Background and Significance

In most mammals, separation of the infant from its mother (or caregiver) drives infant vocalization (such as a baby's cry in humans) (1-8). Infant crying in many mammalian species share similar acoustic tonal structures [2]. Illustrating these common features, mother deer respond similarly to infant cries of several mammalian species as she would to her own [2,6,12]. The leading hypothesis is that infant vocalization emerged early in mammalian evolution as a behavioral adaptation for the necessity of mammalian infants to rely on their mother for survival. An alternative view would be that a great deal of convergent evolution occurred among a variety of different mammals (3), meaning that this same behavior evolved among species independently. One way to formally test these assumptions would be to uncover the neuronal circuits and the genetics involved in infant vocalization. For example, having a common neural pathway that gives rise to these infant vocalizations across species would make a parsimonious argument that infant vocalization and caregiver responses are highly conserved. In other words, it would suggest that infant vocalization appeared once in early mammalian evolution [2,6,13]. During my PhD, my overall goal is to bridge this fundamental gap in our knowledge by studying the emission of separation-induced vocalization in mice (*Mus musculus*).

When socially isolated, infant mice emit ultrasonic vocalizations in the frequency range between 30 - 110 kHz (**Figure 1A-B**) (9-11). The genetic and neuronal mechanisms by which infant mice produce these ultrasonic sounds are mostly unknown. Previous studies have mostly used other animal models to identify the neural circuits involved in vocalization, including guinea pigs and nonhuman primates (3). Several brain regions were shown to modulate or mediate the emission of vocalizations, including the periaqueductal gray region (PAG) in the brainstem, the amygdala, and the anterior cingulate cortex (3, 12-16). However, most of these studies were performed in juvenile or adult animals, but not in infants. An exception seems to be the PAG, which mediates ultrasonic vocalizations by infant rats as well (17).

Evidence for the evolutionary conservation of the mechanisms of vocal emission in mammals is illustrated by the anatomy of the vocal organ, the larynx (18). The gross anatomy of the larynx is conserved among mammals, including mice (19, 20). Premotor output to the larynx is transmitted via the superior and recurrent laryngeal nerves. In mice, transection of the recurrent laryngeal nerves dramatically reduces the emission of ultrasonic vocalizations in adults (21), similarly to other rodent species (22-25). The recurrent laryngeal nerves extend to the chest and then return to innervate the larynx. In large mammals, such as the giraffe, these nerves can reach five meters long, a finding that supports the conservation of the motor innervation of the larynx. Here, I propose that - in addition to these premotor circuits - modulatory circuits in the brain involved in the emission of infant vocalizations are highly conserved across mammals. *I will use mice as a tractable model organism to identify for the first time the neuronal circuits and the*

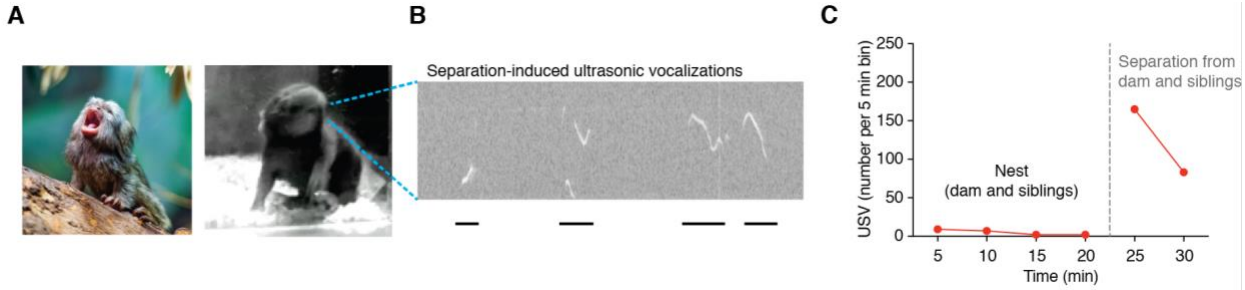

Figure 1: Separation-induced vocalization in infants is conversed in mammals. (A) Illustration of a nonhuman primate vocalizing when separated from its caregiver (left) and a laboratory mouse vocalizing when separated from its dam and siblings (left). (B) Spectrogram illustrating the emission of ultrasonic vocalizations from the infant mouse; these vocalizations present a variety of shapes and durations. (C) Quantification of the rate of emission of ultrasonic vocalization; initially, vocalizations were recorded in the nest (very low levels); upon removal of the dam and siblings the mouse pup started to vocalize at high rate.

genetics involved in the emission of infant vocalizations. To accomplish my overall goal, we plan to develop the following specific aims:

Aim 1. Elucidate the neuronal circuits involved in separation-induced vocalizations.

1.1 Introduction. Here, we seek to identify neuronal circuitries involved in the emission of infant vocalization in mice. In preliminary experiments, we found that Agrp neurons in the hypothalamus are critical for the emission of separation-induced infant vocalization. In this objective, we plan to use Agrp neurons as a starting point to identify downstream neuronal circuits involved in infant vocalization. To accomplish this goal, we will combine viral tracing, optogenetics, and in vivo calcium imaging applied to the study of mouse infant behavior. At its conclusion, this objective will reveal circuit-level mechanisms that govern infant vocalization behavior in mice.

1.2 Background and preliminary data. When isolated, most mammalian infants vocalize, a behavior that attracts the caregiver. The neuronal circuits involved in infant vocalizations are mostly unknown. We reasoned that we could identify brain circuits involved in infant vocalizations by studying *Mus musculus*, a more tractable organism. In preliminary experiments, we used unbiased whole-brain mapping of activated neurons in ten days old mice upon isolation from mother and siblings. We found the arcuate nucleus of the hypothalamus to be a region highly labeled by social separation (not shown). We further identified a population of neurons in this brain region labeled with c-Fos that were positive for the gene *Agrp* (**Figure 2**). These results suggested that Agrp neurons could be involved in the physiological and behavioral adaptations of ten days old mice to social separation, including the emission of vocalizations.

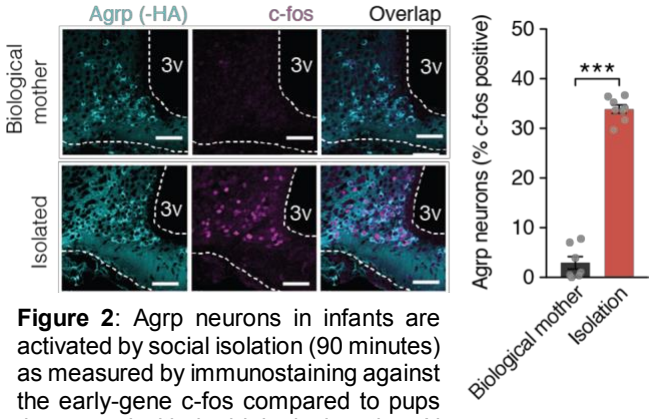

Figure 2: Agrp neurons in infants are activated by social isolation (90 minutes) as measured by immunostaining against the early-gene c-fos compared to pups that stayed with the biological mother. N = 7 per group.

The neurons in the mammalian hypothalamus that produce Agouti-related peptide (Agrp neurons) also produce GABA. Because Agrp neurons were active upon social separation, we tested the extent to which the release of the neurotransmitter GABA from Agrp neurons was involved in the emission of vocalizations upon isolation. In mice lacking the GABA transporter Vgat specifically in Agrp neurons (*Agrp*$^{Vgat\text{-}KO}$ mice) – i.e., unable to release GABA in postsynaptic targets – we found a suppression in the emission of vocalizations upon social separation (**Figure 3A-B**). In these preliminary studies, we also performed a gain of function experiment, activating Agrp neurons in ten days old mice immediately after separation and found an increase in the emission of vocalizations (**Figure 3C**). We also found that the artificial activation of Agrp neurons led to an increase in the emission of vocalizations in fifteen days old pups, but not in twenty-one days old pups (**Figure 3D**). This last result reinforces the importance of studying infant animals to understand the mechanisms involved in infant vocalization, instead of inferring from the work in adult animals.

1.3 Research Design

1.2.1. Anterograde and Retrograde Viral Tracing of Agrp Neurons and the Larynx Muscle: My first step is to identify what inputs of the laryngeal muscles overlap with projections downstream Agrp neurons in neonatal pups (postnatal day 15; PND15). To do this, I will use both anterograde and retrograde viral approaches. Specifically, I will inject Cre-dependent anterograde herpesvirus (H129DTK-TT) in Agrp-Cre mice at PD13, this will ensure that the infection of the virus is specific to Agrp neurons and that it is fully expressed by PND 15. This virus will allow

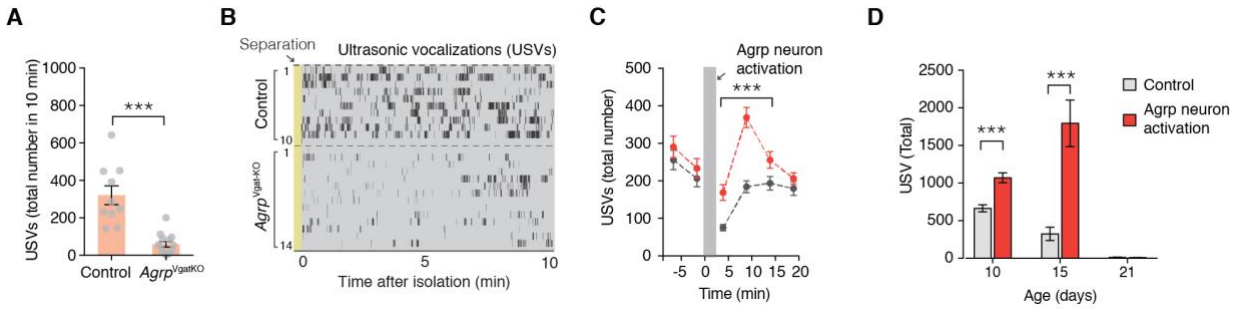

Figure 3: Agrp neurons in infants modulate separation-induced ultrasonic vocalizations. (A) Upon separation from mother and siblings, ten days old Agrp$^{Vgat\text{-}KO}$ showed impaired vocalizations during a 10 min test. (B) Raster plot of the data from A. (C) Activation of Agrp neurons potentiated the emission of ultrasonic vocalizations in 10 days old mice. (D) Time course of activation of Agrp neurons induced vocalizations (in 10, 15, and 21 days old mice). N = 7-35 per group. P < 0.001 (t-test).

polysynaptic labeling of neurons that Agrp neurons projects to. At the same time, I will also inject retrograde viral tracers (GFP-Pseudorabies Bartha strain) directly into the laryngeal muscles to label neurons that project onto these muscles. Once injected, I will wait 48 hours to collect brains of the pups and estimate connectivity between the larynx and Agrp neurons. *Regions with overlapping fluorescent labels (tdTomato for Agrp neurons, and GFP for larynx) will become candidate targets for circuit analysis.* These experiments will be executed in collaboration with Dr. Ivan de Araujo (Moun Sinai Hospital, New York), who has extensive experience in using viral vectors to study neuronal circuitries and has collaborated with our research group before. Our goal is to have at least five mouse brains labeled to compare.

1.2.2. <u>*Use optogenetics to activate candidate projection sites:*</u> My next step will be to activate candidate projections downstream Agrp neurons using optogenetics. Guided by the tracing studies, we will use optogenetics to activate Agrp neuron projections in pups while measuring their USV emission rate. Optogenetics requires the expression of light sensitive receptor in the neurons of interest, in order to use light to activate the neurons during behavior experiments. The lab has been successfully using Cre-dependent adeno-associated virus (AAV) to express the light sensitive receptor (ChR2) in mouse Cre lines tested as young as PND 14**.** I will use our established protocol and inject Cre-dependent AAV in ARC of Agrp Cre mice at PND 0 to ensure full viral expression by PND 15. At PND 13, I will insert fiber optics into brain regions containing candidate Agrp projections. Our lab has done fiber optic insertions in ARC of PND 13 pups successfully in the past. Pups are able to recover from the surgery for the insertion of the fiber in a day. Therefore, at PND 15 we will activate projections using pulses of blue light delivered through the fiber optic to activate ChR2 while USV emission is measured. We will compare ChR2 mice against mice with GFP injections, and against themselves without activation. Our goal is to have a sample size of 5 mice per condition for control and experimental groups.

<u>Feasibility:</u> We do not foresee any limitations with our viral tracing experiments. I have done similar procedures at these ages so far and it has worked well. However, optogenetic activation of Agrp projections may have some difficulties. Our experiment set up of viral infection and cannula placement in pups of young age is of low yield. So far, 10% of the animals used in these experiments have presented good viral expression and probe location. An alternative method to optogenetics is to ablate candidate projections using diphtheria toxin. Mice with diphtheria toxin receptors are available in the lab; however, I am still in the process of learning the protocol for correct use.

<u>***Aim 2. Identify candidate genes for modulation of ultrasonic vocalization emission in pups through the Mouse Collaborative Cross***</u>

2.1 Introduction. In this aim, my *overall objective* is to uncover the neuronal and genetic substrates involved in the emission of separation-induced vocalizations in infant mice by studying genetically distinct mouse strains. My *working hypothesis* is that the study of genetically distinct

mouse strains that present ample variability in infant vocalization will allow the identification of novel neuronal networks and the genetics underlying this behavior. We will *approach* this working hypothesis by taking advantage of modern tools of whole-brain activity mapping and the resources provided by the Mouse Collaborative Cross Consortium. The Mouse Collaborative Cross generated hundreds of recombinant inbred (RI) strains of mice based on random intercrosses between eight genetically diverse founder lines (26-29). We will phenotype a portion of these strains for separation-induced infant vocalizations to identify the genetic loci involved in this behavior. The research in this aim is *necessary* as by using *Mus musculus* and the new technologies to study neuronal networks and genetics, we will uncover the mechanistic details underlying mammalian infant vocalizations. We expect our *overall outcome* will be the identification of new neuronal circuits that are activated in the brain of mice that vocalize upon separation. We also expect to identify novel genomic loci that are involved in the manifestation of this conserved behavior. The results of this intentionally exploratory aim will open many new research directions, benefiting my long-term goal of studying the evolution of infant vocalizations in mammals.

2.2 Background and Preliminary Data. To profile the emission of vocalizations in infant mice from diverse genetic backgrounds, we took advantage of the genetic heterogeneity of the Mouse Collaborative Cross (CC) (26-28, 30) (**Figure 4**). The eight founder lines present ~90% of genetic diversity, similar to what is observed in the human population (30). These founder lines include three wild-derived subspecies - WSB/EiJ from *Mus musculus domesticus*, PWK/PhJ from *Mus musculus musculus*, and CAST/EiJ from *Mus musculus castaneus* – and five inbred strains that are mostly from a *Mus musculus domesticus* origin (A/J, C57BL/6J, 129S1/SvImJ, NOD/ShiLtJ, and NZO/HILtJ) (31). Using thousands of 'eight-way funnel breeding design', recombinant inbred strains (RI) were generated with random contributions from the eight founder lines (26-29). RI strains are genetically homogeneous and can be used to investigate the genetics of complex phenotypic traits. However, one essential characteristic of the trait to be studied is the presence of significant phenotypic variability in the population. To test whether the Collaborative Cross would be a useful resource to understand the genetic basis of infant vocalizations in mice, we first characterized the founder strains. In these preliminary experiments, we observed substantial variability in the emission of vocalizations upon separation across the seven strains tested (**Figure 4**). Thus, these results support the use of the Mouse Collaborative Cross and its recombinant inbred strains to search for the differential neuronal networks involved in the emission of vocalizations and the genetics of this widespread mammalian behavior.

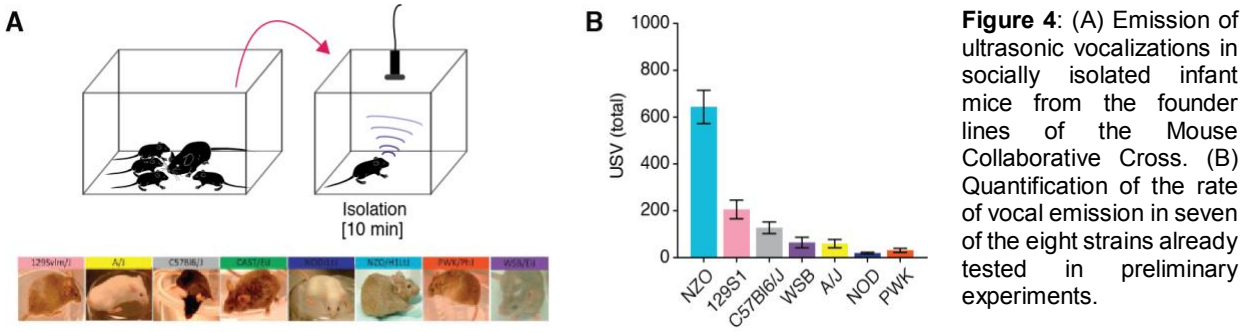

Figure 4: (A) Emission of ultrasonic vocalizations in socially isolated infant mice from the founder lines of the Mouse Collaborative Cross. (B) Quantification of the rate of vocal emission in seven of the eight strains already tested in preliminary experiments.

2.3 Research Design

2.3.1. <u>Quantitative trait loci (QTL) mapping:</u> We will use the available genetic and sequencing information available for the Collaborative Cross RI strains to investigate the genetic basis of infant USV control using QTL mapping. There are currently 69 RI strains that have been sequenced with their data freely available (Zenodo accession no. 377036; ENA accession PRJEB14673). Genotyping array for the Collaborative Cross mice is also available, consisting of

150,000 single nucleotide polymorphisms (SNP) that can be used to fine-map gene loci influencing ultrasonic vocalization (http://csbio.unc.edu/CCstatus/index.py). Following the same protocol used for founder strains, we will record and measure vocalization rate of the RI strains in 90-minute isolation tests at the age of PND 10. We expect to initially record vocalization of at least three pups for each strain, totaling in close to 200 samples. These mice are available for use through UNC Systems Genetics Core Mice.

Our preliminary data shows strains that increase vocal rate with time, decrease vocal rate with time, and continuous rate with time. Therefore, after recording RI strains, we will select 5% strains that show the most significant increase and decrease of USV emission with time, as well as the bottom 5% of low and continuous vocalizers and increase our sample size of vocal emission with two more pups for each of these strains. Next, we will obtain each of their genotypes (which is publicly available). The genotypes will be combined, and after quality filtering (GATK best practices: quality depth > 5; genotype quality > 20) and label of identical genotypes, the high confidence markers will be used as our genetic map representing candidate markers influencing vocalization rates. The genome sequence of each RI strain will be used for genotype calling with the GATK software (https://software.broadinstitute.org/gatk). Using the genetic map and the genotype data together with the vocalization rate as our phenotype data, we will perform QTL mapping with the R/qtl2 software (available at http://kbroman.org/qtl2/). We expect to find multiple candidate genes or gene loci that influence ultrasonic vocalization in infant pups during isolation in small portions. This is based on the fact that behavior studies with QTL analysis typically show that individual loci contribute to less than 10% of the variance in the behavior trait. Identified QTLs will allow us to select genes of interest to further study their role in infant vocalization and test whether they are conserved in other mammalian species.

Feasibility: It is possible that some RI strains will have highly variable vocalization rates during isolation and should not be considered for QTL mapping. An alternative to QTL mapping would be to perform allelic specific expression (ASE) analysis. ASE is used to find differential abundance of allelic copies of a given transcript. Combining ASE analysis and RNA-seq of founder and recombinant inbred strains would provide a comprehensive source to find to which extent small changes in the genetic information between alleles influences vocalization. With this approach, we would be able to estimate the haplotypes segregating strains that vocalize under different categories and generate a new genetic map that could be used as input for QTL mapping.

2.3.2. Whole-brain mapping of activated neurons in the founder strains of the collaborative cross. Differential ultrasonic vocalization emission may be explained by differences in neuronal activity. We can compare these differences in neuronal activity using new imaging techniques. iDisco is a brain imaging technique that uses solvents to preserve the cellular structure of the tissue and permits immunostaining of various proteins with antibodies. This tool allows for visualization of the brain in 3D, rather than through the staining of individual brain slices. Our group has already used iDisco successfully for early gene mapping of mice in other studies. In this case, we will use iDisco to discover neuronal networks that are activated upon isolation of infant mice. As described previously, we will isolate PND 10 pups of each founder strain for 90 minutes while recording their vocalizations. Afterwards we will collect their brains and use iDisco to label activated neurons using immunohistochemistry for c-Fos gene, a proxy for neural activation. Brain will then be imaged using light sheet microscope (Neuroscience Imaging Facility). We will have a sample size of six mice per strain and per group (control animals and animals that were isolated), totaling in 96 animals to be tested. Images will then be analyzed using high-performance computers and the ClearMap software. ClearMap is publicly available and developed for whole-brain c-Fos mapping. Given significant differences in vocal data, we expect to find differences in brain activity during isolation between strains. Areas that show consistent differences among the categories of ultrasonic vocalization emissions described before will become candidate brain regions for future studies and will be informative for further analysis of the neural circuit for modulation of crying.