = 23.636$, $p < .000$), as well as for the interactions of Condition x Day ($F(2,18) = 5.008$, $p < .019$), Condition x Frequency ($F(18,162) = .342$, $p < .000$), Condition x Day x Frequency ($F(18,162) = 2.683$, $p < .001$), Condition x Block ($F(6, 54) = 3.293$, $p < .008$), Condition x Day x Block ($F(6,54) = 3.145$, $p < .010$), Frequency x Block ($F(27,243) = 1.963$, $p < .004$), Condition x Frequency x Block ($F(54,486) = 1.363$, $p = .05$), Day x Frequency x Block ($F(27,243) = 1.649$, $p < .026$), and Condition x Day x Frequency x Block ($F(54,486) = 1.634$, $p < .004$). All other variables and interactions were found to be not significant. Based upon these results, data was collapsed, and repeated ANOVA for 60 dB SPL background shows significance for the main effects of Frequency, Condition ($F(2,16) = p < .019$), and for Frequency x Condition interaction ($F(18,144) = 2.920$, $p < .000$) (Table 12). Post-hoc tests for paired T-Student

Table 9. Post-hoc paired T-student tests for PPI in the presence of a 48 dB SPL background sound: Comparisons for salicylate condition (S) versus after salicylate condition (A) for each frequency tested.

Variables	T stat.	df	p value
S4-A4	1.381	8	.205
S5-A5	.612	8	.557
S6-A6	.352	8	.734
S8-A8	-1.50	8	.172
S10-A10	-.686	8	.512
S12-A12	-.126	8	.903
S16-A16*	-4.175	8	.014
S20-A20	-4.63	8	.656
S25-A25	-.560	8	.591
S32-A32	1.50	8	.172

Table 10. Post-hoc paired T-student tests for PPI in the presence of a 48 dB SPL background sound: Comparisons for baseline condition (B) versus after salicylate condition (A) for each frequency tested.

Variables	T stat.	df	p value
B4-A4	2.104	8	.069
B5-A5	-1.43	8	.191
B6-A6	.431	8	.678
B8-A8	-.961	8	.365
B10-A10	-.316	8	.760
B12-A12	-.860	8	.415
B16-A16	-1.307	8	.228
B20-A20	-.865	8	.412
B25-A25	1.993	8	.081
B32-A32	1,972	8	.084

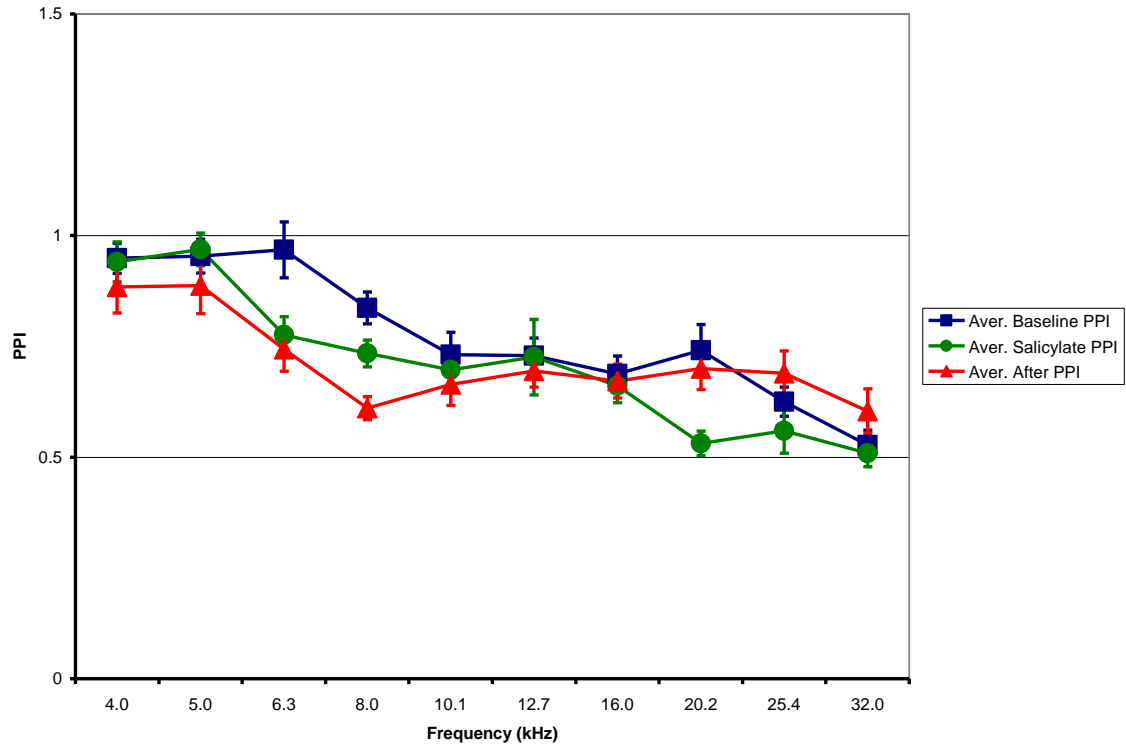


Figure 17b. Average PPI responses seen over the course of the three testing conditions (Baseline [square], Salicylate [circle], After Salicylate [triangle]) in the presence of a 60 dB SPL background stimulus for rats selected to be included in final data analysis.

Table 11. ANOVA with repetitions for PPI in the 60 dB SPL background narrow band noise centered on range of frequencies.

Variables	F Sat.	df	p value
Condition*	4.602	2/18	.024
Day	2.809	1/9	.128
Frequency*	23.636	9/81	.000
Block	.437	3/27	.728
CxD*	5.008	2/18	.019
CxF*	.342	18/162	.000
DxF	1.910	9/81	.062
CxDxF*	2.683	18/162	.001
CxB*	3.293	6/54	.008
DxB	1.789	3/27	.173
CxDxB*	3.145	6/54	.010
FxB*	1.963	27/243	.004
CxFxB*	2.045	54/378	.05
DxFxB*	1.649	27/243	.026
CxDxFxB*	1.634	54/486	.004

Variables: Condition (baseline/salicylate/after salicylate), Day, Frequency, Block, x (interaction between variable(s)).

Table 12. ANOVA with repetitions for PPI evoked by a gap in the presence of a 60 dB SPL background stimulus – data collapsed.

Variables	F stat.	df	p value
Frequency*	27.018	9/72	.000
Condition*	5.118	2/16	.019
FxC*	2.920	18/144	.000

Variables: Frequency, Condition (baseline/salicylate/after salicylate), x (interaction between variable(s)).

tests were conducted and found that the administration of salicylate had a significant affect over baseline measurements at 6 kHz ($t = 3.497$, $df = 8$, $p < .008$) and 20 kHz ($t = 4.390$, $df = 8$, $p < .002$). However, upon inspection of the raw data (Figure 17b), it is observed that this change in PPI is a result of an increase rather than a decrease of PPI. All statistical interactions for baseline and salicylate for 60 dB SPL are presented in Table 13. Additional statistical analysis is shown for the interactions between salicylate versus after salicylate (Table 14) and baseline versus after salicylate (Table 15).

The same results for difference of PPI between the various conditions are presented as a difference from baseline condition in Figures 18a and 18b, which show the same trends. It is interesting to note that these trends appear even more distinct, especially for the 60 dB SPL stimulus. As is seen in Figure 18b, it appears that as testing progressed in time, not only did PPI not decrease, but it remained stable for the salicylate conditions (as evidenced by the difference values of 0), and increased for the after salicylate conditions (difference values in the negative range). Baseline PPI is clear for 8 kHz and above; however, there is no indication of a decrease of PPI due to salicylate. Rather, PPI appears to increase for most frequencies with the administration of salicylate.

ABR Results

ABR measures were conducted to monitor the hearing thresholds of the rats both prior to and with the influence of salicylate. ABR testing consisted of tone bursts from 4k to 20k Hz in 1/3 octave steps. Results showed that for individual rats, ABR responses were systematically lower with the presence of salicylate (Figures 19a and 19b), as well as when examined as a group (Figure 20).

Table 13. Post-hoc paired T-student tests for PPI in the presence of a 60 dB SPL

background stimulus: Comparisons for baseline condition (B) versus salicylate condition

(S) for each frequency tested.

Variables	T stat.	df	p value
B4-S4	1.145	8	.285
B5-S5	-.195	8	.850
B6-S6*	3.497	8	.008
B8-S8	2.126	8	.066
B10-S10	.798	8	.448
B12-S12	.976	8	.358
B16-S16	.994	8	.349
B20-S20*	4.390	8	.002
B25-S25	1.628	8	.142
B32-S32	.357	8	.730

Table 14. Post-hoc paired T-student tests for PPI evoked by a gap in the presence of a 60 dB SPL background stimulus: Comparisons for salicylate condition (S) versus after salicylate condition (A) for each frequency tested.

Variables	T stat.	df	p value
S4-A4	.276	8	.789
S5-A5	.994	8	.349
S6-A6	.478	8	.645
S8-A8*	2.763	8	.025
S10-A10	-.067	8	.948
S12-A12	-.618	8	.554
S16-A16	-.174	8	.866
S20-A20*	-3.246	8	.012
S25-A25	-2.136	8	.065
S32-A32	-1.579	8	.153

Table 15. Post-hoc paired T-student tests for PPI in the presence of a 60 dB SPL background stimulus: Comparisons for baseline condition (B) versus after salicylate condition (A) for each frequency tested.

Variables	T stat.	df	p value
B4-A4	.668	8	.523
B5-A5	.920	8	.384
B6-A6*	3.509	8	.008
B8-A8*	7.206	8	.000
B10-A10	.817	8	.438
B12-A12	.272	8	.793
B16-A16	.630	8	.546
B20-A20	.582	8	.577
B25-A25	-.934	8	.378
B32-A32	-1.798	8	.110

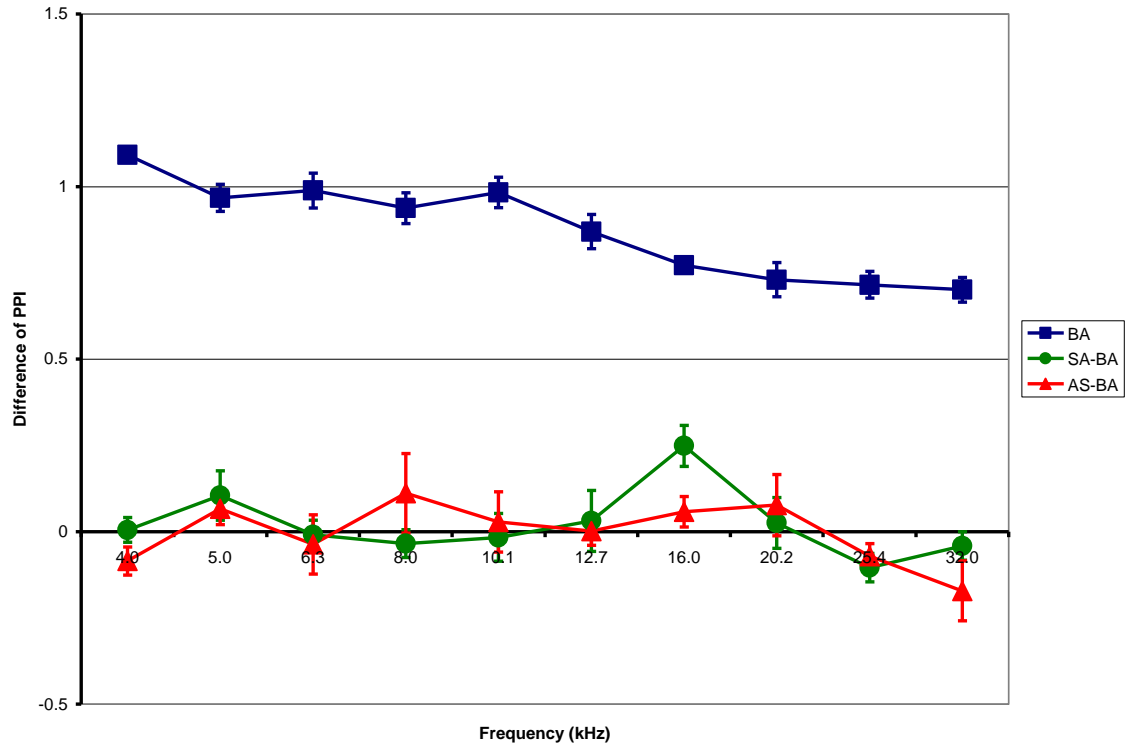


Figure 18a. Differences in averaged PPI measurements for all rats in the presence of a 48 dB SPL background stimulus that were selected to be included in the final data analysis seen when each subsequent condition after baseline is subtracted from baseline. Differences of zero indicate no change, whereas positive differences (above zero) indicate a decrease in PPI and negative differences (below zero) indicate an increase in PPI. Like Figure 16a, there is a clear indication of a salicylate affect at 16 kHz.

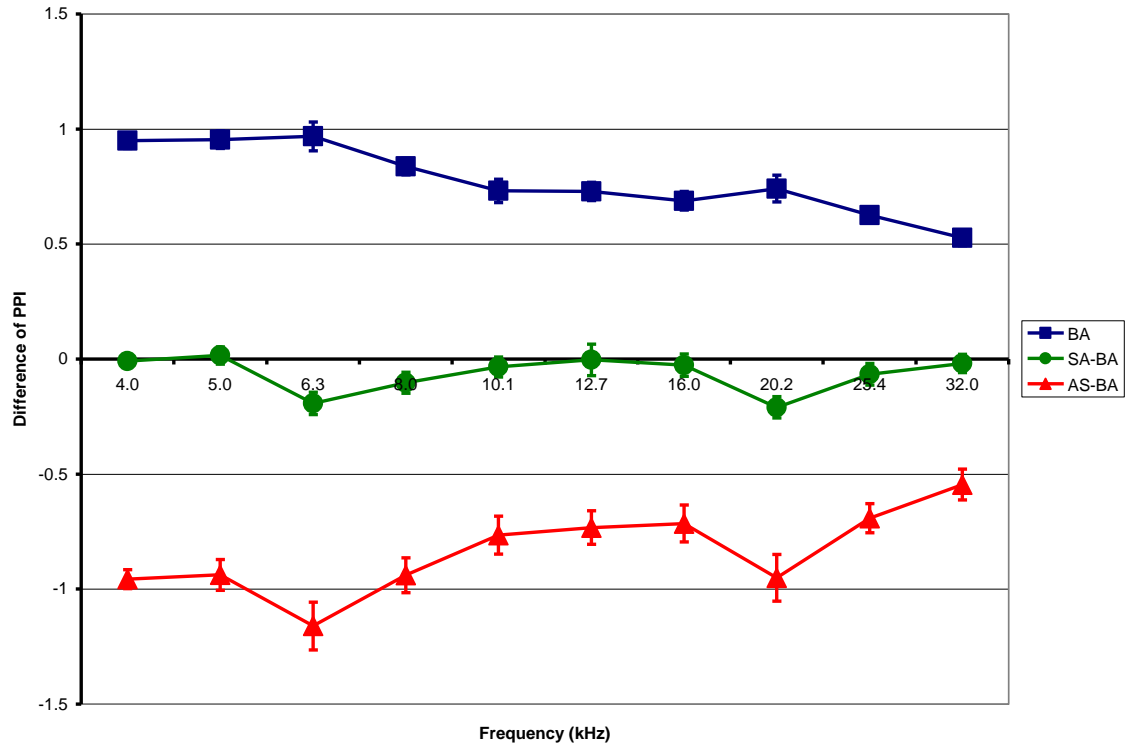


Figure 18b. Differences in averaged PPI measurements for all rats in the presence of a 60 dB SPL background stimulus that were selected to be included in the final data analysis seen when each subsequent condition after baseline is subtracted from baseline. Differences of zero indicate no change, whereas positive differences (above zero) indicate a decrease in PPI and negative differences (below zero) indicate an increase in PPI. While baseline PPI (square) is strong, PPI was not affected at all by salicylate (circle) and increased as testing continued through the last condition (triangle).

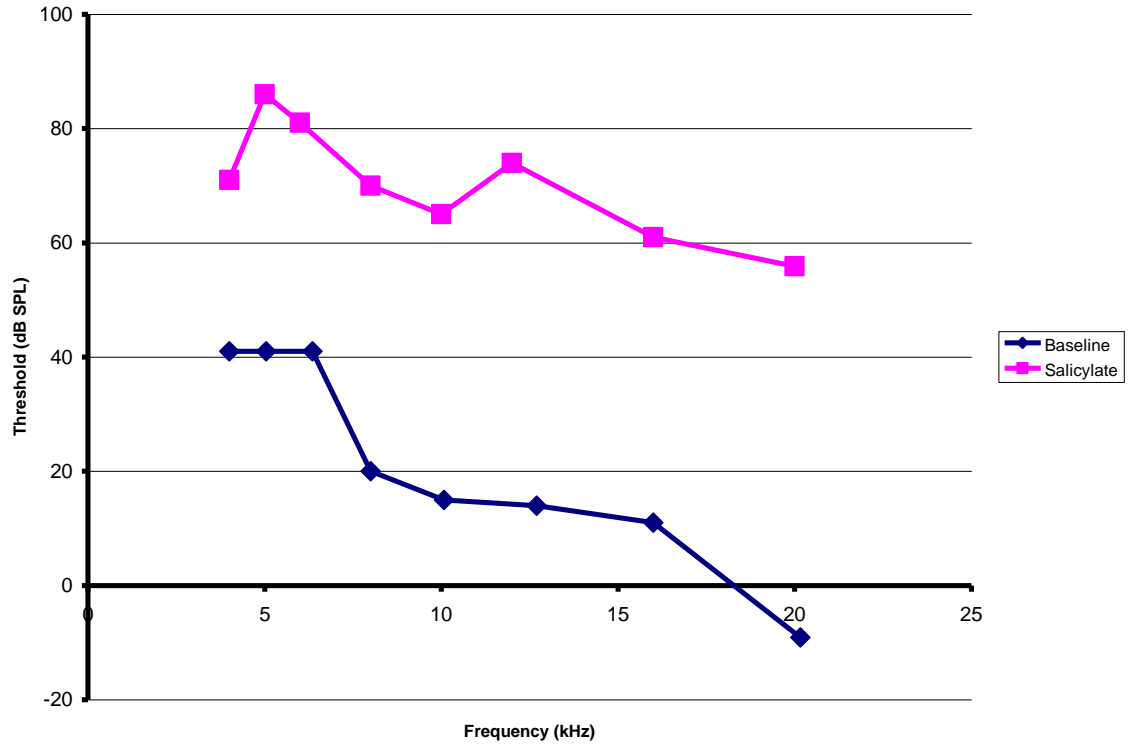


Figure 19a. ABR thresholds for Rat 1 measured before salicylate (diamonds) and with salicylate (squares). Thresholds are frequency dependant in that rats have better hearing in the higher frequencies verses the lower frequencies. It is noted that salicylate affected thresholds at all frequencies due to thresholds being higher.

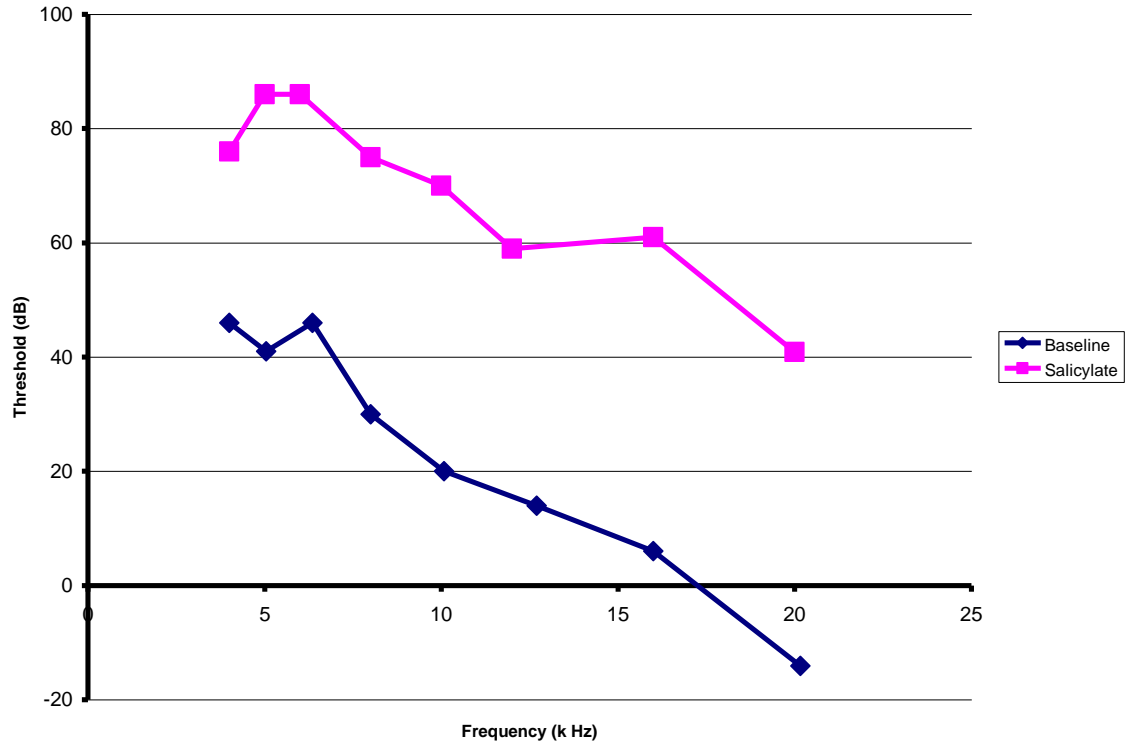


Figure 19b. ABR thresholds for Rat 2 measured before salicylate (diamonds) and with salicylate (squares). Thresholds are frequency dependant in that rats have better hearing in the higher frequencies verses the lower frequencies. In comparison with rat 1, rat 2's thresholds are quite similar, yet some variability is present.

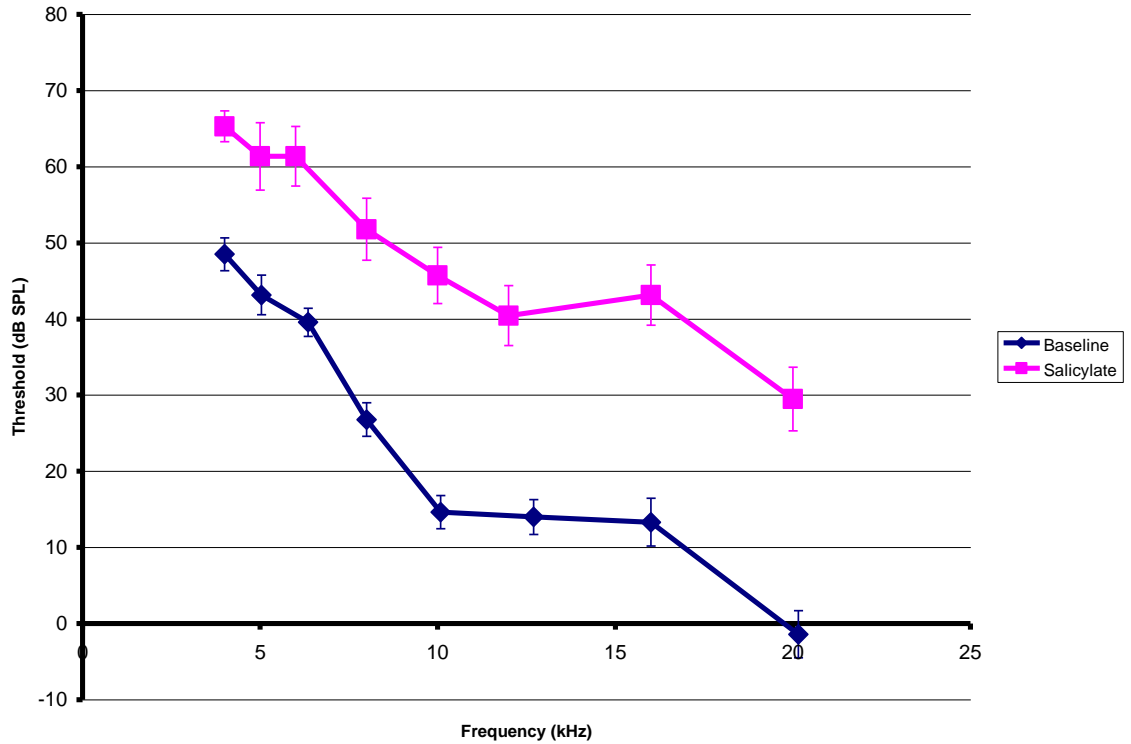


Figure 20. ABR thresholds for all rats measured before salicylate (diamonds) and with salicylate (squares). When all rats are seen together, the baseline tracing and salicylate tracing follow a similar pattern, with the baseline thresholds being lower than salicylate thresholds, indicating that salicylate systematically affected ABR thresholds at all test frequencies.

Repeated measure ANOVA showed a significant level for the main effects of Condition ($F(1,12) = 54.357, p < .000$), Frequency ($F(7,84) = 10.128, p < .000$), and for the interaction of Condition x Frequency ($F(7,84) = 5.213, p < .000$). Post-hoc paired T-Student tests for all data showed both conditions (baseline and with salicylate) statistically significant at all frequencies. Tables 16 and 17 show all ABR statistical findings. However, statistical analysis did not show a high level of correlation for the 48 dB SPL condition between the change seen in PPI and the change in ABR thresholds for 16 kHz, the frequency which was significant for a change in PPI for this background level ($R^2 = .2553, df = 6, p > .05$) as seen in Figure 21.

DPOAE Results

DPOAE testing was conducted to monitor the functional status of the outer hair cells before and during the administration of salicylate. DPOAEs were measured for the frequency range of f_2 from 4 to 20 kHz, 6 points per octave, and primary levels of 65 and 55 dB SPL, with a f_1/f_2 ratio of 1.2. Similar to ABR results, DPOAE levels decreased from the baseline measurement to when they were measured with the influences of salicylate for both individual animals (Figures 22a and 22b) and for the group as a whole (Figure 23). Repeated measures ANOVA showed significance for both the main effects of Condition ($F(1,12) = 27.274, p < .000$) and Frequency ($F(12,144) = 8.475, p < .000$), but no significance for their interaction ($F(12,144) = 1.475, p > .05$) (Table 18). Moreover, post-hoc paired T-Student tests showed a high level of significance for the baseline measurement versus the salicylate measurement for all frequencies except 15984 Hz, which are presented in Table 19. Additionally, statistical analysis did not reveal a high

Table 16. ANOVA with repetitions for ABR thresholds before salicylate administration and with salicylate.

Variables	F stat.	df	p value
Frequency*	10.128	7/84	.000
Condition*	54.357	1/12	.000
CxF	5.213	7/84	.000

Variables: Condition (baseline/after salicylate), Frequency, x (interaction between variable(s)).

Table 17. Post-hoc paired T-student tests for ABR thresholds before salicylate (B) administration versus with salicylate (S).

Variables	T stat.	df	p value
B4-S4*	5.524	13	.000
B5-S5*	3.517	13	.004
B6-S6*	6.523	13	.000
B8-S8*	5.784	13	.000
B10-S10*	10.140	13	.000
B12-S12*	6.433	13	.000
B16-S16*	6.225	13	.000
B20-S20*	6.861	13	.000

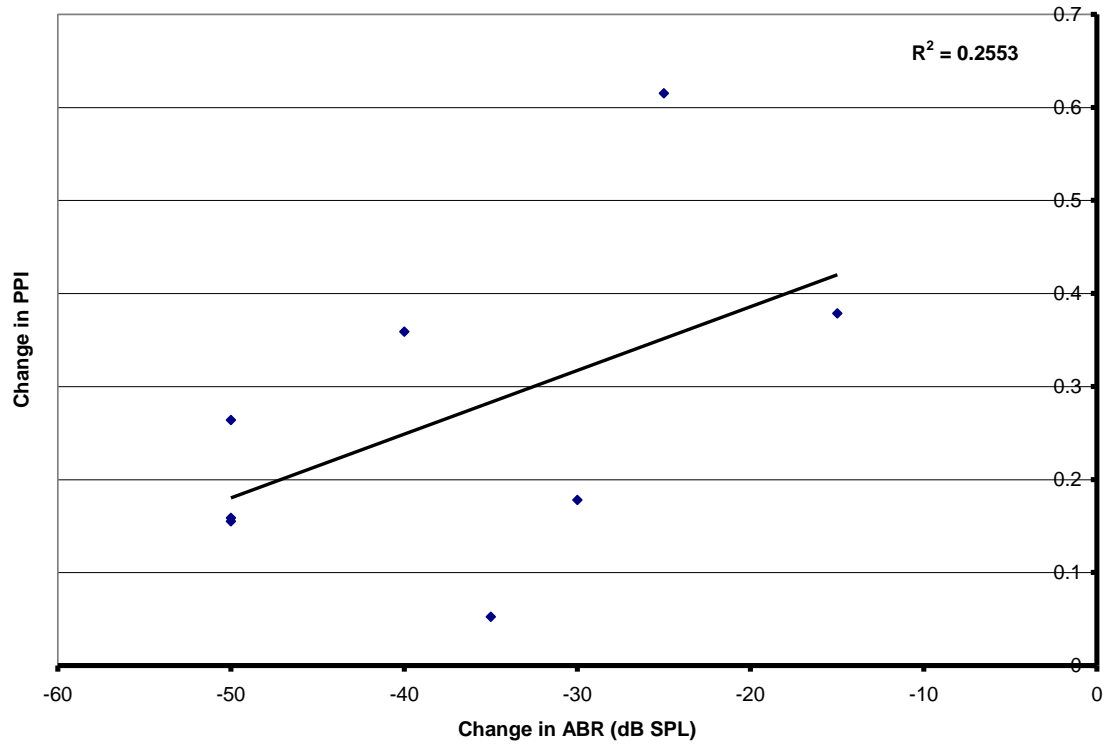


Figure 21. Correlation between the change in ABR and PPI (48 dB SPL background condition) for 16 kHz. The level of correlation for these two conditions is not significant ($R^2=.2553$, $df=6$, $p>.05$).

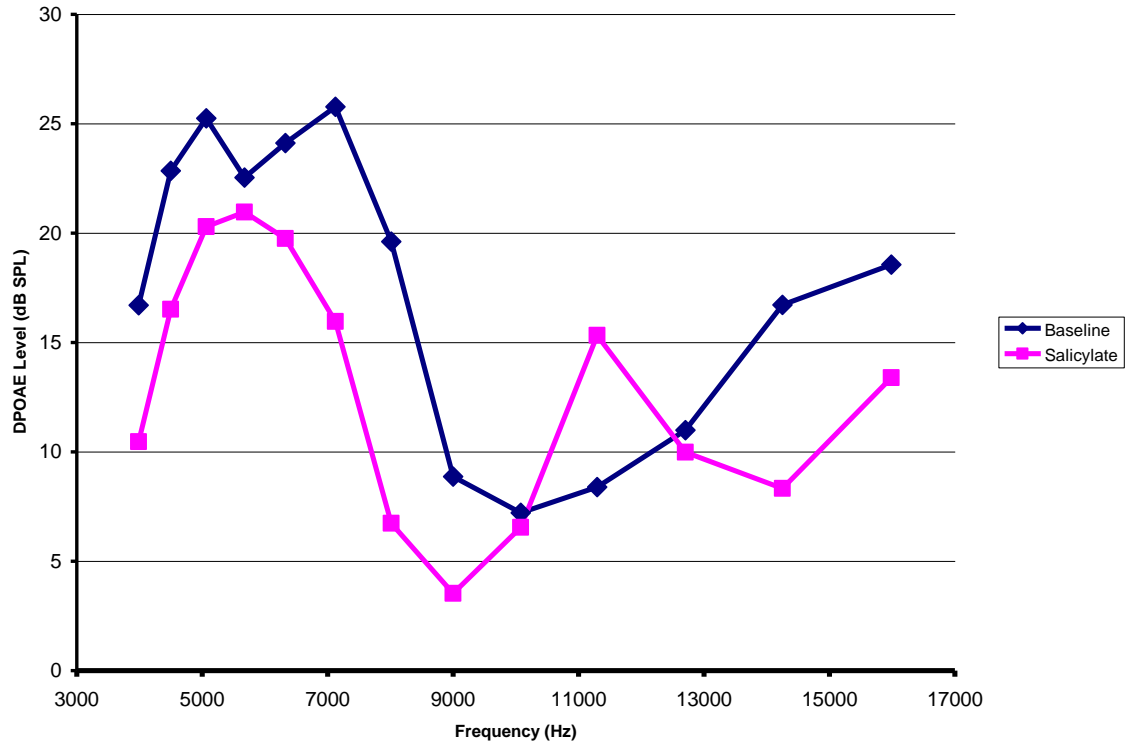


Figure 22a. DPOAE levels as measured in Rat 1 at baseline (diamonds) and during administration of salicylate (squares). DPOAE levels are higher at all frequencies except 11 kHz for baseline measures, indicating greater outer hair cell function at baseline versus with salicylate.

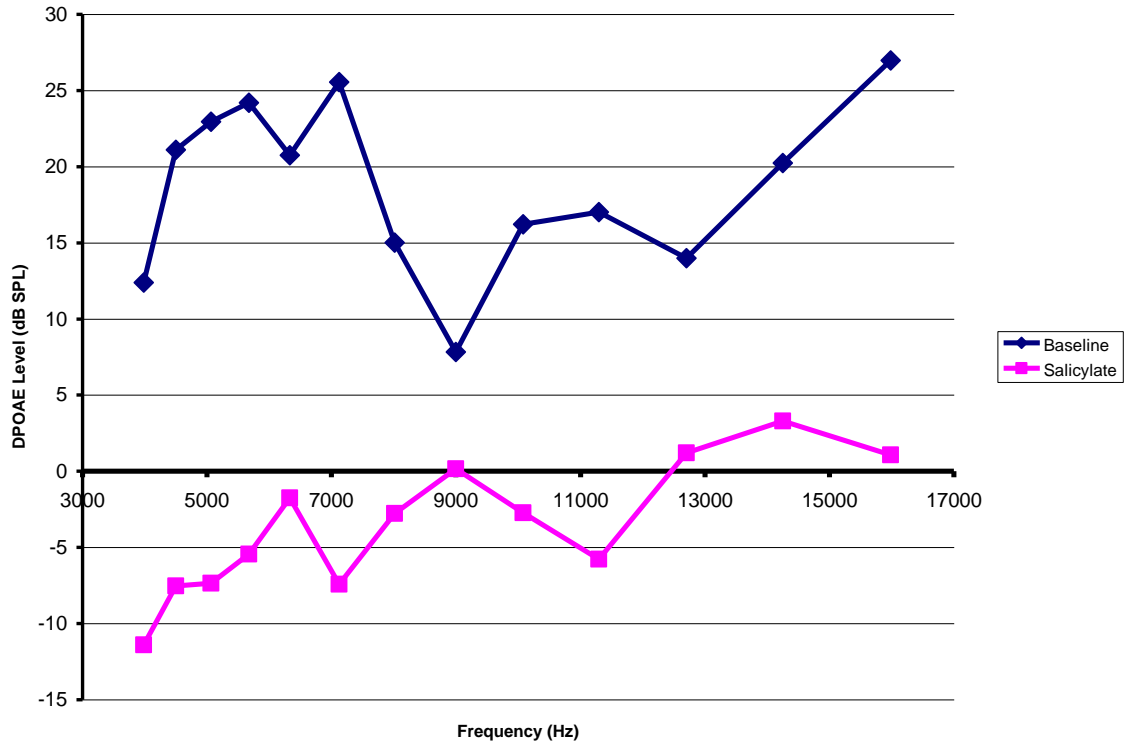


Figure 22b. DPOAE levels as measured in Rat 2 at baseline (diamonds) and during administration of salicylate (squares). DPOAE levels are higher at all frequencies, indicating greater outer hair cell function at baseline versus with salicylate. Note the vast amount of variability for rat 1 versus rat 2.

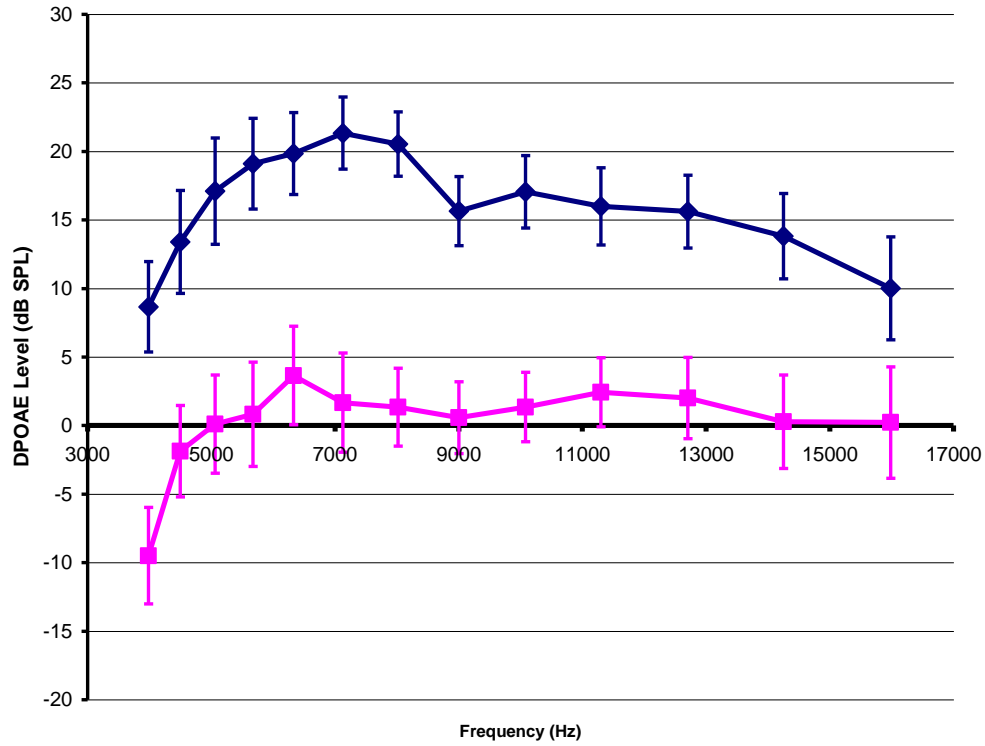


Figure 23. DPOAE levels as measured in all rats at baseline (diamonds) and during administration of salicylate (squares). DPOAE levels are higher at all frequencies, indicating greater outer hair cell function at baseline versus with salicylate.

Table 18. ANOVA with repetitions for DPOAE measures before salicylate and with salicylate.

Variables	F stat.	df	p value
Frequency*	8.475	12/144	.000
Condition*	27.274	1/12	.000
CxF	1.475	12/144	.140

Variables: Condition (baseline/after salicylate), Frequency, x (interaction between variable(s)).

Table 19. Post-hoc paired T-student tests for DPOAE measures before salicylate (BA) and with salicylate (SA).

Variables	T stat.	df	p value
BA 15984-SA 15984	1.987	12	.070
BA 14250-SA 14250	4.667	12	.001
BA 12703-SA 12703	4.556	12	.001
BA 11296-SA 11296	3.406	12	.005
BA 10078-SA 10078	5.068	12	.000
BA 9000-SA 9000	5.093	12	.000
BA 8015-SA 8015	5.973	12	.000
BA 7125-SA 7125	4.654	12	.001
BA 6328-SA 6328	4.233	12	.001
BA 5671-SA 5671	4.212	12	.001
BA 5062-SA 5062	4.429	12	.001
BA 4500-SA 4500	5.078	12	.000
BA 3984-SA 3984	4.993	12	.000

level of correlation ($R^2=.3726$, $df= 4$, $p>.05$) between the change seen in PPI and the change in DPOAEs for 16 kHz (Figure 24).

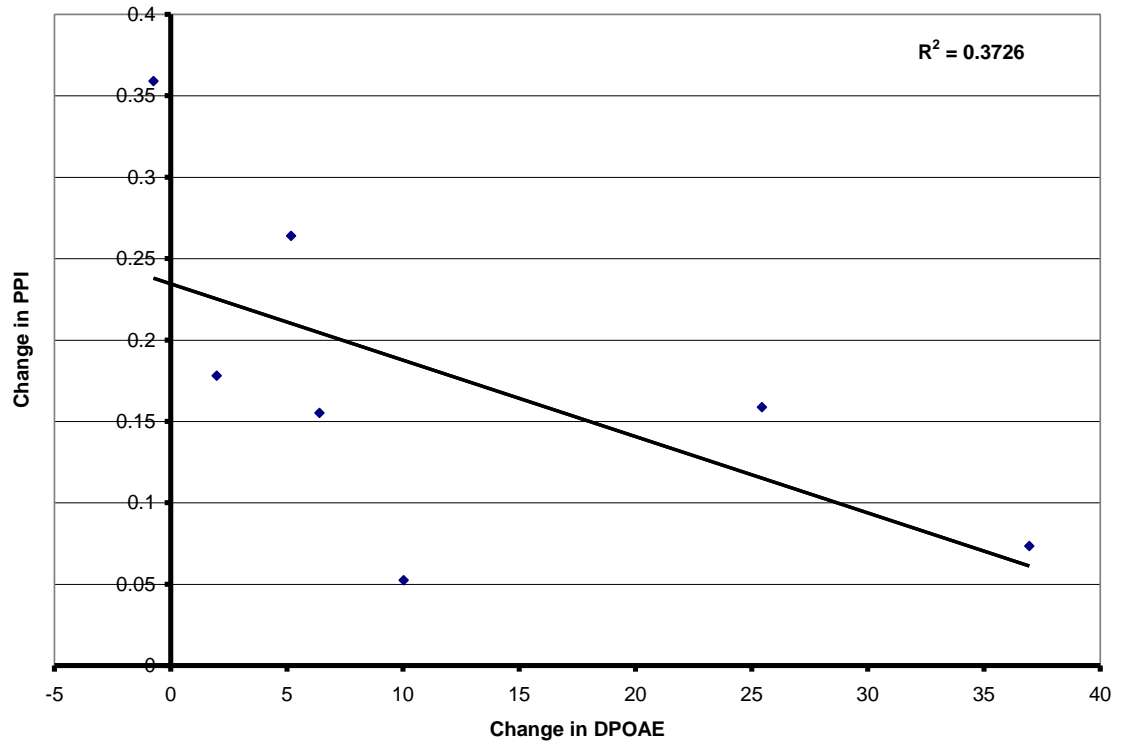


Figure 24. Correlation between the change in DPOAEs and PPI (48 dB SPL background condition) for 16 kHz. The level of correlation for these two conditions is not significant ($R^2=.3726$, $df=4$, $p>.05$).

CHAPTER 5

DISCUSSION

The goal of this investigation was to estimate the pitch of salicylate induced tinnitus in a group of Long-Evans pigmented rats utilizing a pre pulse inhibition (PPI) (or gap) model (Turner et al., 2006). In general, the PPI method utilizes the observation that when a weak stimulus is presented prior to a stronger sound that evokes a startle reaction, then this reaction will be decreased. In the case of tinnitus, it is thought that a short gap embedded in a continuous low level (e.g. 60 dB SPL) narrow band noise can be used as a pre-pulse stimulus. When tinnitus is introduced or present, the gap gets partially “filled” by the noise heard by the subject (tinnitus) and thus the inhibitory effect of the gap is decreased. Consequently, the startle reaction becomes closer to the PPI without a gap present. In other words, PPI decreases. Tinnitus evoked change of PPI should occur for the frequency of the continuous noise level which is the closest to the pitch of tinnitus as perceived by the rats. Two different background sound levels were used in this work in attempt to increase the sensitivity of the paradigm.

It was expected that the salicylate induced PPI change would be found in the range of 12-16 kHz, as reported previously (Brennan & Jastreboff, 1991; Turner et al., 2006), i.e., larger PPI would be seen at frequencies far from the tinnitus pitch. It was expected as well that the lower background stimulus level of 48 dB SPL would yield a more precise PPI curve than the 60 dB SPL background stimulus.

A number of observations can be made based on our results. First, there is a large variability between the rats in their responses, as well as from day to day for a given rat. This was seen based upon initial startle responses and when comparing the individual test days with one another. The high variability suggests the need for increasing the number of days for a given experimental condition from two to at least three days, as well as keeping at least 16 animals in the experimental group.

Second, there was a systematic dependence of startle reaction (alone and with a gap) on the frequency of the background stimulus. In general, the PPI responses of the rats showed a lack of PPI for the lower frequencies, which might reflect the increased threshold of hearing for these lower frequencies. This suggests that the background sound may be more effective if presented at a constant sensation level (SL) rather than a constant sound pressure level (SPL) as it was done in the current protocol. Overall, low values of PPI were observed during not only baseline measures, but at additional testing days as well, which indicates the need to modify parameters of the paradigm to achieve higher PPI. Methodological manipulations can be made to the level of the startle stimulus and background sound. Checking the presence of statistically significant PPI at the baseline would allow for the selection of animals exhibiting sufficient PPI.

Third, various responses seen for the different levels of background noise support the initial prediction that the level of background noise plays an important role in the utilization of the gap method for tinnitus detection. Lastly, there was a clear indication of decreased PPI for 16 kHz for the 48 dB SPL background stimulus as compared to baseline, which is consistent with the postulate of the presence of salicylate evoked tinnitus with pitch corresponding to this frequency.

There were some unexpected results seen from this investigation as well. For example, there were a number of frequencies that showed a significant difference in the presence of the 60 dB SPL background level for baseline versus PPI when animals were injected with salicylate. Yet, when the data were examined for these frequencies, the difference in PPI was due to an increase and not, as expected, to a PPI decrease. There is a possibility that salicylate-induced hearing loss resulted in a shift of PPI in the opposite direction than expected from tinnitus presence due to unknown factors. Indeed, salicylate increased the general level of startle reaction (Figure 11b) and increased PPI (Figure 16b). These trends were seen in parallel experiments aimed at the estimation of tinnitus loudness (Alper, 2008) and at the determination of threshold of hearing (Nguyen, 2008). Unlike previously reported results using this same general method (Turner et al., 2006), the 60 dB SPL background level did not prove to be a good measure for the presence of salicylate induced tinnitus. Rather, the 48 dB SPL background condition proved to be more sensitive for the potential manifestation of salicylate induced tinnitus. While an attempt was made to closely follow the method proposed by Turner et al., there is a possibility that small changes in the parameters of startle and background noise may have influenced PPI.

Of particular concern is that for a number of animals, there was a very small value of PPI seen during both baseline measures and subsequent testing days. This strongly suggests the need to modify the methodology to increase PPI. Large fluctuations from day to day and lack of significant correlation of PPI values from one day to another suggest the need to increase the number of testing days. A further modification to reduce variability could be to increase the number of repetitions per block from four to eight.

Doubling the number of repetitions should decrease the variability by a factor of 1.41 (square root of 2).

In conclusion, the use of the PPI model of animal tinnitus has proven to be successful in detecting the presence of salicylate evoked tinnitus this and previous investigative endeavors (Turner et al., 2006). The optimal parameters for the detection of tinnitus were, however, different. Further investigations using this model should aim at increasing the sensitivity of the method and decreasing its variability. Particularly, for future experiments investigating noise induced tinnitus, it will be critical to have a methodology which would allow for the determination of the tinnitus presence in individual animals. As there is a consensus that for work which would have clinical implications, it is necessary to study noise induced tinnitus. It has been documented that after noise exposure only approximately 50% of subjects (animals and humans) experience tinnitus; therefore, the ability to detect tinnitus in individual animals is vital. Our results indicated directions for further modifications needed to achieve this goal. Last, but not least, once the PPI method has been shown to work reliably for individual animal subjects, it should be possible to use its modification in human research and for routine clinical work, potentially offering an objective method for detecting tinnitus presence in humans.

APPENDICIES

APPENDIX A

IACUC LETTER OF APPROVAL



DATE: August 30, 2005

TO: Drs. Margaret Jastreboff & James Brennan

Department of Biological Sciences
Smith Hall
Towson University
8000 York Road
Towson, MD 21252-0001

FROM: Institutional Animal Care and Use Committee

Department of Biological Sciences

Towson University
8000 York Road
Towson, MD 21252-0001

t. 410 704-3042
f. 410 704-2405

RE: IACUC PROTOCOL #SP0506RPR.01
"Investigation of the Mechanisms of Tinnitus in Rats"

This is to certify that the Institutional Animal Care and Use Committee met on 8/30/05 to review the changes to your protocol and granted FULL APPROVAL. Your approval date for this protocol is August 30, 2005.

Your protocol is approved for a period of 3 years; an annual report must be submitted to the IACUC one month before each anniversary of the protocol. Please note that your protocol will expire on April 07, 2008. If you need to extend the protocol beyond this date, you must submit an Animal Care and Use Form at least 3 months prior to the expiration.

If you have any questions, please do not hesitate to contact the IACUC Chair by email (detolla@vetmed.umaryland.edu) or by phone (at 410-706-8537).

A handwritten signature in black ink, appearing to read "Louis J. DeTolla".

Louis J. DeTolla, VMD, PhD
Chairman, IACUC

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