AN ONTOGENETIC PROFILE OF INFANT ULTRASONIC VOCALIZATIONS USING WHOLE LITTER RECORDINGS: THE TRANSITION FROM INFANT TO ADULT CALLS IN RATS

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ABSTRACT

Rodent ultrasonic vocalizations (USVs) have been recorded during drug exposure in adults, play behaviour in juveniles, and the mother-infant interaction in pre-weaniling rats. The major finding is that USVs in infants and adults are different. It is hypothesized that there should be a transition in vocal communication that parallels the transition from infancy to adulthood, that parallels the development of their social autonomy. The method used in the present experiments was a whole litter recording technique in which vocalizations were sampled from an entire litter periodically. Calls from postnatal day 7 to postnatal day 21 were analyzed using Luscinia software for duration and frequency. Calls change from an infant to adult patterns around postnatal day 18 in typically developing rats, and earlier in prenatal treatment of valproic acid that shows precocious development. The findings from this thesis suggest that whole litter recordings are a valid model of studying USV development.

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ASD	autism spectrum disorder
BAT	brown adipose tissue
DD	double dose
G	gestational day
GABA	γ-aminobutyric acid
Hz	hertz
kHz	kilohertz
LDT	laterodorsal tegmental nucleus
LME	linear mixed effects model
ms	millisecond
NST	nonshivering thermogenesis
P	postnatal day
SD	single dose
se	standard error of the mean
USVs	ultrasonic vocalizations
VPA	valproic acid
VTA	ventral tegmental area

Chapter 1

General Introduction

Rodent ultrasonic vocalizations (USVs) have been used to assess affective states induced by various pharmacological and situational stimuli. USVs assessed in the literature can be broadly categorized to represent three different affective states in rats. Two adult states, a positive and negative state, and one infant state, distress. Current research uses infant distress calls as a model of neurodevelopment for prenatal and postnatal evaluations of drugs and for induced neurodevelopmental disorders (Branchi, Santucci, & Alleva, 2001). Distress calls are an innate stress response as separation from the mother or loss of body temperature induces these distress calls at high rates. Providing further evidence that these are distress calls being measured, studies have shown that anxiogenic compounds induce and increase the rate of distress vocalizations in infant rodents. Moreover, Brudzynski and colleagues (1999) have found that distress calls change and develop as infant rodents age. The purpose of this thesis is to examine the typical development of infant vocalizations over five time points in the pre-weaning period. The method proposed and used in this thesis removes the stress caused by isolation-induced vocalizations by recording vocalizations from the whole litter.

1.1 History of ultrasonic vocalizations

In 1941, James Gould and Clifford Morgan published the first paper establishing that rodents can hear well above the human frequency. Humans can hear frequencies between 20 and 20,000 Hz, whereas he found that rodents can hear frequencies above 20,000 Hz. These high frequency sounds are termed ultrasounds or ultrasonic indicating that they are above the human hearing limit.

The experimental method that Gould and Morgan used was a signal detection method. A rat was placed in a two-compartment testing chamber, and taught to run from one chamber to the other to avoid a foot shock after the presentation of an 8 kHz tone. They then changed the frequency of the tone, raising it above the human threshold to 40 kHz (the limit of their tone generating device). They found that the rats were able to successfully avoid the foot shock at this frequency.

Shortly after the Gould and Morgan study was published, other investigators using bat detectors began studying whether rodents can also emit ultrasounds (in addition to hearing them). Anderson found that rodents can emit sounds up to 80 kHz (Anderson, 1954). Together, the findings that rats can hear in the ultrasonic range as well as emit sounds in that range set the stage for research into the functional significance of these sounds.

The first theory tested was that ultrasounds produced by the rats were used for orientation, as seen in bats. Rosenzweig and his coworkers (Rosenzweig, Riley, & Krech, 1955) took blinded rats and placed them in a two-armed maze with a food reward at the end. During the trials, one of the two arms of the maze was blocked and the goal was to determine whether the rats could detect the block. They found that the rats were successful at choosing the correct path to the food reward and so were obviously detecting the block. To further uncover if the rats' successes were in fact due to auditory cues and not olfaction, they occluded the animal's ears and found that the rats' ability to choose the correct path was reduced to chance. This experiment provided evidence that the rats were using auditory cues to choose which path to take to the food reward.

During the testing phases, they recorded ultrasounds in the maze in order to confirm that the rats make ultrasonic sounds. There was a problem, however, as the ultrasounds did not seem to be related to maze performance. In fact, they noted that the ultrasounds were rarely made when the rat was in the maze. It was therefore hypothesized that other sounds in the environment, such as sniffing, clicking of teeth, or footfalls, may be used in a similar manner to how bats use echolocation. The ultrasounds, themselves, must therefore have had some other function.

Further research into the use of ultrasounds by rats for orientation has generally supported the idea that they can echolocate. Consequently, Smith (1979) argued that the sounds produced could be used as both sonar and social signals.

1.2 The role of ultrasonic vocalizations in adult social behaviour

Adult rats produce calls that can be separated into two categories: 22 kHz and 50 kHz calls. Figure 1.1 gives a detailed overview of the difference between the two call categories and the subcategories that have been identified (Wright, Gourdon, and Clarke, 2010). Fifty kHz calls are short in duration and can vary between 30-100 kHz range. These calls can subsequently be broken down into 14 different call categories. These categories represent differences in the acoustics of the call and their frequency pattern. The 22 kHz calls are longer in duration and have a narrow frequency range, being emitted at 22 kHz. There is a single call in this category with no or little modulation of frequency (flat).

These two categories of calls are used in different affective states. Twenty-two kHz calls are produced by rats in negative affective states, both with

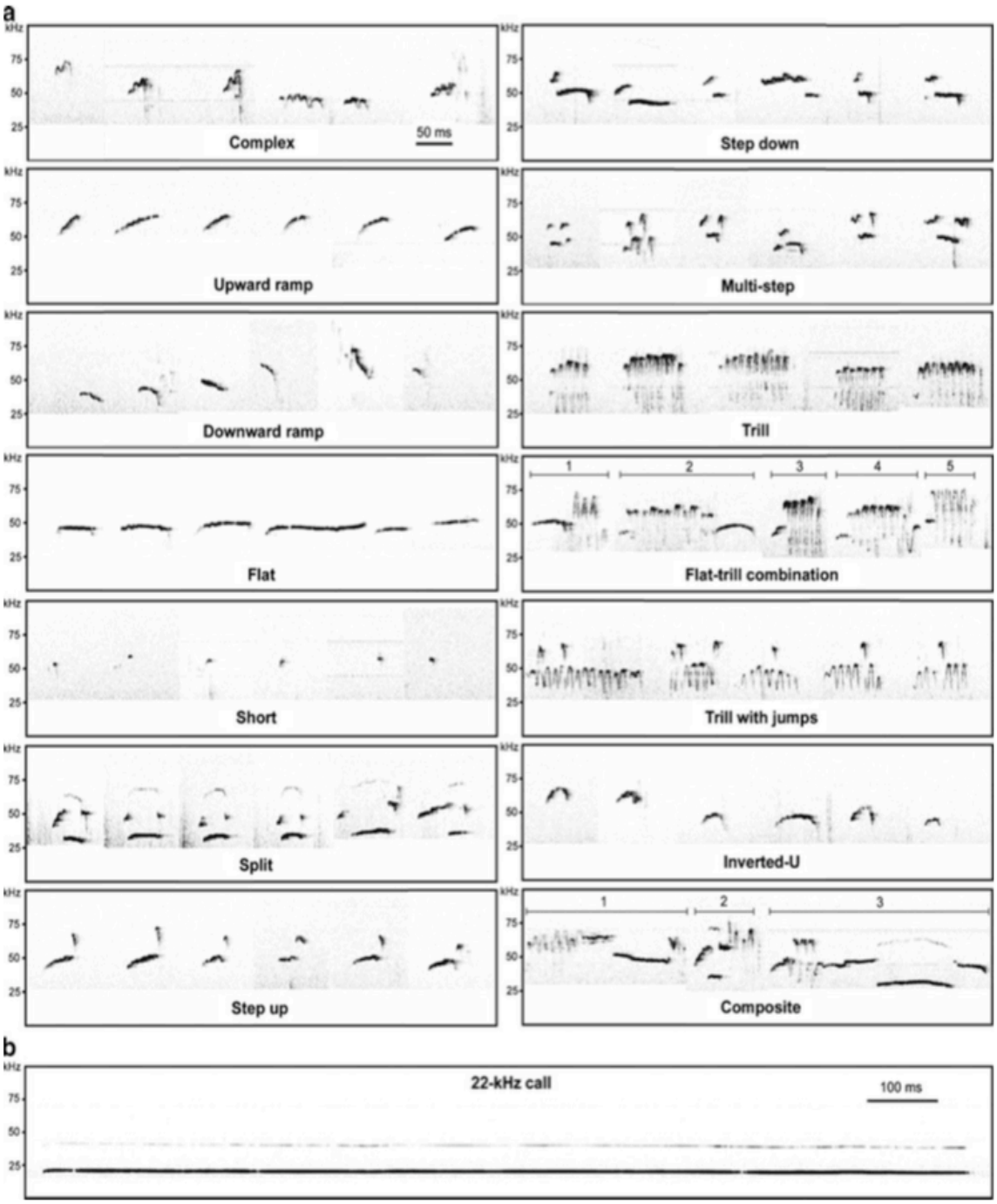

Figure 1.1: Examples of the calls that adult rats make. There are 14 distinct call categories in the 50 kHz range (a) and one type of 22 kHz vocalization (b). The time scale for the 50 kHz calls can be found in the top left panel (50 ms), while the scale for the 22 kHz call can be found in the top right of the panel labeled (b) (100 ms). The 50 kHz calls range from complex acoustic features, as seen in the trills and composite calls, to simple, as seen in the flat category. Adapted from Wright, Gourdon, and Clarke (2010).

respect to internal cues such as hunger or nausea, and external cues, such as approach by aggressive conspecifics, defeat in fighting, or painful stimuli. On the other hand, 50 kHz calls are generally produced in positive affective states, such as approach of familiar conspecifics, during play behaviour, and in response to food rewards. Because negative and positive states serve an adaptive function to the animal it follows that the ability to signal such a state to other animals would be adaptive. That is, positive and negative states are important for survival and can trigger approach or avoidance behavior; behavior that might be helpful to others in the group. The acoustic characteristics of vocal production thus can be important in guiding other members in the group (Brudzynski, 2007).

It comes as no surprise that with two different calls serving two different functions, the underlying neural mechanisms of the call types must also differ. There are two underlying neural systems that have a large impact on behaviours associated with vocalizations. The 22 kHz system is proposed to involve the ascending cholinergic pathway of the brain that has its origin in the laterodorsal tegmental nucleus (LDT) and its terminations in the hypothalamic-preoptic region. Stimulation of the LDT with the neurotransmitter glutamate, an excitatory neurotransmitter compound, induces 22 kHz vocalizations. These 22 kHz vocalizations are attenuated with the application of a cholinergic antagonist, scopolamine, to the hypothalamic-preoptic region (Brudzynski & Barnabi, 1996). Injection of carbachol, a cholinergic agonist, into the hypothalamic region of the brain induces 22 kHz calls. Along with the calls the treatment produces widely open eyes, lowered head, crouched body posture, decrease in locomotion, and increases in freezing. Accompanying autonomic changes include increased heart rate and

blood pressure. Thus, the behaviour and autonomic changes induced by the drug are similar to that which may occur in a real situation that is dangerous or threatening (Brudzynski, 1994). This portion of the ascending cholinergic system is behind the activation and maintenance of the negative affective state in the rat, which is associated with the 22 kHz vocalizations.

Conversely, 50 kHz vocalizations have been produced following stimulation of the dopaminergic pathway that has its origins in the ventral tegmental area (VTA) of the midbrain and its termination in the nucleus accumbens and the frontal cortex. Animals treated with flupenthixol, a dopamine receptor blocker, in the nucleus accumbens, show a significant decrease in 50 kHz calls (Burgdorf et al., 2007). Activation of the nucleus accumbens by amphetamine induces 50 kHz calls and increases locomotion. The release of dopamine in the nucleus accumbens is proposed to be associated with positive states, such as consumption of novel food or sucrose and mating activity. The increase in locomotion is proposed to be associated with approach behaviour (Brudzynski, 2007). Taken together, these results suggest that 50 kHz vocalizations signal a positive state in rats.

Of course, these regions of the brain are not exclusive to the reception and production of USVs, because the animals have an auditory system that perceives them and a motor system that produces them. What they do indicate, however, is the USVs are correlated with specific types of behavior and likely play a role in the instigation of behavior.

There is other evidence that positive states are associated with 50 kHz vocalizations. During play, a positive social situation for rats, it has been demonstrated that rats will emit 50 kHz ultrasonic vocalizations (Burgdorf et al., 2008). Other recent research has found that 50 kHz calls may be used to mediate playful encounters. Rats emit more 50 kHz calls when initiating a play bout, especially one that leads to contact with the nape, than when terminating a playful encounter (Himmler et al., 2014). This work suggests that 50 kHz calls mediate social encounters.

To further investigate the hypothesis that 50 kHz calls mediate playful social encounters, Kisko et al. (2015) devocalized rats and assessed the use of USVs as play signals and play modulators. They found that pairs of devocalized rats had fewer play encounters and the specific repertoire of behavioural signals was altered when compared to intact control animals. Thus, they suggest that USVs are important social signals in encouraging play (Kisko et al., 2015). These studies show that the 50 kHz calls are important in play facilitation, perhaps by maintaining a positive state in the rat.

Perhaps there is a teeter-totter arrangement between the cholinergic system activating a negative state and the dopamine system in activating a positive state. Maintenance of a positive state has been shown to decrease brain acetylcholine levels, thus suppressing the ascending cholinergic pathway (Brudzynski, 2007).

Positive and negative states are rather broad categories of behavior and for this reason other investigators have begun looking at correlating specific behaviours with specific call types. Using the call categories from figure 1.1 (Wright, Gourdon, & Clarke, 2010) Burke and colleagues (2017) correlated 50 kHz call types with behaviours of rats

anticipating a play partner. They found that rats frequently vocalize when performing some type of locomotion, i.e. running, walking, or jumping, whereas non-movement activities, such as sniffing and rearing, were negatively correlated with vocalizations. This work, in conjunction with that done by Himmler et al. (2014), shows that vocalization occur more frequently at the onset of behaviour as opposed to the offset of the behaviour. The finding of an increase in vocalizations before play and a reduction in vocalizations at the end of the play supports the view that vocalizations may be use to initiate social interactions (Burke et al., 2017; Himmler et al., 2014).

1.3 The role of ultrasonic vocalizations in rodent development

A third vocalization category in rats is the infant 40 kHz call. The function of these calls has received less attention than the study of adult 22 Hz and 50 Hz calls. Understanding the relationship between behavior and infant calls may be as productive as studying the relationship between adult behavior and adult calls. The study of infant calls could have multiple positive outcomes: (1) They could provide information concerning the ethology of the vocalizations (Smith, 1979; Takahashi, Kashino, & Hironaka, 2010); (2) an understanding of the effects of early hormone exposure and genetics on physiology and behaviour (Hofer, 1996) and; (3) a better understanding of the mother-infant interaction (Bowers, Perez-Pouchoulen, Edwards, & McCarthy, 2013; Brunelli et al., 2015; Farrell & Alberts, 2002). Of interest to the current thesis, is the work that utilizes infant USVs to study various aspects of neurobehavioural development (Barron & Gilbertson, 2005; Branchi, Santucci, & Alleva, 2001; Cox et al., 2012; Zimmerberg & Germeyan, 2015).

Forty kHz calls are proposed to mediate some aspects of mother-infant interactions, as their calls have been shown to elicit retrieval of a pup and ano-genital licking of the pup by the mother. The USV can also induce prolactin release in the mother and this facilitates milk let down for suckling (Brudzynski, Kehoe, & Callahan, 1999). Wöhr and Schwarting (2008) found that playback of 40 kHz calls induced searching behaviour in mothers. Searching behaviour was not induced by a 40 kHz tone (sine wave) or white noise (Wöhr & Schwarting, 2008). This study provides evidence that 40 kHz calls are used as a signal to the mother from a pup in distress. Moreover, that a pure tone is ineffective provides evidence that there is information in the complexity of USVs beyond simple frequency.

Studies have used infant USVs as a measure of anxiety, as there is an increase in pup vocalizing rates in distressing situations. These calls by pups can be attenuated by anxiolytic (anxiety-reducing) compounds and increased by anxiogenic (anxiety-provoking) compounds (Insel, Hill, & Mayor, 1986). It makes sense that infants would vocalize when in distressing situations and it further makes sense that their calls would stimulate retrieval by their dam. Blumberg and Stolba (1996) reported that in their second week of life, infant rats vocalize more than in the first week of life, when subjected to moderate and extreme cold temperatures. The increase in the vocalization rate seen in the second week of life can be attributed to the increased sensitivity of older infant rats to temperature changes. Research has shown that within the first week of life infant rodents are more resilient to extreme cold temperatures. It has been suggested that the call rate increases significantly during the second week of life because the threat of hypothermia is

at its highest, the pups are becoming mobile but they still cannot thermoregulate (Blumberg & Alberts, 1990; Blumberg & Stolba, 1996).

Another hypothesis as to why infant rodents vocalize is that USVs are a byproduct of laryngeal braking. Laryngeal braking is an acoustic by-product of forced air out of the lungs, as commonly heard during coughing or sneezing. This theory was initially proposed as USVs were noted to increase when infant rats temperature decreases. Since rodents cannot thermoregulate until the second week of life they lose temperature rapidly. There are mechanisms to combat heat loss. The majority of heat is generated through nonshivering thermogenesis (NST), which mainly comes from brown adipose tissue (BAT). BAT is concentrated near vital nervous and systematic structures to protect against cold exposure. Originally postulated, the decrease in temperature compromises gas exchange, therefore USVs would increase as BAT begins producing heat in order to increase oxygen to the lungs, and decrease as the pup is retrieved and its temperature normalizes (Blumberg & Alberts, 1990). This theory is not considered to explain the main factor or mode of USV production in rats as temperature regulated infants still produce USVs and artificially inducing NST does not produce USVs (Blumberg & Alberts, 1990; Blumberg & Sokoloff, 2001; Burke et al., 2017; Hofer & Shair, 1993).

There are some complexities to the ease of interpreting infant USVs. One of the biggest limitations is individual differences between animals, which may have a genetic basis. Brunelli and colleagues (1997) were able to selectively breed rats into high calling (High USV) and low calling (Low USV) lines. Based on calling rate on postnatal day 10 (P10), rat pups were separated into either a high calling group or a low calling group and later bred with another rat from the same group. After 5 generations, analysis of infant

isolation-induced USVs revealed success in separating the lines (Brunelli, Vinocur, Soo-Hoo, & Hofer, 1997). Subsequent research on these lines reveals co-selection of other behaviours. Specifically there are alterations in acoustic patterns in USVs (Spence et al., 2016), in maternal behaviours (Brunelli et al., 2015), and in play behaviour (Brunelli et al., 2006) in the separated lines. In addition, relative to control rats, the high calling line shows a more anxious phenotype as adults and the low calling line shows more aggressive behaviour as adults (Brunelli & Hofer, 2007).

1.4 The valproic acid model of autism

Autism Spectrum Disorder (ASD) in humans is diagnosed early in life and diagnostic criteria rely on observable behavioural characteristics as outlined in the DSM-5. ASD symptoms are marked by changes in social and communication behaviors.

As the underling etiology of ASD remains unclear it is important to have ecologically valid animal models of the disorder. The valproic acid (VPA) model involves treating mothers with valproic acid while pregnant in order to produce offspring that display analogues of human autistic behavior. The model has gone through extensive testing over the past 10 years and it is well accepted as a valid model of autism in rats and mice (Roullet, Lai, & Foster, 2013). VPA treated animals are less likely to engage in defensive tactics that promote bodily contact and had an overall reduction in USV calls during play bouts (Raza, 2015). Thus they display alterations in social and communication behavior that is analogous the symptomology of human autistic behavior.

1.5 Purpose of the thesis

The studies in the present thesis are aimed at evaluating and creating a developmental profile for USVs in infant Long-Evans rats using a novel whole-litter approach. In doing so I hope to determine the typical vocalization patterns that develop without resorting to distressing the animals.

1.6 Theory and hypothesis

A **Theory of USV development** in rats proposes that infant isolation-induced USVs gradually transition into adulthood. Generally, USVs start of short and at 40 kHz, their duration peaks at postnatal day 14 before reaching the short adult typical levels, while the frequency gradually increases to adult levels. Following from this theory, I **hypothesize that USVs recorded from the whole litter will follow a similar pattern of development.** I will investigate USV development during whole litter recordings. I will compare my results to the findings others using the isolation-induced method (Brudzynski, Kehoe, & Callahan, 1999). Furthermore, I will assess whether or not whole litter recordings is a valid model for assessing the VPA model of autism. This is a neurodevelopmental disorder that has been shown to change USVs, this will serve as a proof of concept that this is a viable method to study USV development (Raza, 2015).

1.7 Objectives of thesis

The main objective of this thesis is to create a developmental profile for typically developing infant vocalizations in rodents using whole litter recordings. Since no such profile has been reported in the literature, unique methodology had to be implemented to capture and assess theses developmental changes. Therefore, several acoustic software

programs were piloted for use in this thesis. Finally, once the vocalization profile had been created, the model was then used to assess changes in acoustics in an autism model in rodents. The specific objectives for each chapter are as follows:

1. To evaluate different acoustic software for the analysis of infant USVs. Various software programs are used throughout the literature and these programs have varying prices. One of the most commonly used programs is the Avisoft SASLab Pro (http://www.avisoft.com/soundanalysis.htm) but it comes with a $2400 price tag. This thesis is limited to review different software programs (3) that are free, and compares them to each other;
2. To assess USVs in typically developing pups using the entire litter as the recording unit as opposed to the popular isolation induced method. A detailed acoustic profile will be created evaluating the changes over the 5 recording days, and;
3. To assess the validity and specificity of the developmental profile created in Chapter 3, the method was used on an autism model, that is known to produce impairments in communications and alteration in acoustic parameters. There are statistical techniques that will be employed in this chapter to account the genetic and litter differences that could otherwise confound the data. By utilizing a linear mixed effects (LME) model the random effects of genetic variance will be taken into consideration and accounted for. This statistical method has been employed in the study of ecology and evolution with success (Bolker et al., 2009).

1.8. Organization of the thesis

The present thesis includes five chapters; chapters 2-4 are presented as individual manuscripts with the intention of later publication. Chapter 2 explores and compares three different software programs that were piloted for use in this thesis, and gives a little methodological background on how each program was manipulated and used for the purpose of studying infant USVs. Chapter 3 discusses the typical development of infant USVs in whole litter recordings as compared to the isolation method. Finally, Chapter 4 is a proof of concept that whole litter recordings can be used when assessing developmental disorders.

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Chapter 2

Comparison of three bio-acoustic software programs for the analysis of rodent ultrasonic vocalizations: Praat, Sound Analysis Pro, and Luscinia

2.1 Abstract

Rodents emit ultrasonic vocalizations (USVs) that can be used to study such behavioural phenomena such as play, fear conditioning, neurodevelopment, and drug manipulation. With the insights gained from studying USVs, more and more researchers are adopting and incorporating USV analyses into their protocols. USV recordings are non-invasive signs of behavior but they produce large amounts of data. With the amount of data gathered from USV recordings there needs to be a way to summarize and interpret it. There are many programs available for purchase or that are free, but a lot of time may be spent learning a program just to find out it does not reveal much about a behavior of interest. In this chapter I review three different free software programs, and their utility for analyzing rodent USVs. Commentary is provided on ease of learning, interface design, segmentation abilities, acoustic parameters, and statistical analysis for each program.

2.2 Introduction

Rats are highly social animals that live in colonies in natural settings and are known to emit ultrasonic vocalizations (USVs). It has been hypothesized that ultrasonic signaling serves as a method of cross-colony communication between rats over short distances and may be responsive to influences such as predatory pressures (Branchi, Santucci, & Alleva, 2001). In 1941, Gould and Morgan published a pioneering paper that established rodents can perceive ultrasounds. This work created an interest that continues to motivate the use of USVs to study rodent behavior. Rodent USVs have been shown to play a role in mother-infant interactions (Hahn & Lavooy, 2005), are sensitive to pharmacological manipulations (Maier et al., 2010), and have implications in modulating play behaviour (Himmler et al., 2014; Kisko et al., 2015). The use of USVs in developmental research is ideal because their recording involves limited handling of the infant rodents while potentially revealing a great deal about their behavior with respect to each other and to their mother (Branchi, Santucci, & Alleva, 2001).

There are many ways to assess rodent USVs. For the purpose of this thesis, I will describe the most common methods for data collection and analysis of infant ultrasonic vocalizations. The most common methods for eliciting USVs in infant rodents is by either isolating the infant or reducing ambient temperature. Drugs also induce or alter USVs, but are commonly used in conjunction with isolation to look at the acoustic affects of the drug treatment. The most common acoustic parameter measured and reported in the literature is call rate. Call rate is simply acquired by taking the total number of calls emitted and dividing by the recording time. Next most commonly reported acoustic

findings are average call frequency, usually measured in kilohertz, and duration, measured in milliseconds.

The acoustic parameters are collected by segmenting the individual sounds. This is achieved through software that takes a sound file and transposes it into a visual representation of the sound called a spectrogram. Spectrograms represent a sound in time and frequency, and software for acoustic analysis allow the user to select the individual sounds in the spectrogram for further processing. The types of processing depends on the software used; this is described in greater detail below.

USVs are challenging to analyze as their frequency falls above the limits of human hearing (20,000 Hz) and must be transformed to be audible to humans. This process obviously distorts the original sounds. In addition, many considerations must go into experimental design, set up, testing protocols, and ultimately the choice of software for analysis. Currently, there is a wide range of commercial and free software available, each with advantages and limitations. This means there is often a tradeoff between ease of use, versatility, and power. The conventional software used for rodent USV analysis are able to capture the basic parameters of the sounds, however there has been an explosion of acoustic software used for other species that offers a more robust analysis of vocalizations. The purpose of this chapter is to give an overview of the three software programs that were sampled for use in this thesis. To accomplish this overview the following were the criteria used to assess each software program: ease of learning, interface design, segmentation abilities, acoustic parameters, and statistical analysis. The software programs compared have a wide range of capabilities and, as will be pointed out,

all have both positive and negative aspects regarding their design. One positive note first; all of the programs are free.

2.3 Praat

Praat was created in 1995 for the analysis of phonetics in human speech and has since been used to analyze vocalizations from dogs, frogs, and many other animals (Riede et al., 2005; Preininger et al., 2013; Vannoni, Torriani, & Mcelligott, 2005). Praat can be run on Windows, OS X, and Linux, and regular updates are made available to its users. Because Praat was created for human acoustics, the spectral settings are set up for audible sounds but can be changed easily to accommodate any sound range. For the purpose of ultrasonic analysis, the frequency was set to a maximum of 80 kHz. This could be adjusted to higher ranges if the microphone in use is able to record at a higher range.

2.3.1 Ease of learning

In my experience, Praat is difficult to master and this can be daunting for a new user. Praat utilizes complex scripts that are used to analyze the sounds. These scripting capabilities allow the user to create custom functions. This is perhaps the biggest upside to using Praat. There is also the ability to gain a great deal of information from built in analysis functions found in the menus. Since Praat is a free software (http://www.fon.hum.uva.nl/praat/) there are open source scripts available for download. Once the scripting capabilities have been mastered, this program becomes an extremely powerful tool, allowing the user to create custom scripts that automate routine operations, or conduct numerical analysis and output the data (Owren, 2008).

2.3.2 Interface design

Sound files as large as 2 GBs can be uploaded and analyzed with ease, which is equivalent to 3 hours of recorded sound. A spectrogram can then be created of the sound, with the horizontal direction representing time and the vertical direction representing frequency. The main Praat object window (Figure 2.1) allows the user to read in sounds and run scripts to analyze the sound files (the working space of the script). This feature allows the user to read in multiple sounds files as well as view, make changes, and even extract data from the different files. The object window also acts as an ongoing workspace and alterations to the sound file can be saved from this menu. Sound files can be opened, using the view and edit feature and the sound can be viewed as a spectrogram (Figure 2.2). The user is able to select sections and zoom in. In order for the spectrogram to be visible for analysis, the sounds must be viewed at a minimum of 10 seconds, but can zoom in as much as 0.000015 seconds. An advance bar can be found at the bottom of the screen to scroll through a sound. Drop down menus along the top allow the user to utilize various functions such as extracting sounds and gaining information at exact points on the spectrum. The design is simple and does not overwhelm the user.

3.2.3 Segmentation abilities

Segmentation on Praat works in a temporal manner. This segmentation style is a disadvantage to studying vocalizations that are recorded from multiple animals, as there may be overlap in sounds in a temporal manner. If the user was to analyze vocalizations from a single animal, this program would be ideal. When performing analysis of the sounds, a script can be utilized to track different features. Scripts can be utilized to collect

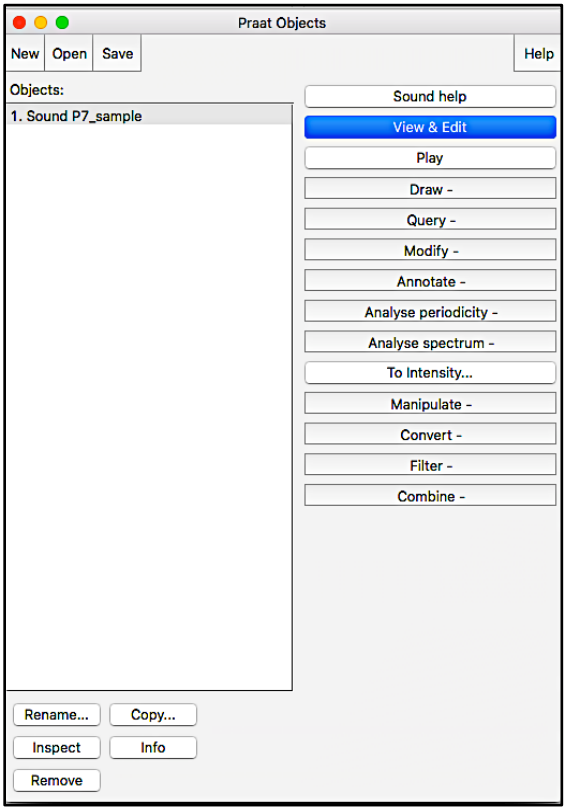

Figure 2.1: Praat object window. This is the main screen the user uses to navigate sound files. Multiple files can be loaded or created in the object window and analyzed or viewed with ease.

a number of different types of information. To accurately measure the duration of a sound, the sound must be segmented precisely, which is the most time consuming aspect of this analysis. Segmentation of a 5 minute sound can take anywhere from 15-75 minutes depending on the number of calls occurring within the 5 minutes.

2.3.4 Acoustic parameters and statistical analysis

Extraction of data can be done directly in the sound file using the drop down menus or by creating and utilizing a script. Scripts to assess amplitude, duration, frequency, mean frequency, bandwidth, and pitch are easily created or downloaded (Owren, 2008). Once the data are extracted they can be analyzed using any software/method.

2.4 Sound Analysis Pro

Sound Analysis Pro (SAP) was created in 2000 for analyzing animal vocalizations. Although only compatible with Windows, SAP has been used with much success. The main uses of SAP have been for bird song analysis (Feher et al., 2009). SAP can accommodate sounds in the ultrasonic range, and upon uploading a sound file, the program automatically changes the sonogram to show the sound.

2.4.1 Ease of learning

As with Praat's scripting capabilities, learning SAP is not easy due to SAPs complex design. For the purposes of using SAP for USV analysis, it was difficult to bring the sounds into focus. Multiple adjustments to the sound must be carefully made. When using this program, it is vital to keep the user manual handy as many of the functions that

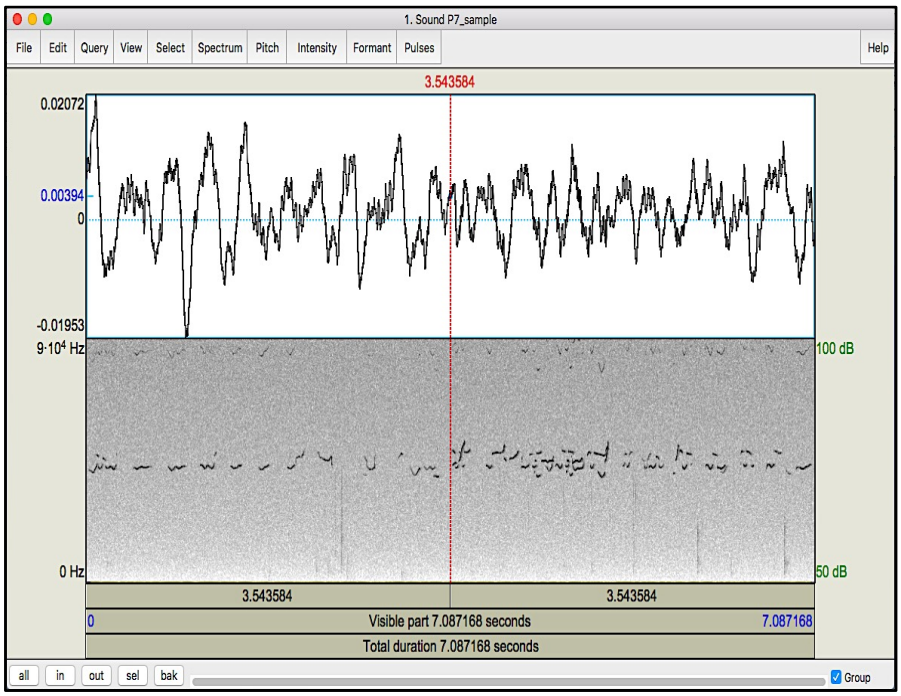

Figure 2.2: Sonogram view of rat vocalizations with Praat. Along the top are drop down menus that allow the user to navigate the program. On the left side of the sonogram is the frequency scale as a function of time, which can be found on the bottom.

help sharpen the sounds (reduce the background noise) are not common features of most sound analysis software.

2.4.2 Interface design

SAP cannot accommodate large sound files. It is therefore recommended that 1-minute segments be uploaded individually. This is obviously a big drawback with respect the analysis of large files. Once the sound is loaded it is represented as a spectrogram. SAP also allows the user to analyze two separate sounds at the same time in separate tabs and to compare segmented sounds. Scrolling through a sound is accomplished fairly smoothly as there is an advance bar under the sonogram. Zooming into a sound poses an issue, however, as the user has to change the Fast fourier transform (FFT) data window and this zooms the sound in and out but on the order of milliseconds. FFT converts sounds into the conventional frequency representation we see in most sonograms. By changing the FFT data window in SAP the user is presenting the sound in a different time-frequency compromise.

Sounds can also be viewed and analyzed using MultiTaper Spectral Analysis. This is an algorithm used by SAP to take the sound information and transform it into a visual representation. Similar to how Fourier Transform (FT) takes sound information and represents it in time a frequency (spectrogram), the MultiTaper (MT) technique takes sound and represents it as a spectrogram. However, traditional sonograms, as in FT represent the power of the sound as intensity (darker parts of a sound have more power), MT represents changes in power. This results in a smoother spectral estimate giving a clearer picture of the sound for analysis with most background noise filtered out.

2.4.3 Segmentation abilities

Segmentation in SAP, as in Pratt, works in the temporal direction. Therefore, the same issues arise, as some sounds overlap and segmentation will give a combined average of the vocalizations. In SAP there is no mitigation for this issue and even counting the number of vocalizations is impossible. This program is designed to analyze sounds from a single animal, not groups.

One advantage to SAP is its auto-segmentation feature. Auto-segmentation allows the user to segment a sound based on several conditions; amplitude, pitch, mean frequency, goodness of pitch, frequency modulation, amplitude modulation, Wiener entropy, and continuity. The user can use a maximum of two categories to segment the sounds and then they must adjust the segmentation bar that appears. It takes considerable time to find the correct settings, but once an appropriate setting is found, it can be used with little tweaking between sound files.

2.4.4 Acoustic parameters

The acoustic features that SAP measures are very detailed and can give a clear picture of the sounds. SAP assesses amplitude, pitch, mean frequency, peak frequency, goodness of pitch, frequency modulation, amplitude modulation, fundamental frequency, wiener entropy, and spectral continuity. If proper segmentation is achieved, then data from all these categories are collected from each sound segment at intervals of 1 ms, and added to a table at the bottom of the scoring window. Manual entries of sounds can be added as well by clicking and dragging over a specific sound. Once the data are collected,

the table can be copied from SAP to Excel, or any other program, and can be analyzed using any software/method.

2.5 Luscinia

Luscinia was created by Robert Lachlan to study communication in birds. For his research, he required bioacoustic software that allows rapid measurement and computational analysis. Luscinia can be run on both Windows and OS X, making it a widely accessible program. Similar to the other programs I have reviewed here, Luscinia can be formatted to accommodate frequencies in the ultrasonic range. For the purpose of studying USVs, we set the maximum to 80 kHz, as in Praat.

2.5.1 Ease of learning

Learning to use Luscinia was easier than the other two programs. The most time consuming part was creating a database. Two options exist: a local database or a network database (Figure 2.3). A local database is sufficient for one person who has all the data in a single place, while a network database would be advantageous if there are multiple users of a single set of sounds or the data is not localized to one area (as is the case with individuals doing fieldwork). Although Luscinia is a free program, it is not in mainstream use and therefore there is little help available outside of the manual.

2.5.2 Interface design

As with SAP, Luscinia has a limit on the size of sounds that can be read into the program, however 1-minute segments were created and loaded with ease. On the main screen there is a dropdown menu that allows the user to organize sounds based on

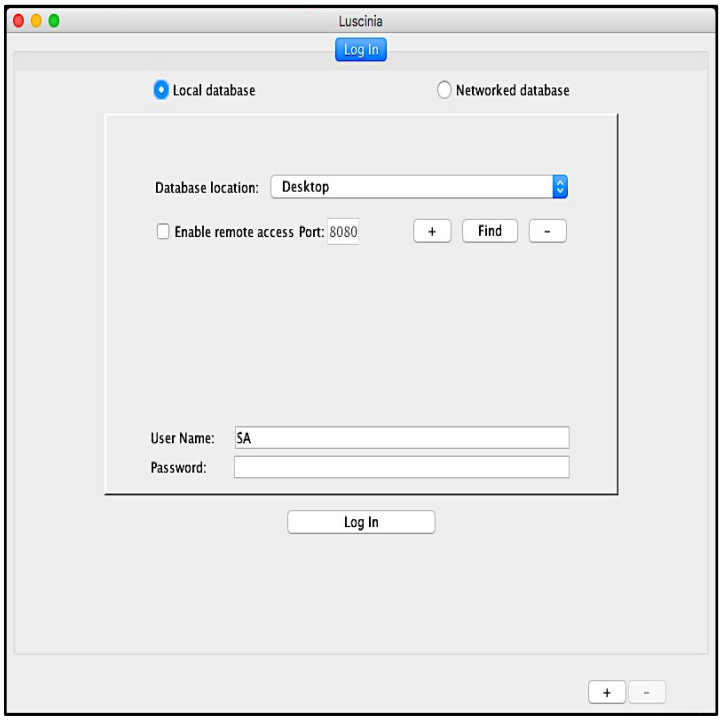

Figure 2.3: Log-in screen of Luscinia. From here a local or network database can be created or accessed.

individuals. This function is useful in keeping track and organizing sounds. Clicking on an individual sound file reveals additional information, including any notes that were added as well as basic information on analysis (number of calls). The sound file can then be made into a spectro-temporal representation of the sound.

Once the spectrogram has been created, the user can view and change many different settings using the tabs at the top of the screen (see Figure 2.4). This is where the default settings can be changed and permanently saved to accommodate ultrasonic sounds. Along the bottom of the viewing window, there is a spectrogram of the entire sound that allows the user to select sections of the sound to be viewed in greater detail.

2.5.3 Segmentation abilities

Segmentation is performed manually, similarly to Praat, but allows the user to segment sounds both temporally and in frequency. Using the cursor, an individual vocalization can be highlighted. There are two key advantages to this method of segmentation. The first advantage resolves the temporal issues encountered with the two other programs; this problem is eliminated and the only sounds that cannot be segmented are those that overlap in both time and frequency. Secondly, this segmentation method allows for accurate representation of the sound as the program is only analyzing the highlighted parts, and any noise that appears above or below the vocalization is ignored. An efficient feature that is built into Luscinia is that once a vocalization is highlighted, the user can view the different parameters (peak frequency, amplitude, bandwidth, etc.) directly on the sonogram. Once the sounds have been segmented, the user can save

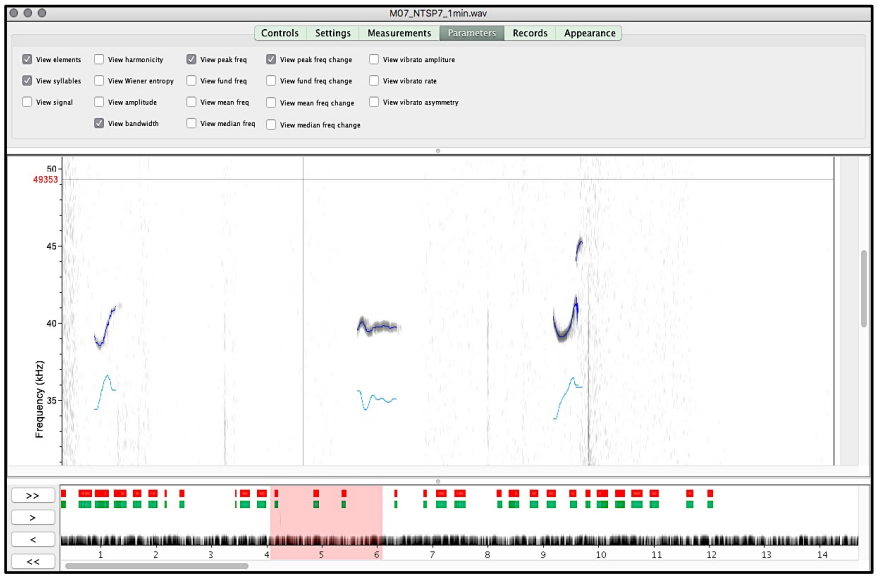

Figure 2.4: Sonogram view of rat vocalization viewed with Luscinia. Along the top are the various parameters that can be viewed directly on the spectrogram. Under the spectrogram is a view of the entire sound file that is used to navigate the sound. The highlighted part is what is viewed in detail on the sonogram.

everything to the database and can then proceed to analyze the results or resume the work.

2.5.4 Acoustic parameters and statistical analysis

An analysis function is built into Luscinia that is accessible from the home screen, which allows the user to analyze multiple sounds. Once the files to analyze have been selected, the next step is to choose what type of comparison will be used. Luscinia has many different features for analysis such as comparison by parameter, comparison by inspection, and dynamic-time warping but it also allows the user the option to export to a spreadsheet.

Comparison by inspection allows the user to subjectively inspect a sound using either visual or auditory inspection. Luscinia will randomize the order the segmented pairs of sounds are presented. The user is then able to categorize the sounds by matching sounds to each other, effectively creating visual or auditory groups (obviously auditory inspection will not work for USVs). Comparison by parameter allows the user to compare segmented sounds automatically using various acoustic parameters (frequency, bandwidth, etc.) and summary stats (mean, maximum, time, etc.). Time Warping changes the length of sounds to match and then calculates how similar or dissimilar sounds are to each other. The algorithm bases similarities or parameters that are weighted by the user. For example, in rodent USVs, frequency of a sound is important, therefore it would be weighted high. Lastly, the user can export the raw data for any acoustic parameter to a spreadsheet. There are many parameters that Luscinia examines, including peak and mean frequency, peak and mean frequency change, harmonicity, bandwidth, fundamental frequency, fundamental frequency change, wiener entropy, amplitude, and duration.

There are two options for data output; one that provides an average over the duration of each highlighted sound, and another that exports the raw data from equally spaced intervals over the duration of the sound. This second option selects as many points across each segmented sound as the user requests; the default is 50 elements per segmented sound, but this can be changed. Both measures can be selected and transferred to an Excel sheet where analysis can be performed using any software/method.

2.6 Discussion

Based on the information provided above, a rating system was used to rank the 3 different programs, as shown in Table 2.1. Because of the power of statistical analysis and the ease of use, in my view, Luscinia is the program best suited to the analysis of rat USVs. Luscinia also has analysis tools that look at similarities of sounds, which may prove useful if the user wants to analyze data into call categories. Luscinia's algorithm is able to objectively sort the sounds, and the user is able to pick the categories on which to base the separation. The unique strength of SAP is its automation feature. If one is able to work it precisely, it could be a time saver and it generates a chart that can be copied into Excel for further analysis. Lastly, the feature of Praat that makes it a powerful acoustic analysis program is its ability to customize and automate many of the functions. But it does not include built-in analysis functions. Another great feature of Praat is that it allows the user to potentially analyze 3 hours worth of recording in one upload, unlike the 1-minute segments needed for Luscinia and SAP.

Table 2.1. Comparison table of three software programs for the use of analyzing rodent ultrasonic vocalizations compared among a variety of categories. 1=poor, 2=good, 3=excellent.

Program	Praat	Sound Analysis Pro	Luscinia
Ease of Learning	2	2	3
Interface Design	3	1	3
Segmentation Abilities	1	1	3
Acoustic Parameters	2	3	3
Statistical Analysis	2	2	3
Total Score	10	9	15

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Chapter 3

Typical development of infant ultrasonic vocalizations

3.1 Abstract

This chapter presents a new method of studying USVs in infant rodents by creating a developmental profile of the natural development of infant USVs. Infant rodents emit ultrasonic vocalizations (USVs) that have been shown to serve as a signal for eliciting maternal behaviour. The current study analyzed the duration and fundamental frequency, at 5 time-points during development, from postnatal day 7 (P7) to P21. The study used an entire litter recording method that allows for analysis of development in a natural context. While the average trends found in the current study support the idea of a gradual USV transition from immature to adult USV, the distribution of the fundamental frequency supports the idea that adult USV calls may in fact replace infant calls.

3.2 Introduction

Rodent vocalizations can be categorized into three types: infant 40 kHz calls, and adult 22 kHz and adult 50 kHz calls. The adult calls have been associated with different affective states. In aversive situations, such as foot shock paradigms, rats will emit 22 kHz calls. In appetitive situations, such as rough-and-tumble play, rats emit 50 kHz calls (Wöhr & Schwarting, 2013). The current literature is replete with studies that hypothesize that as infant rodents' mature and transition into adulthood, their USVs become more complex. This period of transition has been shown to be sensitive to pharmacological manipulations, with reports of alterations to acoustics and call rate, thus, serving as a sensitive measure for neurodevelopment (Branchi et al., 2001; Brudzynski et al., 1999).

The most common protocol for studying infant USVs is the isolation-induced method. Infants vocalize when separated from their dam and littermates (Hahn & Lavooy, 2005). Rodents do not develop the ability to hear and thermoregulate until the second week of life, and thus being separated poses a threat to the life of the pup via hypothermia. Studies have shown that the presentation of an anesthetized sibling, which provides heat, will decrease the vocalizing rate in 3-day-old rat pups. Furthermore, when the anesthetized sibling is cooled to ambient temperature vocalizing rates still decrease. Therefore, it has been proposed that the reduction in vocalizing rates could also be attributed to the mere presence of a littermate, making the experimental animal no longer "isolated" (Carden & Hofer, 1992).

To date, the most thorough investigation of infant USV development was done by Brudzynski et al. (1999). Using the isolation-induced method they recorded distress

vocalizations from 235 Sprague-Dawley pups on postnatal day (P) P10, P15, and P17. They analyzed the USVs for duration, peak frequency, bandwidth, and by call type. They found that call duration, frequency, and bandwidth increased as the pups aged. Furthermore, as pups age, they tended to use more complex calls (Brudzynski et al., 1999).

The methods in this chapter mitigates the stress from the isolation induced calls by recording the infants USVs from the undisturbed litter. This method will give a picture of typically developing infant rats when undisturbed. Based on the findings by Brudzynski et al. (1999) it can be hypothesized that infant USVs will follow a gradual trajectory into more adult typical calling patterns. Because the methods used in the present study are noninvasive, this study should prove to be a strong test of Brudzynski's developmental model.

3.3 Methods

3.3.1. Subjects and experimental setup

The experimental protocol was approved and carried out in accordance with the Canadian Council of Animal Care and the University of Lethbridge Animal Care Committee. All animals were housed in the vivarium, and maintained at a constant 21-23°C on a 12:12 light-dark cycle. Standard polycarbonate shoebox cages were used (26cm x 25cm x 20cm) with corncob bedding. Twelve naïve female and twelve male Long-Evan rats born in-house were used as the parents of the offspring. During mating, one male and one female rat were paired in a standard cage and allowed to mate for seven days. The first day of pairing was considered gestational day (G) 0. After seven days,

males were removed and females were pair-housed for the remainder of gestation; these females were weighed every day to monitor pregnancy. Twenty days after pairing with the male, G20, females were separated in expectation of parturition. Neonates were counted and sexed on postnatal day three (P3), and weighed daily to monitor growth. Overall 131 pups were born to the 12 mothers, 66 males and 65 females. On P21, animals were weaned from their mother and paired with a same-sex sibling.

3.3.2. Apparatus setup and vocalization collection

The vocalization chamber consisted of a Plexiglas box (50cm x 50cm x 50cm) encased in a soundproof chamber (see Himmler et al., 2014 for details). The soundproof chamber was made of fiberboard lined with sound-attenuating foam (Primacoustic, Port Coquitlam, British Columbia). Ultrasonic sounds were recorded using a high frequency microphone (Model 4939, Brüel & Kjaer, Denmark) that was suspended from the ceiling of the box so that it was 15 cm above the center of the Plexiglas box. The microphone was set to record sounds from the range of 4 Hz to 100 kHz. It was connected to a Soundconnect amplifier (Listen, Inc, Boston, MA). Recordings were processed through a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, CA) using an in-house developed MATLAB acquisition program. Sound files were then exported and converted to .wav files and analyzed using Luscinia software (Lachlan, 2007).

For the purpose of recording ultrasounds from pre-weanling pups, a heating pad was placed under the Plexiglas box set to LOW to ensure proper thermoregulation of the pups. On P7, P11, P14, P18, and P21, the dam and pups were transported to the testing room. The rats underwent a 4 part testing procedure, each part consisted of a 5 minute

recording period: 1) The pups and mother recorded together, 2) the mother is removed and the pups are recorded alone, 3) the mother is returned to the pups and recorded all together, and, 4) the pups were removed and the mother is recorded alone. This testing procedure was chosen as it limits the amount of time the mother spends away from the pups, thereby limiting any potential for distress in the pup. For the purpose of this thesis, the second condition, where the pups were recorded alone, was analyzed. After each set of recordings per litter, the Plexiglas box was cleaned with Virkon to remove any odors from the previous litter. After the testing procedure was completed, the mother and pups were returned to their home cage in the vivarium.

3.3.3. Acoustic and statistical analysis

Using Luscinia, the first 60 vocalizations or the full 5-minute recording (whichever came first) were analyzed for duration and fundamental frequency. These parameters were chosen as they are the most commonly used in the literature (Hahn & Lavooy, 2005), and were used by Brudzynski and colleagues (1999). As the Brudzynski paper is the most comprehensive to date, a direct comparison between their parameters are ours is advantageous. Luscinia allows for segmentation of sound in two dimensions, the time and frequency, and thus the only exclusion criteria for sounds were those that overlapped in a temporal/frequency manner. Using the built-in analyze function on Luscinia, the data was exported as a .txt file. The .txt files containing all the acoustic features were analyzed using R Studio, where the 12 litters of 131 pups, were combined and the raw data was plotted for observation. The results described below are descriptive in nature, to tease out overarching patterns in the development. Therefore no statistical measures will be reported.

3.4 Results

3.4.1. Duration

Vocalization duration follows an inverted U shape pattern (Figure 3.1), with peak duration occurring on P14 and the shortest duration, on average, being on P21. The mean and standard error for vocalization duration for each of the days is 59.96±1.68 (standard error), 88.69±2.24, 139.88±3.63, 70.12±4.71, and 32.54±3.43 on P7, P11, P14, P18, and P21 respectively.

3.4.2. Fundamental Frequency

Fundamental frequency is the lowest frequency wave in a periodic wave. Luscinia averages the fundamental frequency over every time bin of each segmented sound. The average fundamental frequency for each day of recording over all the litters is seen in Figure 3.2. On P7, the average fundamental frequency was 43.3±162.50 kHz, the average frequency decreased on P11 and 14 to 39.6±207.70 kHz and 39.3±263.29 kHz, respectively. The fundamental frequency then increased to 53.8 ±582.12kHz on P18 and continued this upward trajectory to 59.3±441.50 kHz on P21.

The distribution of the fundamental frequency was then viewed to get a better picture of the range of the frequency over the 5 recording days. Figure 3.3 shows that on P7 the most prevalent frequency of vocalizations is in the 40-50 kHz range. This is also seen on P11 and P14, with the exception of a small number of vocalizations in the 65-75 kHz range on P14. On P18, there is a clear bimodal distribution of calls with the peaks being around 40 kHz and 65 kHz but with the majority of vocalizations in the 40 kHz

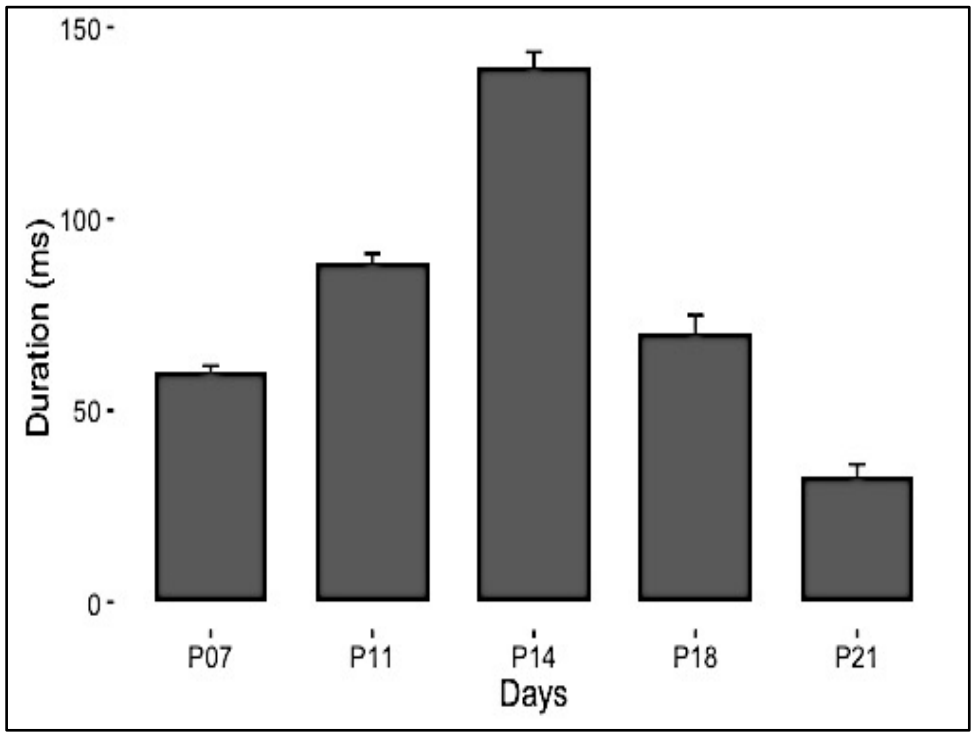

Figure 3.1: Average duration of ultrasonic vocalizations produced by developing rat pups over 5 different days. Vocalization length begins relatively short on P7 averaging 59.96 ms, the length peaks of P14 at 139.88 ms, and then drops to an average of 32.54 ms on P21. Error bars represent standard error of the mean.

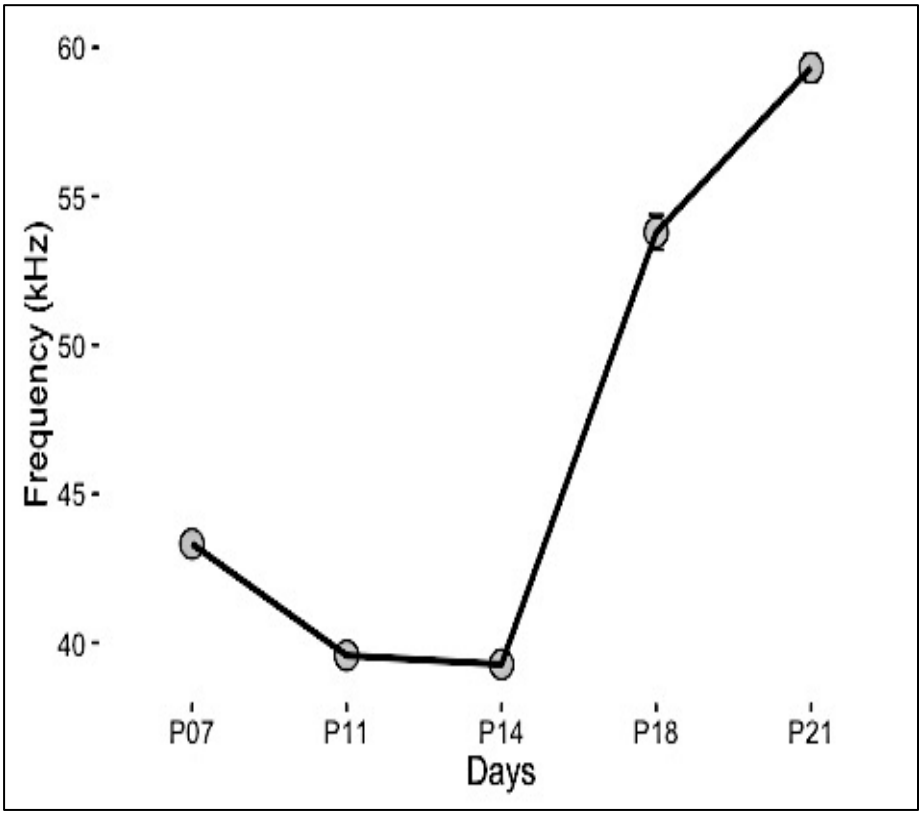

Figure 3.2: The average fundamental frequency of ultrasonic vocalizations produced by developing rat pups over 5 different days. The fundamental frequency on P7, P11, and P14 is relatively low, staying within the typical 40 kHz infantile range. On P18 and P21, there is an increase in frequency to adult typical ranges of 50 kHz and above. The error bars represent standard error of the mean.There are no error bars????

range. This trend is reversed on P21, with a smaller distribution around 40 kHz and the majority of vocalizations in the 50-80 kHz range.

3.5 Discussion

Analysis of the acoustic parameters of 7- to 21-day-old rat pups shows an ontogenetic profile of the development of ultrasonic vocalizations from infancy into pre-adolescence. The average duration and fundamental frequency of the vocalizations changed over the 14-day recording period from an infant to an adult pattern. In adult rodents, 22 kHz calls are long in duration, well exceeding 100 ms (Brudzynski, 2006), while 50 kHz calls are generally on the order of a few milliseconds (Sales, 1972).

The average duration change of vocalizations, seen in Figure 3.1, is consistent with other reports in the literature (Brudzynski et al., 1999; Sales, 1972). Figure 3.1 shows that infant USVs start off with a short duration and increase in duration to peak around P14. After this, the average duration decreases to well below 50 ms on P21. Brudzynski et al (1999) recorded USV development, using the isolation induced method, on P10, P15, and P17 and reported a peak duration, of 144.03 ms, on P15 with no significant decrease in the USV duration on P17 when compared to P15, although the average duration did decrease. The observed differences between that study and the current study may be related to the age of sampling of the pups. In the current study there is a 4-day sampling gap between P14 and P18, whereas there is only a 2-day age gap (P15 to P17) in the methods from Brudyznski and colleagues. Nevertheless, the relatively short duration of vocalizations recorded on P21 fits with the hypothesis that, infant USVs are becoming more adult typical, as adult 50 kHz calls are short in nature.

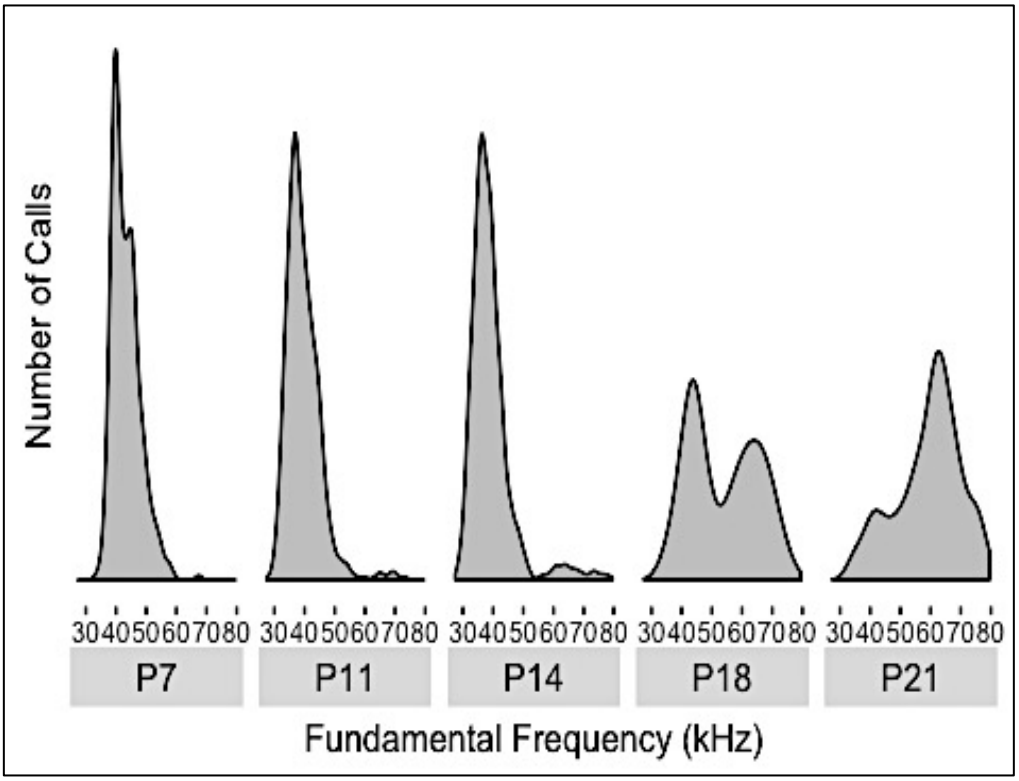

Figure 3.3: The distribution of the fundamental frequency, the y-axis represents the number of vocalizations produced with in a given frequency range (x-axis). As can be seen on P7, P11, and P14 the majority of vocalization occur in the 40 kHz infantile range. While on P14, more calls can be seen in the upper adult typical range, but majority of calls are in the 40 kHz range. On P18, there is a bimodal distribution with majority of calls clustering around the infantile range. This trend is reversed on P21 with majority of calls in the 60 kHz range.

The frequency of USVs is important in signaling affective states. Playback studies of adult USVs reveal that, when given the choice, rats will self-administer the playback of 50 kHz calls while avoiding playback of 22 kHz calls (Burgdorf et al., 2008). Closer analysis of the animals behaviour reveals that playback of 50 kHz calls leads to approach and searching behaviour whereas playback of 22 kHz calls elicits freezing behaviour (Sadananda et al., 2008). Thus the response of the animals in these studies provides evidence that the frequency of a vocalization provides information to the receiver.

When looking at the distribution of the fundamental frequency, a gradual transition into adulthood is not seen. Figure 3.3 shows that during the time between P14 and P18, the adult typical call pattern emerges and it replaces the infant typical calls. This bimodal distribution, seen in Figure 3.3, suggests a new theory for the development of the frequency of rat USVs. I call the new theory "the replacement theory of rodent ultrasonic vocalizations".

There are several considerations that need to be taken into account when looking at the replacement of calls. The first is the social changes taking place in the pups between the 14^{th} to 18^{th} days of development in infant rodents. Frequency of USVs is important in signaling affective states, thereby playing a role in communication. Playback studies of adult USVs reveal that, when given the choice, rats will self-administer the playback of 50 kHz calls while avoiding playback of 22 kHz calls (Burgdorf et al., 2008). Closer analysis of overt behaviours shows that playback of 50 kHz calls leads to approach and searching behaviours, while playback of 22 kHz calls elicits freezing behaviours (Sadananda et al., 2008). Thus, the change in behavior and call type go together.

Interestingly, the 14th to 18th day of life is an important milestone in the development of a rat's social structure. Tactile contact is important in the survival of a newborn rat, from the time they are born pups are maintained in a cluster for warmth (because they cannot thermoregulate) and nursing. Newborn infants are termed to be *physiological huddlers* because the cue used to maintain and elicit huddling is the need for heat. As the pups age the mother leaves the nest more often and for lengthier intervals as the pups become more mobile. At 2 weeks of age rats are able to thermoregulate, and development of their olfaction becomes much more robust. It is at this age that there is a shift in the cue used to huddle, and infants huddling behaviours become *filial*. At this age there is a stronger preference to huddle with a sibling than with a non-sibling, unlike during the first 2 weeks of life where the main criteria was warmth. This preference for filial huddling is displayed by postnatal day 15 (Alberts & Brunjes, 1987; Alberts, 2007).

What guides this transition to filial huddling preference? As infant rodents gain the ability to hear, see, and thermoregulate they become more self-sufficient. This is reflected in the development of other behaviour. During the first 2 weeks of life an infant rat spends most of its time sleeping, and in contact with its huddle. Social behaviours, such as play, begin emerging between the 15th and 16th day of life and increase to peak playfulness at 30-35 days of age. The bimodal distribution seen to appear between the 14th and 18th day of life in Figure 3.3 could represent the changes to the social dynamic of the pups, hence, follow the emergence of more adult typical behaviours (Baenninger, 1967).

Further analysis could be done to assess the specific characteristics of the calls in each mode of the distribution seen in Figure 3.3. The call catalogue created by

Brudzynski et al. (1999) would give a clearer picture of potential functionality of the calls. Studies of USVs can serve as a new sensitive means to measure development. Using a non-invasive and sensitive model that is proposed in the current study for monitoring rodent development will alter the way infant USVs are analyzed and should enable greater precision in determining the impact various experimental treatments have on development (Spear, 1990).

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Chapter 4

Development of infant ultrasonic vocalizations in an autistic model of rats: Proof of concept

4.1 Abstract

Ultrasonic vocalizations (USVs) in infant rats are hypothesized to serve as a signal to the mother to elicit behaviour such as retrieval and grooming. As such they have been used as a measure of neurodevelopment. The purpose of this chapter is to evaluate the usefulness of the whole litter USV recording model, described in Chapter 3, as an assessment tool for the neurodevelopmental disorder, autism spectrum disorder. A core charateristics of autism spectrum disorder (ASD) is an impairment in social communication. Whereas many researchers are investigating animal models of ASD, one area of study that is particularity challenging is social communication. The present study looked at the development of ultrasonic vocalizations (USVs) in infant rodents who had prenatal exposure to valproic acid (VPA). Prenatal VPA exposure has been proposed as an animal model of autism. Pregnant dams were treated with either a single (SD), double (DD), or no dose (controls) of VPA and the offspring's USVs were recorded. Analysis of the number of calls, length and fundamental frequency show the VPA groups displayed a precocious development of USVs. This anomaly is discussed with respect to understanding the symptoms of VPA model and humans with autism spectrum disorder.

4.2. Introduction

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by repetitive and stereotyped behavior and impairments in social interactions and communication. ASD is usually diagnosed within the first 3 years of life (Kahne et al., 2002). The specific etiology of ASD is unknown but it may have a genetic link as it has been associated with many genetic abnormalities. These genes are then hypothesized to be acted on by teratogens in the environment (Levy, Mandell, & Schultz, 2009). A teratogenic cause is proposed to be modeled by the impairments produced by valproic acid administered to infant rodents during the prenatal period (Roullet et al., 2013).

Valproic acid (VPA) is an anticonvulsant agent that has been shown to increase the incidence of autism in the human population to an absolute risk of 4.42% (Rodier et al., 1996). Thus, it has been used to create a rodent model of autism (Schneider & Przewlocki, 2004). The VPA model has been shown to produce behavioural and neuroanatomical abnormalities that can be detected throughout the lifespan of the rat (Mychasiuk et al., 2012).

Neonatal rats are born deaf and blind, gaining the ability to hear and see at approximately the 12^{th} and 14^{th} day of life, respectively. Thus, complex behavioural tasks generally used to assess impairments in higher order functions normally cannot be given until after weaning. As such, tasks that are sensitive enough to pick up possible differences in infancy are rare, and the majority of tasks employed generally rely on simple reflexes (Spear, 1990). Neonatal rodents, however, begin to emit ultrasonic vocalizations (USVs) soon after birth in the 40 kHz range. Forty kHz calls have been

shown to serves a communicative role in eliciting pup retrieval, lactation, and grooming (Brudzynski, et al 1999; Farrell & Alberts, 2002; Hahn & Lavooy, 2005). For adults, USVs change and can be categorized into two groups: 50 kHz appetitive calls, and a 22 kHz aversive calls. These two broad categories of calls are proposed to communicate the animal's emotional state (Brudzynski, 2013).

The purpose of this study was to assess the development of USVs in the VPA autistism model using rats. The study used the methods described in Chapter 3 of this thesis. Operating under the theory that USVs serve a communicative function, it is proposed that changes in acoustic features of USVs might be analogous to the communication deficits described in the clinical definition of ASD.

Whereas USV analysis has been used to assess affective states of neonatal rodents, mainly by using isolation or temperature induced methods, these vocalizations are distress calls (Hahn & Lavooy, 2005). As these distress calls are thought to be reflexive in nature they may not be entirely useful in characterizing USVs and their neuroanatomical underpinnings. The transition from the use of the USVs used in typical development to the use of complex social calls of adults may provide insights into the differences in development in an animal model of ASD. For the study USVs were recorded from the entire litter to reduce stress as is described in the previous chapter.

4.3 Methods

4.3.1. Subjects

Experimental protocols were approved by and carried out in accordance with the Canadian Council of Animal Care and the University of Lethbridge Animal Care

Committee. Animals were kept in standard 26cm x 25cm x 20cm polycarbonate shoebox cages, with corncob bedding, while the vivarium was maintained at a constant 21-23°C on a 12:12 light-dark cycle. Fifteen naïve female and 15 male Long-Evans rats born at the Canadian Centre for Behavioural Neuroscience, University of Lethbridge, were used. One male and one female were paired in the standard shoebox cage and mating behaviours were observed for 20 minutes. If successful mating was observed during the 20-minute interval this was considered gestational day one (G1). If no mating behaviour was observed, the male was removed, and the mating procedure was repeated the next day at the same time, and continued until successful mating of all pairs had occurred. For the duration of the pregnancy, the dams were pair-housed to reduce stress. On G19, the females were singly housed in preparation for birth.

4.3.2. VPA administration

Three days prior to initial VPA administration (G9-11), all dams, including controls, were individually spoon fed 1.5g of peanut butter per day. On the respective treatment days, pregnant dams received a dose of 800 mg/kg of VPA (Raza et al., 2015) mixed into the peanut butter, and controls were given peanut butter alone. Pregnant dams received the treatment of VPA and peanut butter on G12.5 for the single dose (SD) condition, and again on G13.5 for the double dose (DD) condition.

4.3.3. Behavioural groupings

Neonates remained with their dam until weaning on postnatal day twenty-one (P21). They were then separated from their dam and housed with a same-sex sibling. Thirty-six animals were born to 4 control dams (17 males, 19 females), 51 animals were

born to 6 SD VPA dams (34 males, 17 females), and 55 animals were born to 5 DD VPA dams (20 males, 35 females).

4.3.4. Enclosure and apparatus setup

The vocalization chamber was set up according to Himmler *et* al. (2014). Recordings were taken in a 50cm x 50cm x 50cm Plexiglas box encased in a soundproof chamber. The chamber itself was made up of a medium density fiberboard with all inside walls lined with a sound-attenuating foam (Primacoustic, Port Coquitlam, British Columbia).

Ultrasonic vocalizations were collected using a high frequency microphone (Model 4939, Brüel & Kjaer, Denmark), mounted from the ceiling of the chamber approximately 15cm above the center of the Plexiglas enclosure. The microphone was set to pick up sounds ranging from 4 Hz to 100 kHz, and was connected to a Soundconnect™ amplifier (Listen, Inc, Boston, MA). Recordings were then passed through a multifunction processor (model RX6, Tucker-Davis Technologies, Alachua, CA) using a self-developed MATLAB acquisition program. All files were subsequently converted to .wav files and analyzed using Luscinia (Lachlan, 2007).

4.3.5. Collection of ultrasonic vocalizations

On P7, P11, P14, P18, and P21, the dam and pups were transported to the testing room. Under the Plexiglas enclosure, a heating pad was placed and set to LOW so the pups could maintain a consistent temperature. The same four-part recording schedule was used as in Chapter 3 to minimize the time the mother spent away from the infants. The mother and pups were placed in the chamber and their vocalizations were recorded for 5

minutes. The mother was then removed and the pups' vocalizations were recorded for 5 minutes. The mother was placed back in the chamber and recorded for another 5 minutes. Finally, the pups were removed and the mother was recorded alone. After the testing procedure was complete, the mother and pups were returned to their home cage. The Plexiglas enclosure was cleaned with Virkon between litters to remove any odors from the previous litter.

4.3.5. Acoustic and statistical analysis

The total number of calls were manually counted using Praat. In order to accomplish this, a script was created with a tiered design to measure the number of vocalizations. These data were extracted and plotted for observation. Using Luscinia (Lachlan, 2007), the first 60 vocalizations or the full 5 minutes (whichever came first) of the second recording session (pups alone) were analyzed for duration and fundamental frequency. The data was then exported and the files were analyzed using R Studio (R Core Team, 2016). To account for the non-independence of data points within a litter, a linear mixed effects model (LME) was used (*lme4*; Bates et al., 2015) to assess the relationship between the acoustic parameter and the day of recording and treatment condition. P-values (set at $p \leq 0.05$) were obtained by using a general linear hypothesis (*multcomp*; Hothorn et al., 2008) that performed a post-hoc Tukey analysis.

The duration of calls did not fit the assumption of linearity, therefore a nonlinear transform was performed to fit this assumption. This was done using a log-transform. The log transformed data fit the linear assumption and the LME was run using the duration as the dependent variable, the day of recording and treatment as the fixed effects, and the

litter as the random error. The relationship of term was set up as follows: LogDuration ~ Treatment + Day + Day*Treatment + (1| Litter). Where treatment and day were assessed separately as well as the interaction between them.

The fundamental frequency expresses a strong bimodal distribution (see Figure 4.5). A log transform was not able to fit the data into a linear model, therefore, the data was transformed into a binary model. The binary model had a cut off of 50 kHz, anything above was classified as a 1, and anything below was classified as a 0. This in turn fit the data into a linear model. The same fixed and random terms were used as in duration. The relationship term was set up as follows: BinaryFundamentalFrequency ~ Treatment + Day + Day*Treatment + (1| Litter).

4.4 RESULTS

4.4.1. Total number of calls

Figure 4.1 displays the number of calls on each day as a percentage of the total calls for each treatment group. Of note is the peak number of calls for each condition: for controls this is on P14, for the single dose group this is on P11, and for the double dose group the peak is on P7. Figure 4.2 represents the vocalizations as a percentage of the controls vocalizations. On P7, the single dose and double dose groups vocalizations are 237% and 233%, respectively, higher than the controls vocalizations. On P11, the single dose group's vocalizations are 743% higher than the controls, while the double dose begins a downward trend (221%). From P14 and on the treatment groups vocalize less than controls, well below 75% less.

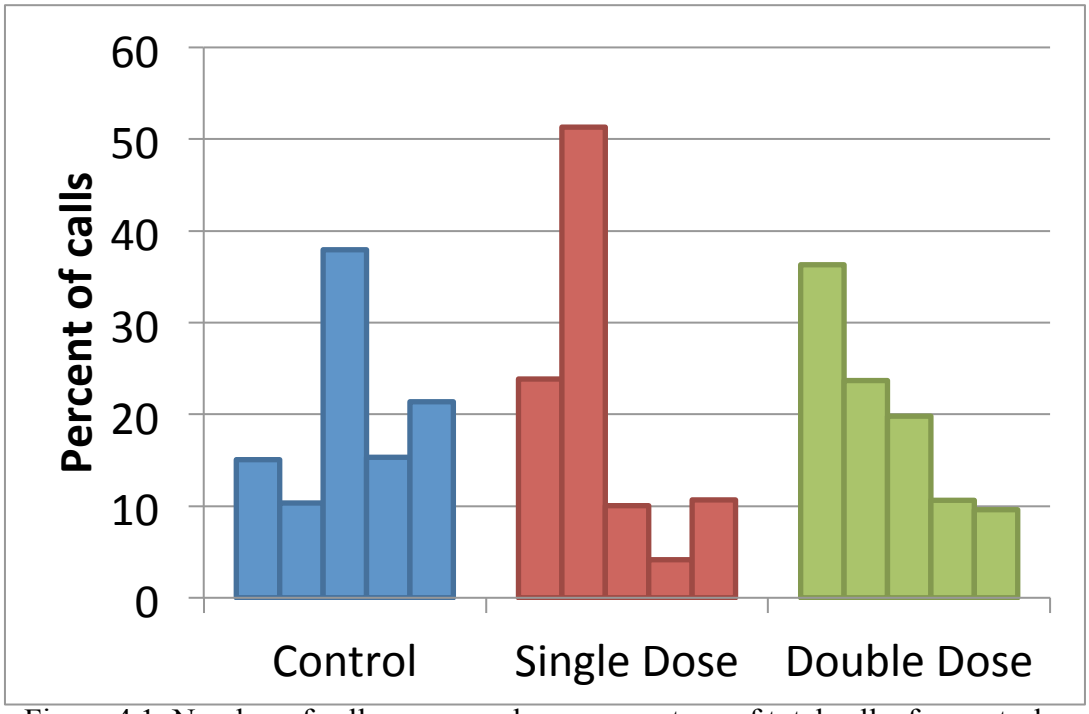

Figure 4.1: Number of calls, expressed as a percentage of total calls, for control (blue), single dose (red), and double dose (green) litters. On the x-axis the bars indicate the respective postnatal day of recording from left to right, P7, 11, 14 18, and 21.

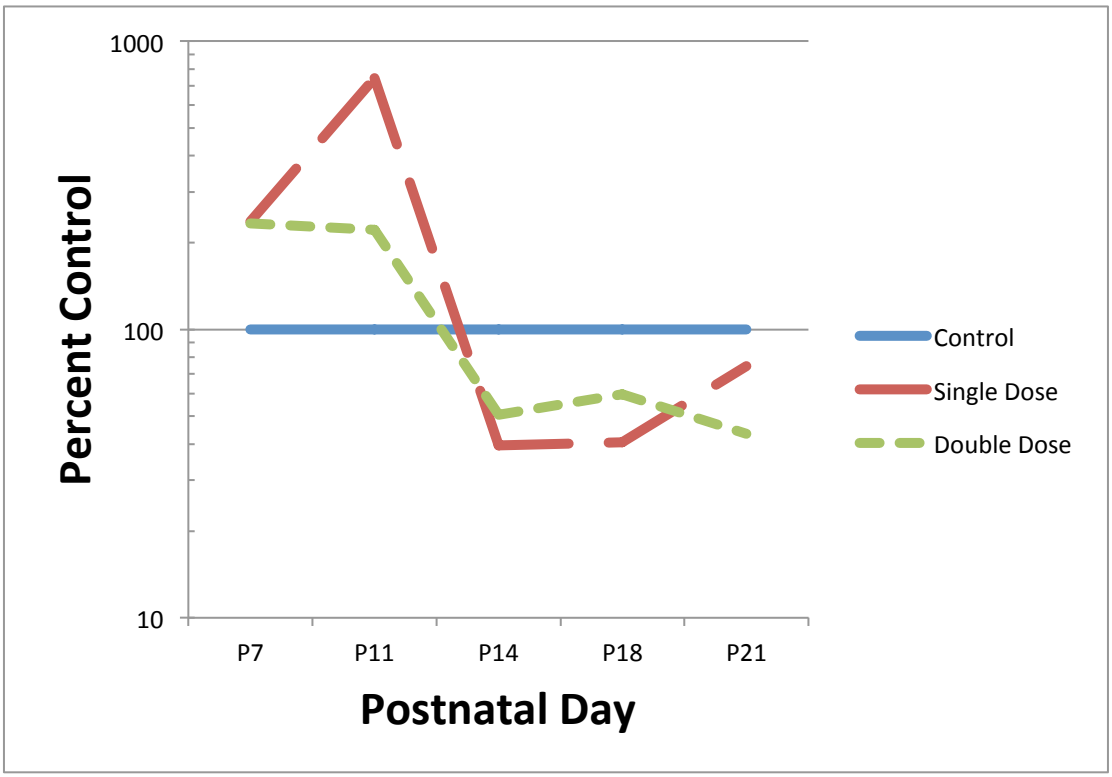

Figure 4.2: The number of vocalizations emitted viewed as a percentage of controls. The y-axis is represented as a logarithmic scale, while the x-axis represents the different days of development. As can be seen both treatment groups vocalize more than controls over the first two days of recording and vocalize less over the last 3 days of recording.

4.4.2. Duration

There was a significant effect of day of recording and treatment on the duration of the vocalizations. The interaction between day and treatment were weakly significant and varied by day. Table 4.1 shows the results of the LME with respect to the control condition on P7. Overall, in Figure 4.3, the duration of the treatments calls are longer in the earlier days of recordings, from P7 ($p = 0.017$) to P14. All groups peak on P14, then the duration of the calls on P18 is shorter ($p = 0.014$) in the treatment groups on average.

Table 4.1: The effects of the treatment and day of recording on vocalization duration. Significance refers to the reference level, which is the control group on P7. A positive estimate means that there was an increase in duration with respect to treatment or day, while a negative estimate means a decrease. Number of observations = 3303, over the 15 different groups (Litters).

Treatment or Day	Estimate	Error z	p-value
Single Dose	0.26	4.02	0.0002
Double Dose	0.23	3.34	0.002
Postnatal day 11	0.21	4.74	<0.0001
Postnatal day 14	0.37	9.06	<0.0001
Postnatal day 18	-0.15	3.44	0.001
Postnatal day 21	-0.32	-7.92	<0.0001

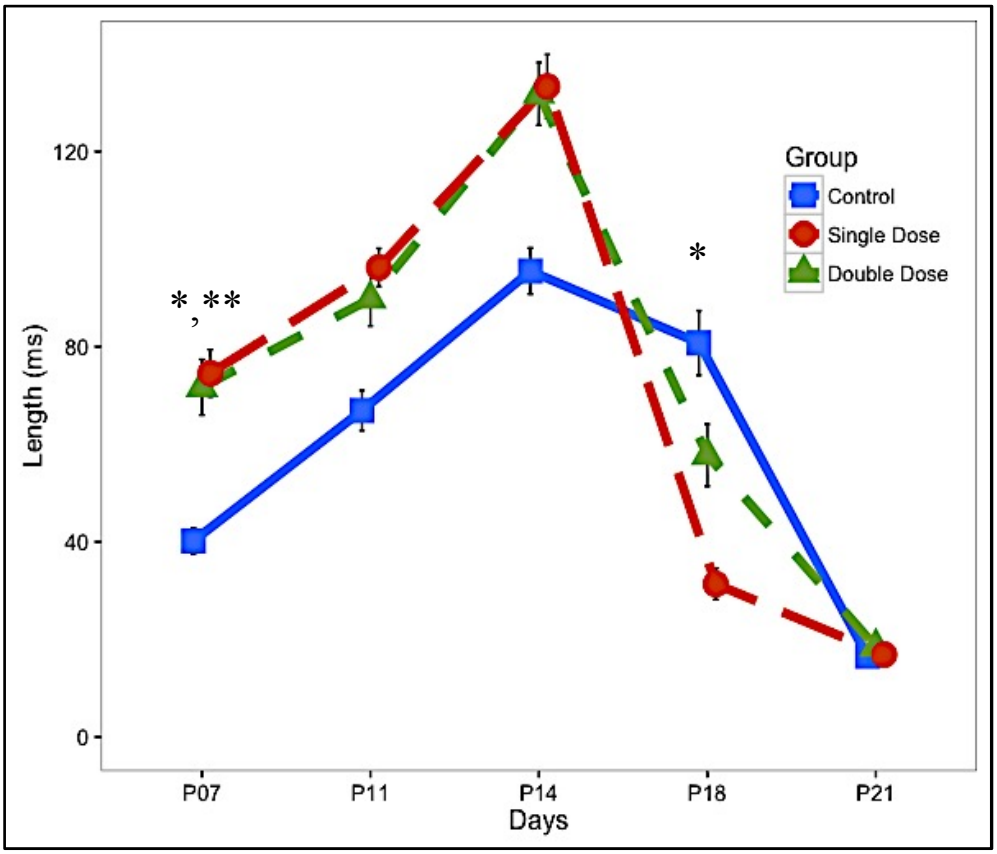

Figure 4.3: Average duration of the vocalizations from the 3 different groups over the 5 days of development. Controls are marked in blue, single dose in red, and double dose in green. Standard error is shown. Significance was found on P7 and 18 for the single dose group compared to controls, as indicated (*), and significance was found on P7 between the double dose group and controls (**).

Figure 4.4.3 Fundamental frequency

The overall trends of the average fundamental frequency reveals that the treatment groups begin with vocalizations in the lower range, seen in Figure 4.4 on P7. The average frequency on P11 is the same in all groups with the vocalization on P14 diverging to be higher in the treatment groups on P14. This trend, to have a higher frequency, continues on P18 and P21.

Closer inspection of the data reveals an interesting trend. The LME model reveals a main effect treatment and day of recording on the binary model of frequency. There is a weakly significant interaction between the day and treatment. Table 4.2 summarizes the findings. Figure 4.5 shows the distribution of the frequency of the calls over the 5 days of recording. On P7 and P11, for all conditions, the vocalizations cluster around 40 kHz, while on P14 there is a clear reduction in the number of vocalizations for both treatment groups around 40 kHz, and a bimodal distribution starts to emerge around 70 kHz. This bimodal distribution is trending but not significant for the single dose group ($p = 0.077$) having more calls in the upper range (above 50 kHz) on P14. On P18, there is a bimodal distribution with the single dose group having more vocalizations above 50 kHz than the control groups ($p = 0.029$). Finally, on P21, the majority of calls from all groups are clustered towards the 65 kHz range, while both treatment groups still show the bimodal distribution.

Table 4.2: The effects of the treatment and days on the binary vocalization frequency. Significance refers to the reference level, which is the control group on P7. A positive estimate means that there was an increase in the number of vocalization in the upper range (above 50 kHz) with respect to treatment or day, while a negative estimate means a decrease. Number of observations = 3303, over the 15 different groups (Litters).

Treatment or Day	Estimate	Error z	p-value
Single Dose	-1.24	0.46	0.008
Double Dose	-1.78	0.54	0.0009
Postnatal day 11	-1.04	0.31	0.0008
Postnatal day 14	-3.88	0.74	<0.0001
Postnatal day 18	1.00	0.24	<0.0001
Postnatal day 21	2.85	0.27	<0.0001

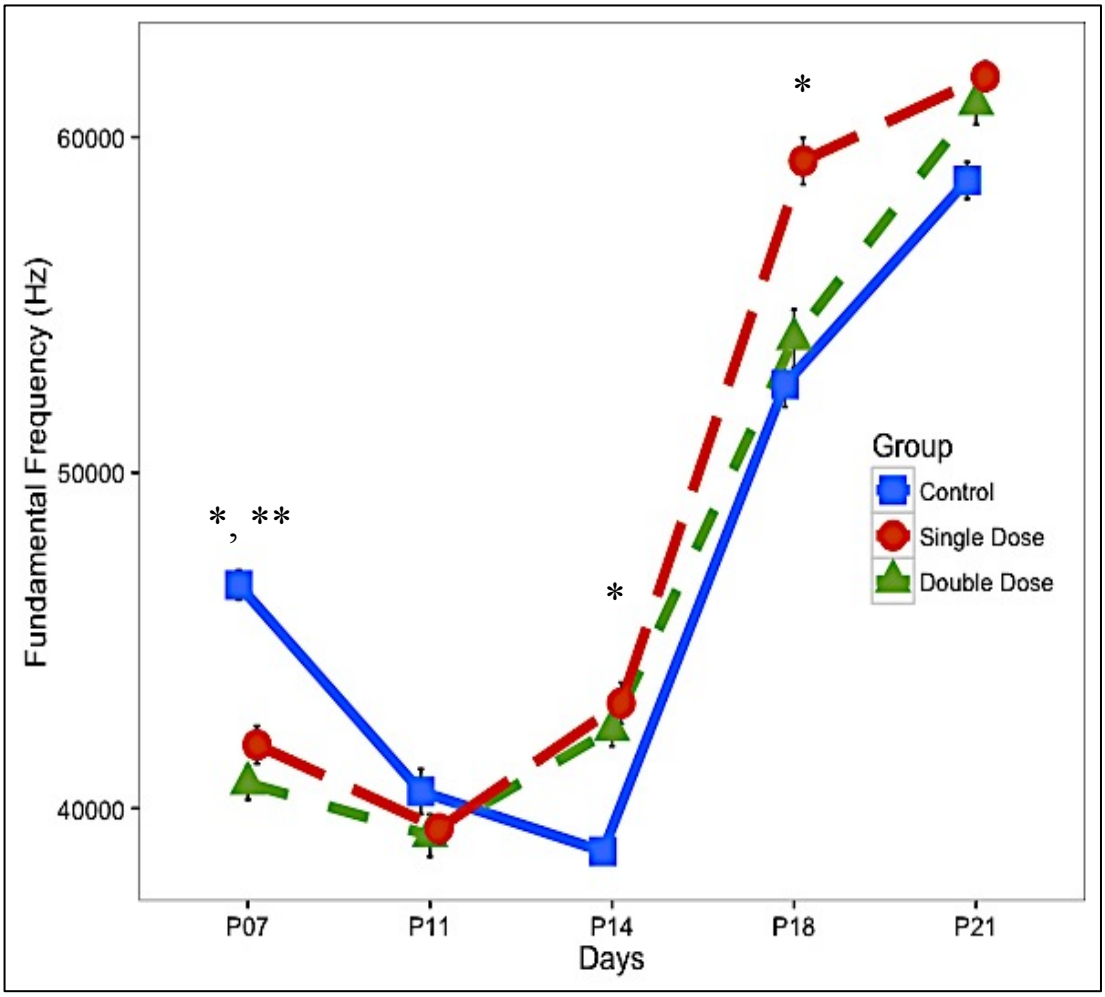

Figure 4.4: The mean fundamental frequency in kHz for each treatment group over the 5 days of development. Significant differences between the single dose and controls were found on P7, P14, and P18 (*), where as differences between the double dose and controls was found on P7 (**).

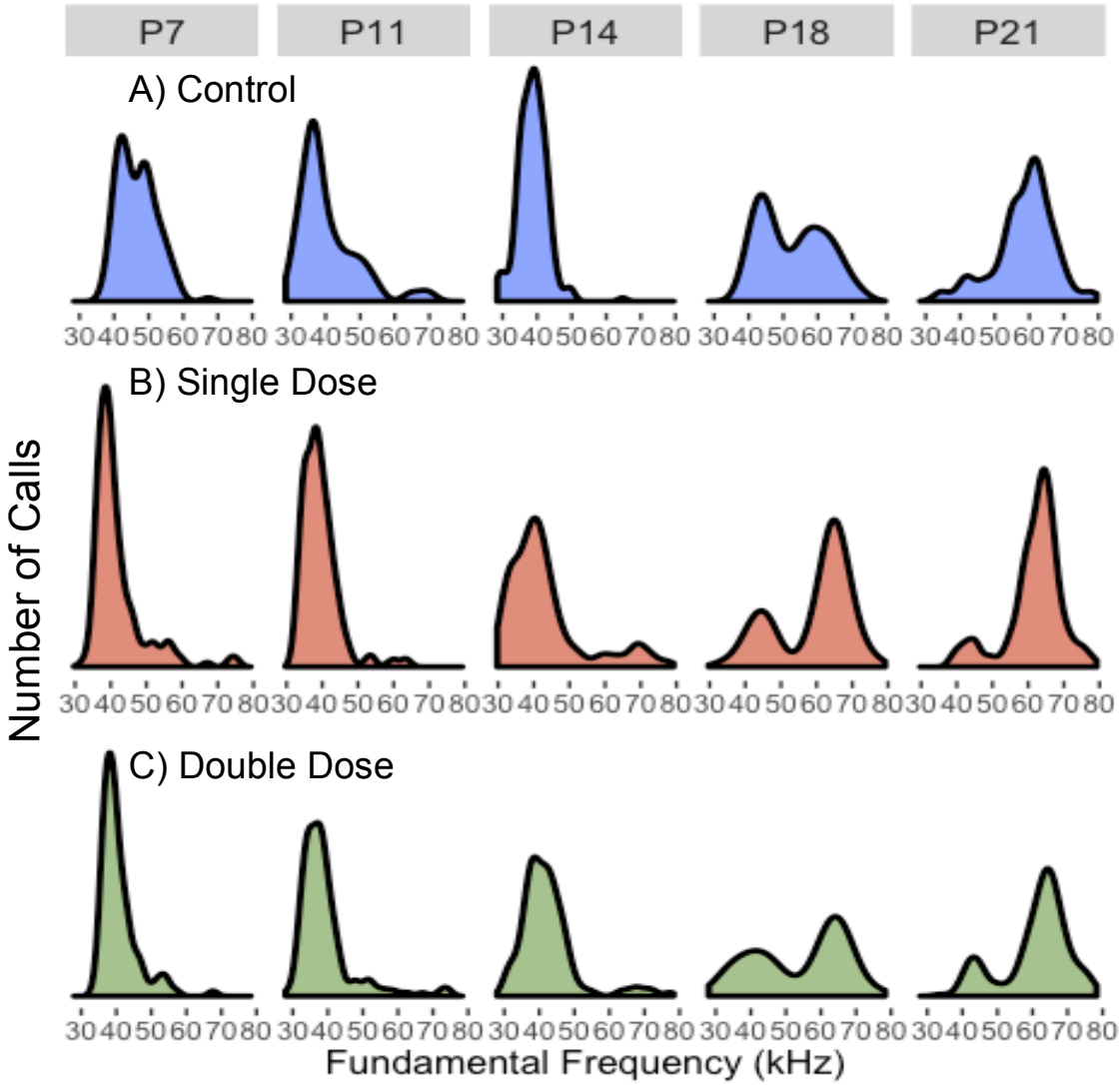

Figure 4.5: The distribution of the number of calls as a function of the fundamental frequency (kHz). A) Shows the distribution of the fundamental frequency for the controls, as can be seen majority of calls cluster around the infantile 40 kHz range from P7 to P14. On P18, a bimodal distribution begins to emerge, and on P21, the majority of calls cluster in the 65-70 kHz adult range. B) Shows the distribution of the fundamental frequency for the single dose litters; the majority of calls cluster around the 40 kHz range on P7, P11, and P14, however a bimodal distribution begins to emerge on P14 at which point the calls switch to around 65-70 kHz. On P18, the bimodal distribution favours calls around the 65-70 kHz range, this trend is continued on P21. C) Shows the distribution of the fundamental frequency for the double dose litters. With the double dose group, a similar trend to that seen in the single dose condition was observed where the majority of calls on P7, P11, and P14 are at 40 kHz, while the bimodal distribution begins to emerge on P14. As with the single dose, the majority of calls on P18 and P21 are at the 65-70 kHz range.

4.5 Discussion

The aim of the present analyses was to assess whether prenatal exposure to a single or double dose of VPA results in changes to the natural development of USVs. The significance and efficacy of *in utero* exposure to a single dose of VPA on offspring behaviour and neuroanatomy is well characterized. The question asked was, can a single or double dose of VPA produce impairments or changes in UVs? If such a change occurred it was proposed that this could be used as a symptom in an animal model of human autism disorder. It is well documented in the literature that autism is a spectrum disorder, with some individuals being highly functioning and others being low functioning (Roullet et al., 2013). Therefore, an animal model that produces a similar varying degree of deficits would be useful in further studying and understanding the etiology of the disorder.

Analyses of USVs have been shown to be a sensitive measure of neurodevelopment, and changes to USV call rate have been shown to be affected by perinatal pharmacological treatments in the absence of changes to other behaviours, such as locomotion (Branchi et al, 2001). While the developmental onset of the peak call rate is debatable, with some papers citing the peak to be around 10-12 days of age (Brudzynski et al., 1999), it is important to note that there is great strain variability (Hahn & Lavooy, 2005). With regards to the present analysis, in controls there is a peak in the number of vocalizations emitted on P14, and this peak decreases in the single dose group (P11) and the double dose group (P7) (see Figure 4.1). The double dose group could possibly peak earlier since USV recording did not start until the 7th day of life.

The current literature, as discussed in Chapter 3, supports a linear model of USV development. Infants vocalize in the 40kHz range; these calls are long in duration and flat (not complex), and as the rodents develop their calls become more complex, shorter, and display the more adult typical 50kHz frequency ranges (Brudzynski et al., 1999). In the present study, the peak number of vocalizations emerges earlier in the VPA groups in a dose-wise manner, however a dose dependency was not seen in the fundamental frequency of USVs. When looking at the distribution of the USVs (Figure 4.5), there is an earlier emergence of the bimodal distribution in the single dose group.

I propose that adult calls replace infant calls. This is supported by the frequency distribution over time data. The control animals begin to show a bimodal distribution somewhere between P14 and P18, and by P18, there is a very clear bimodal distribution of calls. For the VPA treated groups, the bimodal distribution begins to emerge on P14 for the single dose group. This points to early development of adult-like USV patterns. This trend is also confirmed by looking at the average frequency of the calls. As shown in Figure 4.4, on both P14 and P18 the single dose groups' average frequency is significantly higher than the control group. This increase in average frequency is the result of an early emergence of the adult calls.

Interestingly, the only significant change in the double dose VPA group is on P7 for the average fundamental frequency. The more general lack of significant change in the double does condition may be due to a neuroprotective effects of valproate when given in large doses. Recent research has found that animals with spinal cord injuries or ischemic stroke have improved recovery after VPA treatment. Specifically, VPA protects surviving neurons from secondary damage and stimulates neurogenesis. The mechanisms

underlying this protection are associated with many signaling pathways mediated by HDAC inhibition and GSK-inhibition which both play an integral role in cell death (Chu et al., 2015). A group of researchers had found that chronic dietary administration of, on average 1400 mg/kg/day, of VPA had neuroprotective benefits against an animal model of neurodegeneration (Eleuteri et al., 2009). Therefore the lack of changes seen in the double dose group could be the result of a neuroprotection obtained from the second dose. More research will be needed to investigate this hypothesis, but it is advanced as a tentative explanation of the results in the present study.

In both human and animal research it has been established, through analysis of ontogenetic profiles, that developmental trajectories can be impacted in several different ways by various pharmacological and environmental factors. The first way is that a trajectory may be increased; in this case experimental development overlaps temporally with control development, however, the mean values are higher. The second way is through a decreased trajectory; this has the same temporal overlap as well, but instead has a decrease in mean value. A third way that a developmental trajectory can change is by precocious development in which mean values are not affected, however behavior begins earlier. A fourth way, delayed development, is similar to precocious development; there generally is no change to mean value, but the developmental period begins later. A fifth way that development can be altered is through an extended or narrowed period, where the time the development occurs is lengthened or decreased. Figure 4.6 shows a summary of the various ways development can be impacted. Finally, to make things more complex, developmental impacts can include a combination of the above-mentioned trajectories.

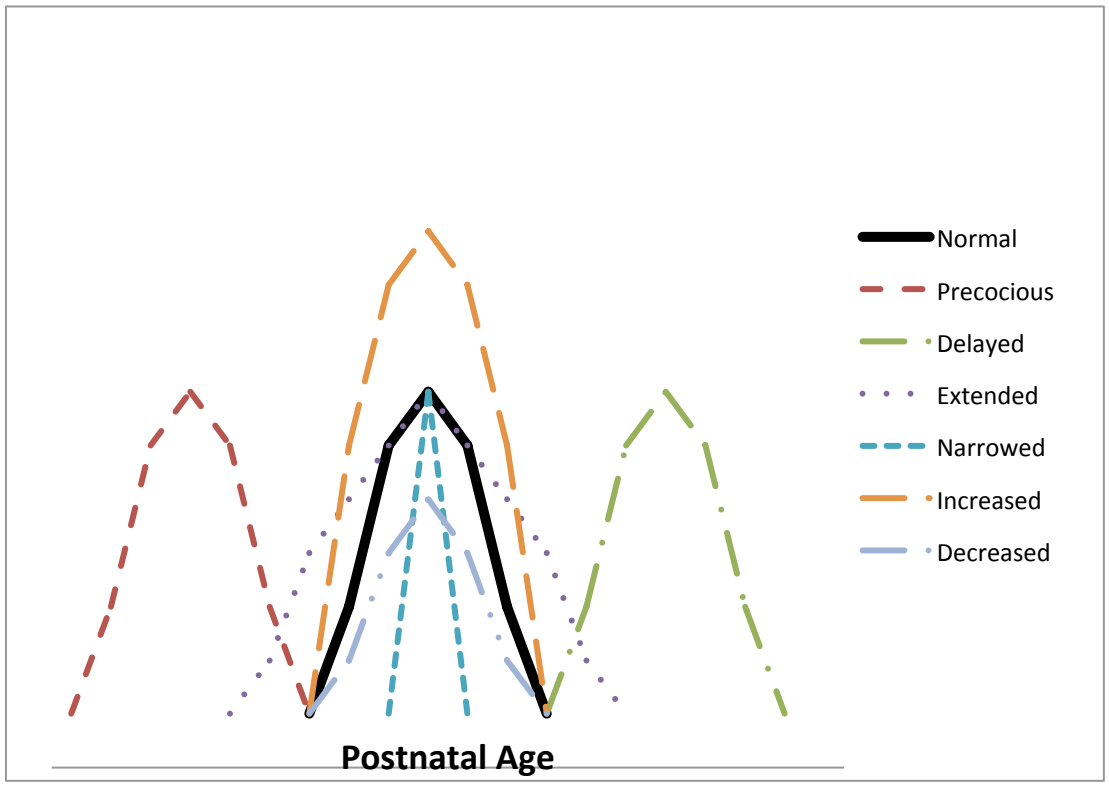

Figure 4.6: Representation of the various ways development can be impacted. The solid black line depicts the typical or normal development. There is a definite start and end point to the development (duration), as well as, a peak in this development. The dotted lines represent the ways in which this development can be impacted. There can be changes to the start and end points, thereby affecting duration, as well as changes to the peak, whether that be increased, decreased, or shifted.

Based on the different ways development can be impacted, the distribution of the fundamental frequency in the development of UVS following VPA seems to be precocious. The bimodal distribution begins on P14 in the treatment litters and later in the control group. Also, in support of the precocious development idea, the number of calls seen on P18 for the control group is clustered around the 40 kHz, infantile call range, while the treatment groups calls similar clustering is in the 65 kHz range. This finding supports the idea that the adult typical calls are emerging earlier and reaching adult typical levels earlier in the treatment litters. Furthermore, it can be seen in Figure 4.4, that on P7 there is a significant reduction in the average frequency of vocalizations in both treatment groups compared to controls. In the control litters, there is a reduction in the frequency on P11 and P14 compared to P7 before increasing on P18. The increase we see in controls on P18 is shifted to P14 in the treatment groups. This suggests that the higher frequency seen on P7 in controls may have shifted to an earlier time point in the treatment groups. Because P7 was when recordings began, future research should examine USVs before P7.

There is the same pattern of precocious development in call duration. On P7, for both treatment groups, the duration is longer, closer to levels seen on P11 in the control group. The effect is not statistically significant, but it is suggestive. On P18 there is a significant reduction in duration in the single dose group, which is more like the adult pattern. It could be possible that for the control group, duration peaks after that of the single dose group, between P14 and P18. This trend would also make sense of the drastic reduction in duration found on P18 in the single dose group. For future studies, recordings on all development days would provide useful information on exact timing of the peaks in

the ontogenetic profile of duration. Others researchers (Tonkiss et al., 2003) have found that prenatally malnourished pups showed increases in call frequency and duration without affecting call rate. They equated this finding to other findings in the human literature where altered crying acoustics are a result of CNS developmental perturbations (Tonkiss et al., 2003).

The altered acoustics reported in the current study are suggestive of alterations to the natural developmental trajectory produced by VPA. According to the synaptic hypothesis of ASD, ASD may be the result of abnormal cellular and synaptic growth and an imbalance between inhibitory and excitatory brain circuitry. This inhibitory/excitatory imbalance is supported by the comorbidity of ASD and epilepsy. Both symptoms may be the result of an alteration in the neurotransmitter γ-aminobutyric acid (GABA). In the mature brain, GABA acts as an inhibitory neurotransmitter, but in the developing brain has an excitatory role. GABA also plays a role synaptic pruning, the loss of synapses during development, and therefore is connected to the timing of critical periods. Thus, an imbalance in GABA could result in altered critical periods (Bourgeron, 2009; LeBlanc & Fagiolini, 2011; Rinaldi et al., 2008). Because other investigators have found a role for GABA in the modulation of USVs (Insel, Hill, & Mayor, 1986), the altered acoustics reported here could be symptomatic of an excitatory/inhibitory imbalance in brain development caused by VPA. The precocious development of UVs in the VPA treated animals could be the result of the alterations GABA. The synaptic hypothesis of ASD may thus help explain the precocious development seen in the age of peak calling and in the fundamental frequency.

As a final note, Hahn & Lavooy (2005) in a literature review, report that 99% of studies of rodent USVs report call rate, with fewer (21%) reporting frequency and duration. A first take away lesson from the present study is that it is important to assess multiple acoustic features because each may be affected by a treatment in different ways. A second take away lesson is that analyses limited to a single day may be miss key developmental information (Branchi et al., 2006).

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Chapter 5

General Discussion

The research conducted and discussed in this thesis adds to the growing body of literature on infant rat ultrasonic vocalizations (USVs). Currently there is a gap in understanding how USVs develop and transition into adulthood. The present work builds on an important study in understanding USV development that was conducted by Brudzynski and colleagues (1999). In that study, the authors assessed the development of USVs in Sprague-Dawley rat pups, specifically looking at three measures of USVs, frequency, duration, and bandwidth. In addition, they created a 10-call category catalogue to assess complexity. There study used the isolation-induced method, wherein a single pup is removed from its dam and littermates for a period of time during which its vocalizations were recorded. They recorded USVs from 24 litters on postnatal day (P) 10, P15, and P17. They found that over time the duration of calls increased from P10 to P15, and the bandwidth and frequency increased over all three days studied. When assessing the individual call categories, they found that the pups use more complex calls as they get older.

The objective of the present thesis was three-fold. The first objective was to determine whether similar changes in USVs to those reported by Brudzynski and colleagues (1999) occurred when recordings were taken from rat pups in less stressful situation. To this end, in the present thesis, USV were recorded from rat pups that remained in the litter. The second objective was to determine whether there was a gradual transition form infant to adult USV patterns or whether there as a discrete change. This question was answered by recording from more postnatal days. The third objective was to assess whether a manipulation of development induced by valproic acid treatment, a

treatment that produces an animal analogue of autism spectrum disorder, could be detected by changes in the infant rat vocalizations.

With respect to the first two questions, in agreement with the findings from Brudzynski and colleagues (1999), the duration of the calls obtained using the whole litter method increases from P7 and peaks on P14 before decreasing to adult typical calling durations (Brudzynski et al., 1999; Sales, 1972). My finding that the average frequency increases over time (reported in Chapter 3) is also similar to that reported by Brudzynski and colleagues. The information from frequency change, average frequency, and the length of the USVs supports the hypothesis that infant USVs develop gradually overtime and transition into adult typical calling patterns.

A different trend is observed, however, when the distribution of the fundamental frequency was analyzed. This finding suggested that infant calls may be replaced by adult calls. This abrupt maturation was reflected in bimodal distribution on P18, of the infant peak of 40 kHz and the adult peak 60 kHz co-occurring that was reversed on P21, at which age the majority of calls were in the 60 kHz range. This trend has not, to my knowledge, been published in the literature. Thus the distribution of fundamental frequency may offer a new method of analysis in the future.

The third question asked in this thesis is whether USV analysis is sensitive enough to examine disruptions to neurodevelopment. The valproic acid (VPA) model of autism has been shown over the past decade to have both face and construct validity as a model of autism spectrum disorder. *In utero* exposure of rats to VPA on gestational day 12.5 has repeatedly been shown to have negative behavioural and neuroanatomical effects that

mimic the clinical manifestations of autism spectrum disorder (ASD). Few animal studies are done, however, on one of the core behaviours used to assess and diagnose ASD, social communication (Roullet et al., 2013). Nevertheless, previous research has found that *in utero* exposure of mice to VPA leads to a reduction in USVs call rates and delays in processing complex auditory stimuli (Gandal et al., 2010). Raza (2015) found that VPA exposure reduced the number of calls during play behaviour when compared to the play of control animals. These studies demonstrated that the VPA model does produce abnormalities in rodent USVs.

Recent research on language acquisition has shown the impact that the environment can have on its development. Weikum and colleagues (2012) examined the development of infant language as affected by prenatal expose to serotonin reuptake inhibitors (SRIs). SRIs are antidepressants and mood stabilizers and have been shown to alter neural plasticity and shift sensitive periods. They tested infants at 6 and 10 months from non-depressed mothers who had not taken SRIs, depressed but non-treated mothers, and depressed mothers who had been treated with SRIs. They found that prenatal SRI exposure accelerated the development of speech perception (Weikum et al., 2012). Further research into SRIs reveals an increased risk for developing ASD with *in utero* exposure, outlining a possible connection between environmental exposures, developmental disorders, and impairments to social communication (Gidaya, et al., 2014).

This thesis adds to the list of deficits in social communication by showing that there are changes in the number of vocalizations made and to the specific acoustic characteristics of the vocalizations in VPA treated rats. Specifically, an early emergence of peak number vocalizations was observed in a dose-dependent manner. Animals that

received a double dose of VPA, display a peak in the number of vocalizations before the single dose group does, while both treatment groups peak before the control animals. Also observed was an alteration to the duration of the vocalizations as well as its frequency. Furthermore, on P18, VPA exposed animals emit more calls in the adult typical frequency while controls are still emitting more calls in the infantile range.

The data presented in this thesis are different from what Gandal and colleagues (2010) found in their VPA exposed mice. They found a reduction in the number of USVs emitted. It is important to note, however, that recent research into critical periods has lead to the idea that impairments underlying neurodevelopmental disorders may have their onset or are the result of altered critical periods (Wang, Kloth, & Badura, 2014). Thus, exactly when a treatment is given will influence the effect that it has. GABA is an important neurotransmitter in the brain as it is linked to synaptic pruning, and is important for developing the necessary excitatory and inhibitory balance in the brain. GABA is likely a key player in establishing critical periods and an imbalance in the GABA-related excitatory and inhibitory synapses may lead to some of the symptoms of ASD. Previous research has shown that VPA exposed rats have impairments in GABAergic transmission (Banerjee et al., 2013). The changes that we see to the acoustics may in fact reflect the underlying changes to the GABAergic synapses that are affected by exposure to VPA.

Looking at the developmental patterns for USVs that were reported in Chapter 3, in combination with the experimental data from Chapter 4, it is proposed that a whole litter study of USVs could provide a valid model for studying neurodevelopment. The question that needs to be addressed is what is the purpose of the USV calls that are made during these recordings. They may reflect anxiety. It is well documented that the

isolation-induced or temperature-induced calls are a measure of anxiety. Previous research has shown that calling rates can be increased or induced by anxiogenic compounds and decreased by anxiolytic compounds (Insel, Hill, & Mayor, 1986). Moreover, isolation-induced USVs have been shown to be affected by other pharmacological manipulations that affect anxiety, such as alcohol (Barron & Gilbertson, 2005) and cannabinoids (Branchi, Santucci, & Alleva, 2001).

In an effort to distinguish whether or not the vocalizations being recorded in this thesis were due to the mother being taken away, a pilot study was run on 2 litters of animals. On all 5 days of recording, vocalizations were counted in the condition where the mother was present and absent. There were no significant differences in the number of vocalizations emitted. This study lends credence to the notion that the USVs recorded are not stress-induced vocalizations. Moreover, a heating pad was used throughout the testing procedures thereby ensuring that the calls that were recorded were not due to temperature decreases. This evidence, supports the idea that the calls being analyzed are not due to anxiety but are the natural sounds produced by the pups during development.

The calling patterns observed over development in the present study also does not support the idea that infants only produce a limited range of calls. Vocalizations graphed in Figure 3.3 show that the majority of the calls are made in the 30-60 kHz range. Nevertheless, there are some calls made below 40Hz and some above 70 kHz, even though they are few. This provides evidence that the pups are physically capable of calling above and below 40 kHz. Because the majority of calls are at 40Hz there must be some adaptive advantage to calling in the 40 kHz range.

One of the limitations of the analysis that is presented in this thesis is the fact that recordings ended at P21. One can offer two obvious hypotheses on how the vocalizations complete their transition beyond P21. The first is that the infant 40 kHz calls are fully replaced by the adult calls. The second is that there is a retention of the infant calls throughout adulthood. There are currently no reports in the literature that supports or refutes either hypothesis (Brudzynski, 2013; Sales & Pye, 1974; Wright, Gourdon, & Clarke, 2010). Clearly, analyzing how calls change after P21 will provide further insight into how infant vocalizations develop into adult calls.

Having an understanding of how USVs develop in ages beyond P21 would also be useful in the assessment of neurodevelopmental disorders. In the VPA model it is likely that there are impairments that continue past P21. This knowledge would be useful in studying treatments for neurodevelopmental disorders and it would allow researchers to assess if treatments are effective and if treatments in the infant period have an effect later in life. In order to gain a better understanding of how neurodevelopmental disorders are impacted, a clearer picture of the natural development of rat pups into adulthood, needs to be developed.

One avenue for future research would be to look at the vocal behaviours of older groups of rat pups in the testing chamber. The emergence of the bimodal distribution seen in this thesis may reflect changes to the social dynamic of the group. As newborns, rats lack the ability to thermoregulate, therefore they huddle in order to maintain a consistent temperature, this is called *physiological huddling*. At around P15 rats are able to thermoregulate and their eyes and ears are open. They no longer need to huddle for warmth but prefer to huddle with a sibling, over another age-matched rat, this is the

transition to *filial huddling* (Alberts, 2007). Also around this time point is where play behaviour begin to emerge (Baenninger, 1967). With the recent literature supporting the hypothesis that USVs play an integral role in mediating, initiating, and maintaining play behaviour combined with the importance of play in rats, it is expected that changes in the patterns of calls occurs (Burke et al., 2017; Himmler et al., 2014; Pellis, 2010)

Another weakness, in the literature on rat pup vocalizations, is the lack of knowledge of the influence of sex differences in USVs. To date no sex differences are reported in the development of infant USVs. Sex differences have been reported as a result of various conditions such as call rates induced by temperature (Blumberg & Stolba, 1996). Naito and Tonoue (1987) report that litters with altered sex ratios showed differences in call rate. Males called more when in a mixed litter than in an all male litter, while the females call rate was relatively unaltered with changes to the sex ratio (Naito & Tonoue, 1987). Nonetheless, the majority of studies use males only and so may be missing some of the finer details of sex-related USV development (Ahrens et al., 2009; Antonelli et al., 2005; Laloux et al., 2012; Lehner et al., 2014; Maier et al., 2010; Tonkiss & Galler, 2007; Willey & Spear, 2013). It would be expected that a more detailed study of sex differences would provide insights into sex-related USV differences because there are differences in other behaviours , i.e. pharmaceuticals or environments may affect one sex differently and that may be picked up in USV analysis even though no sex differences are reported in typical development (Gillies & McArthur, 2010; McCarthy, Vries, & Forger, 2017).

One of the biggest limitations is the individual differences seen between animals, this may be due to differences in genetic backgrounds. Brunelli and colleagues (1997)

were able to selectively breed rats into high calling (High USV) and low calling (Low USV) lines. Based on calling rate on postnatal day (P) 10 rat pups were separated into either the high calling groups or low calling groups and later bred with another rat from the same line. After 5 generations, analysis of infant isolation induced USVs revealed success in separating the lines (Brunelli et al., 1997). Subsequent research on these lines reveals co-selection of other behaviours. Specifically there are alterations to acoustic parameters (Spence et al., 2016), maternal behaviour (Brunelli et al., 2015), and play behaviour (Brunelli et al., 2006) in the two lines. The high calling line shows more anxious phenotypes and the low calling line shows more aggressive behaviour as adults relative to control animals (Brunelli & Hofer, 2007). Possible individual differences may be missed when recording from the entire litter, creating a sampling error. This effect was mitigated by using a linear mixed effect model in the present analysis. This statistical model is able to factor in random errors along with fixed effects. In the case of this thesis, while the fixed effects were controlled for in the study design, such as the days that we recorded on and what litters got what treatment, there is no way to account for specific genetic phenotypes.

Another possibility for future work is to examine neural pathways underlying the production of infant USVs. Although not as much research has been devoted to looking at infant USV pathways as adult ones, previous research into the production of isolation-induced USV's reveals influences from the limbic system. Cox and colleagues (2012) found that rats with prenatal exposure to cocaine, which they argue affects the limbic system, show altered vocalizing rates. In addition, they found alterations to the ventral medial hypothalamus and central amygdala that correlated with the altered acoustics of

USVs (Cox et al., 2012). The main question here with regards to the findings in the current thesis is: is the neural basis of vocalizations obtained using the litter method the same as that for isolation-induced calls? Examining the answer to this question will lead to a better understanding of the current results and potentially the contributions of neurodevelopment to USVs.

Finally, it is important to understand the adaptive function of infantile calls is. It was originally believed that the USVs produced by pups were a byproduct of laryngeal braking (Blumberg & Alberts, 1990). Although this is now an outdated view, the initial hypothesis stated that the USVs were a byproduct of a decrease in oxygen due to a rapid cooling of the body. To combat the heat loss support of brown adipose tissue (BAT) thermogenesis occurs (as pointed out in Chapter 1), along with an increase in oxygen to the lungs via increased USVs. This seems to not be the case, as pointed out by Hofer (1996). Hofer argues that in the initial study there were no manipulations to BAT or the USVs and subsequent investigations have failed to draw a relationship between the two. Furthermore in the current study, pups were kept on a heating pad and were able to huddle to keep warm, and they still vocalized.

5.1 Conclusions

Rodent USVs have been proposed to serve a communicative purpose. Infant distress USVs have been shown to be a signal to the mother that stimulates their retrieval when they stray from the huddle or nest. The present thesis examines USVs emitted by infant rodents in a more natural context; in a temperature-controlled environment and in a non-isolated condition. The attempt was to create a developmental profile for the

transition of infant typical calls into adult typical calls. The litter recording method offers a new way of examining the neurodevelopment of pups and can serve as a sensitive method of studying development. Using the VPA model of autism, it was demonstrated that acoustic changes could be detected and this finding adds to the growing understanding of the etiology of ASD. In conclusion, the method that is used in this thesis provides a time-effective, noninvasive measure of neurodevelopment that can be used to study neurodevelopmental disorders.

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