Combined Behavioral and Neural Investigations of Pup Retrieval

A neural code for pup call representations in the mouse auditory cortex

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To the little helper mice and Oberon.

ABSTRACT

The ability to adequately adapt to a dramatically changing environment is crucial for an animal's survival. When female mice give birth to their offspring, their environment changes drastically and they immediately need to care for the offspring, thereby ensuring the offspring's wellbeing. Pups completely transform the environment around the mouse, triggering a number of new behaviors, as they provide a slew of new sensory inputs, including tactile and olfactory, but also auditory. Pups emit ultrasonic vocalizations (USVs) when isolated outside the nest, triggering retrieval behavior in mothers (MTs). After pups have returned to the nest and are cared for, the USV emission ceases. Interestingly, not only MTs but also virgin mice can perform pup retrieval, provided that they either have experience with pups in their home cage or are repeatedly exposed to pups in a pup retrieval task. Those two animal groups are referred to as experienced (EVs) and naive virgins (NVs). Studies have shown that excitatory neurons in the auditory cortex of MTs and EVs respond more strongly to pup calls over time. However, these studies have been performed under head-restrained unnatural conditions. Here, we provide a framework in which MTs, EVs and NVs retrieve pups in a semi-natural, freely behaving setting. During the experiment, they carry a head-mounted miniscope that allows for imaging neural activity in multiple neurons in the auditory cortex. The entire multisensory scenery is therefore accessible to mice, which was shown to impact auditory responses to pup calls.

In our study, we show differences in behavioral performances of these three groups, with MTs displaying the most skilled and fine-tuned pup retrieval behavior, already highly effective during the final pregnancy stage. EVs show slightly reduced pup retrieval abilities, but superior to NVs, which retrieve pups effectively only after a few days. Additionally, we discovered that not only pups emitted USVs, but also adult mice vocalized. Intriguingly, they vocalized significantly more when pups were present in the behavioral arena, as compared to when they were alone.

Clear pup call responsive neurons in the auditory cortex of all groups were scarce. Nevertheless, the overall neuronal population showed significant responses to pup calls at least in MTs, less so in EVs and least pronounced in NVs. Strikingly, other more global and behaviorally relevant events, such as pup retrievals and nest entries and exits, showed a distinct neural signature.

Despite the scarcity of clear single cell responses to pup calls, the population of auditory cortex neurons carried information about pup call presence throughout all sessions in all groups, measured by a decod-

ing analysis. This population code could be described as a sparse and dynamic code containing a few highly informative neurons, i.e. high weight neurons, that carried most of the decoding weight in a given session. This sparsity was most pronounced in MTs and least so in NVs. Besides, these high weight neurons were largely non-overlapping with high weight neurons for other non-pup call related event types. When relating single trial pup call decoding accuracies with the associated behavioral performance in a given trial, we could identify a significant relationship in EVs that was absent in MTs and NVs, suggesting that improved single trial decoding accuracies were linked to improved pup retrieval abilities.

Altogether, this study shows how different pup exposure regimes can affect the learning of an essential offspring caring behavior and, that these different learning types differently enhance the neural representations of associated sensory cues.

CONTENTS

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INTRODUCTION

In this doctoral thesis, I investigated the neural foundations of social learning in mice. To study social learning, I employed the behavioral paradigm of pup retrieval, in which adult females learned to care for pups over time, which is reflected in a pup retrieval behavior. In this paradigm, mouse pups emit calls that trigger maternal animals to retrieve pups to the nest. These pup calls have been shown to be processed differentially in mothers (MTs) and naive virgins (NVs) in studies of head-fixed and anesthetized animals. Here, I aim to investigate the neural processing of pup calls and pup related events under natural conditions. For this purpose, I utilized miniaturized microscopy to simultaneously record from hundreds of neurons in the auditory cortex of mice, while animals were engaged in such a pup retrieval task.

In the introduction, I will first explain pup retrieval behavior, its purpose and differential manifestation in different animal groups. This will be followed by an overview of the mouse vocalization repertoire that is employed for communication. In the second part of the introduction, the neural components of parental behavior in general and pup retrieval behavior in particular will be discussed, focusing on circuits in the auditory cortex governing parental behavior. I will provide a synopsis of the mammalian auditory system and finally I will discuss plasticity during motherhood in the auditory cortex.

1.1 PARENTAL BEHAVIOR

Investing energy into the raising of offspring provides the basis for successfully passing on traits that are advantageous for survival, to the next generation. It is therefore crucial for an animal to have adapted its offspring-directed caring behaviors in such a way that it can respond efficiently to particular needs of the young. Facilitating the early development of the offspring by protecting it from prey, adequately nurturing it, and keeping it healthy is of foremost priority for parents, as it sets the basis for future coping and prevailing of the young in its environment.

1.1.1 *Studying parental behaviors*

Why are parental behaviors relevant and what can we learn from studying them? Parental behaviors in the animal kingdom involve feeding, protecting the offspring and teaching skills. Typically, mothers take over the feeding role by nursing, however, in some species, fathers can also be involved to different degrees in some of the offspring caring tasks, such as in the giant water bug (Smith, 1979) or the silverback mountain gorilla (Rosenbaum et al., 2011). Since these caring behaviors are greatly influenced by environmental factors as well as the state of the mother, under certain circumstances, maternal care can also go awry, resulting in neglect of the young. This phenomenon occurs in not less than 10% of American mothers, and is referred to as postpartum depression, short PPD (Wisner et al., 2013). In the laboratory, one can make use of a mouse model to study PPD, attempting to understand the neural mechanisms behind it and eventually treat this condition in humans. In 2008, Maguire Mody used GABA receptor delta subunit deficient mice, which displayed a lethargic and a lack of pleasure-seeking state, very similar to PPD in humans (Maguire & Mody, 2008). While some studies point towards the involvement GABA receptor dysregulation, the neural signature during PPD remains largely unknown. It is therefore crucial, to first study the neural coding under natural and healthy conditions to ultimately be able to treat the pathological state of maternal behavior. Moreover, studying natural behaviors such as parental behaviors, can provide insight into basic neural mechanisms and principles that might be unexplored with conventional experimental approaches. Together with classic experimental approaches, studying natural behaviors and its neural correlates can add valuable insight into preexisting knowledge and understanding of the mammalian brain.

1.1.2 *Pup retrieval in mice*

1.1.2.1 *Pup retrieval in adult female mice*

One of the main maternal behaviors in laboratory mice, which is the focus of this study, is pup retrieval. More specifically, mothers retrieve pups that have fallen outside the nest back to it, in which they can feed, warm up, and protect the litter (Ehret et al., 1987). This behavior is initially triggered by ultrasonic vocalizations (USVs) or distress calls emitted by the pups (Sewell, 1970). Isolated pups experience a drop in body temperature, causing them to emit these high frequency calls and thereby alert the mother. Interestingly, this retrieval behavior can persist up to 80 days after the initial maternal experience (Scanlan et al., 2006). In contrast, naive virgin (NVs) females - mice that did not have any contact with pups during adulthood - usually do not show any maternal behavior (Ehret et al., 1987; Ehret, 2005). It is worth noting that occasional pup retrieval can be observed in naive females, both spontaneously (Svare & Mann, 1981), and upon repeated exposure to pups (Okabe et al., 2017), indicating that some preexisting hard-wired brain circuit may trigger this behavior. Further experimental evidence is needed to support the notion that preexisting hard-wired brain circuits trigger pup retrieval behavior in adult females. However, virgin mice that are co-housed with a mother and her litter, show robust retrieval behavior (Ehret et al., 1987). These so-called experienced virgins (EVs) adopt the maternal behavior from the mother and eventually retrieve the pups back to the nest and display general nursing behavior. One study addresses the question of how EVs learn this behavior, and find that EVs are shepherded to pups in the nest by the co-housed mothers (Carcea et al., 2019). More specifically mothers ensured EVs are in the nest, warming the retrieved pups, and demonstrate the execution of that behavior by self-generated retrieval episodes (Carcea et al., 2019). From these experiments, one can therefore deduce that EVs learn this behavior to some degree by observational learning. Thus, it is sufficient that the behavior of a pup-retrieving mother is visible through a glass wall to an observing virgin mouse (Carcea et al., 2019).

1.1.2.2 *Pup retrieval in adult male mice*

As mentioned before, fathers are not always involved in parental care. Male mice can also retrieve pups back to the nest, but only under particular conditions (Liu et al., 2013). First, the male has to be the biological father of the pups. Secondly, pup retrieval occurs only in the home cage of male mice (Liu et al., 2013). Thirdly, the corresponding mother has to be present in the same cage (Liu et al., 2013). Intriguingly, the mother sends out so-called encouraging calls, which prompt the fathers to care for pups by retrieving missing ones back to the nest (Liu et al., 2013). This is understandable from an evolutionary perspective, as fathers have to support their offspring, and if in doubt,

they prefer aggressive rather than caring behaviors. Besides, it has been shown that different mouse strains can differ in their parental care, such that C57BL/6 and CBA/CaJ mice lick and groom their offspring more than Balb/c mice do (Anisman et al., 1998; Shoji & Kato, 2009; Pedersen et al., 2011). These differences might be due to stronger stress sensitivity in Balb/c mice (Beuzen & Belzung, 1995). Cross-fostering experiments revealed that Balb/c mothers raised by CBA/CaJ mice showed more pronounced pup caring behaviors than Balb/c mice raised by mice of the same strain, indicative of the experience dependent component of offspring care (Shoji & Kato, 2009). Ultimately, these differences can be ascribed to differential genetic makeup in these species (Francis et al., 2003). Contrastingly, wild mice display a much higher degree of aggression than laboratory mice do (Chalfin et al., 2014). The study from Chalfin and colleagues shows that not only males, but also wild female mice exhibit aggression towards pups to a higher extent than inbred laboratory mice (Chalfin et al., 2014). Such behaviors are rather detrimental to the domestication of laboratory mice, as this hinders successful breeding. Therefore, certain traits might have been erased from the behavioral repertoire of inbred laboratory mice. How much this extends to other behaviors is unknown but it is crucial to keep this in mind when investigating putative natural behaviors.

1.1.3 *Senses involved in pup retrieval*

Which are the main sensory modalities that play a role in initiating pup retrieval behavior in mice? First, pup USVs are the initial triggers that guide adult mices' attention towards the isolated pup (Sewell, 1970; Smotherman et al., 1974). Acoustic cues therefore serve as the main initial guidance to the pups, and after the pups are located, auditory cues are not needed anymore (Ehret, 2005). When pups are cold and silent, retrieval behavior is much less frequent, as compared to when pups are warm and vocalizing (Schiavo et al., 2020). Nevertheless, auditory signals are only part of the multisensory scenery triggering maternal behavior: Among others, also olfactory cues from the pups contribute substantially to this behavior by guiding social approach behavior and assisting mothers in pup retrieval (Otmakhova et al., 1992; Lévy et al., 2004; Lévy & Keller, 2009). Interestingly, pup odors play a different role in mothers and naive females, such that outside of motherhood these cues can inhibit maternal behaviors and conversely, can act as a potent stimulus during motherhood by partly affecting the motivational center of mothers (Levy et al., 1983; Lévy et al., 2004). Pup odors can occasionally be informative about the identity of the offspring, i.e. whether it is a mother's own or a foreign pup, as shown in rats (Beach & Jaynes, 1956). In 1995, Rosenblatt and Mayer proposed a biphasic model, according to which naive females are rather repelled by pups and lactating mothers are attracted to pups (Beach & Jaynes, 1956).

When animals try to locate an isolated pup, the visual system probably further aids in spotting pups, even though clear experimental evidence for this is currently lacking. Another study in rats showed, that even blind, anosmic or anaptic rats retrieve pups, with different combinations of depleted senses having different effects (Beach & Jaynes, 1956). The loss of the tactile and the olfaction sense alone reduces retrieval behavior, however when both senses are lost simultaneously, pup retrieval behavior is affected more strongly than in single sensory deprivation scenarios. This is indicative of synergistic effects among these sensory modalities (Beach & Jaynes, 1956). Altogether, this suggests, that mothers utilize a plethora of sensory modalities in a meaningful order and combination to locate isolated pups. This highlights the existence of a strong multimodal component in this behavior.

1.2 MOUSE COMMUNICATION BY ULTRASOUNDS

Rodents widely employ ultrasonic frequency (higher than 20 kHz, the human perceptual limit) vocalization as means of communication. Rats have three main types of vocalizations: Pups emit 40 kHz calls when separated from their mother, and adult rats emit calls at either short (30 – 50 ms) and higher frequencies (50 kHz), or long (0.3 – 4 sec) lower (22 kHz) frequencies to signal a non-aversive, positive state or an anticipatory aversive state respectively (Portfors, 2007). Mice on the other hand emit USVs in non-aggressive situations (Sales & Pye, 1974) - primarily during mating - and infants emit distress calls when isolated from the nest, as described above for rats. Emitted USVs can signal identity, group status, context or mood of the sender, such as a signals of distress in isolated pups.

1.2.1 *Male USVs*

Which types of communication calls are emitted by mice? In adult mice, males are the most vocal by emitting primarily mating calls. These mating calls are emitted by males when in proximity to potential female partners, have dominant frequencies between 30 and 110 kHz, and are organized in a song like fashion (Holy & Guo, 2005). These songs consist out of single syllables, which are units of sounds separated by silence, and they can come in a variety of spectro-temporal incarnations, depending on the individual sender. The entire song can last from 0.5 s up to 7 s, with each syllable lasting between 50 to 300 ms with a repetition rate of roughly 5 Hz (Sales, 1972). From an ethological point of view, these male calls attract females to males and generally promote social interaction.

1.2.2 *Female USVs*

Females rarely vocalize, but were reported to vocalize in three contexts: (1) Either when other females are around (Portfors, 2007), (2) when they are encouraging co-housed male mice to retrieve their pups (Liu et al., 2013), or (3) when their offspring was removed from their cages, placed back into the arena and immediately after successful retrieval taken out of the behavioral arena again (Ehret, 1975). In the original paper, these latter calls are defined as confusion calls. The name originates from the fact that pups were held above the cage, sending out calls, while the adult female ran around the cage searching and in a confused state. Therefore the ethological purpose of these calls is not completely clear.

1.2.3 *Pup USVs and behavioral implications*

Infant mice, or pups, are quite vocal as they aim to trigger maternal behavior via these vocalizations. They emit three types of calls in different situations. Firstly, they emit low multiband frequency sounds (wriggling calls) when struggling in the nest, which causes a change in nursing position of the mother (Ehret & Bernecker, 1986). Secondly, pain calls, covering a large frequency bandwidth, can be recorded when pups are handled roughly by either the experimenter or the mother (Ehret, 1975). Thirdly, very prominent and ubiquitous distress calls are send out by pups that find themselves isolated from the warm nest, separated from the other littermates and their mother (Sewell, 1970; Ehret, 2005). In the remainder of this thesis, this subclass of vocalizations will be referred to as 'pup USVs'. As mentioned above, temperature plays an important role in the emission of these calls. In particular, pup USVs can be emitted because pups experience a drop in body temperature outside the nest (Okon, 1970). Interestingly, the amount of calls increases, as the pup's body temperature deviates from the nest temperature, and also depends on the nutritional state of the pup (Allin & Banks, 1971; Hunt et al., 1976). Additionally, a change in the emotional state of pups can also cause pups to emit USVs (Ehret, 2005).

As mentioned in the section above, these calls are alarming, and cause a phonotaxic approach of mothers that will direct their attention and physical orientation towards the acoustic cue (Sewell, 1970; Noirot, 1972; Ehret, 2005). This approach is mainly guided by the pup USVs, shown by a study from Haack et al. in 1983, in which pup USVs were played back via a loudspeaker, causing an approach behavior in mothers (Haack & Ehret, 1983). When mothers and EVs were confronted with pup calls and pure tones in a two-alternative choice maze, both groups approached the pup call speaker much more frequently than the pure tone speaker (Lin et al., 2013), further supporting the attractiveness of these sounds. When mothers have already retrieved some pups and are caring for those pups in the nest, they are less likely to approach a single isolated and calling pup from outside the nest. More specifically, their latency to approach that pup is reduced when mothers are in the nest as compared to when they are outside the nest and orient towards pups (Ehret, 2005). Interestingly, these pup calls elicit an active avoidance behavior in naive virgins, enough to turn off the playback of pup calls after multiple sessions of exposure in a paradigm allowing them to (Schiavo et al., 2020). In general, USVs elicit an arousal mediated approach behavior in lactating mothers, which is supported by Bell's motivation hypothesis (Bell, 1974), proposing that USVs reflect a state of arousal in the sender, and elicit on their own an aroused state in nearby receivers. In this scenario, mothers close the communication loop by retrieving the calling offspring into the nest. A further processing and feedback step in the pups can be excluded, as pups are deaf up to postnatal day ten (Ehret, 1983).

1.2.3.1 *Pup call characterization and discrimination from adult calls*

Typical pup calls have frequencies above 35 kHz, mostly between 70 and 110 kHz and are emitted from pups between postnatal day 3 and 13 (Noirot, 1972; Ehret, 1975). However, the emission rate of pup calls decreases as the pup ages, and they are entirely absent at P13 (Haack & Ehret, 1983). Adult mice have the strongest hearing sensitivity at around 50 kHz, thus making adult mice especially sensitive to pup calls (Ehret, 1975). These calls have a high (above 80 kHz) and a low (below 80 kHz) frequency component. The spectral content of the low frequency component changes systematically across days (Liu et al., 2003), such that at P5 this can cover a larger range of frequencies for different pups, and starting from P9 to P12 this component converges at about 67 kHz, creating more stereotypic calls from different pups. The high frequency component stays roughly constant at about 94 kHz. Also, at the age of P3 to P4, mouse pups emit the highest amount of calls, after that the emission rate goes down as well as the loudness of single calls (Noirot & Pye, 1969).
Moreover, single pup syllables can be relatively long (in mouse vocalization terms), so that they oscillate around 65 ms with a broad distribution around that value (Liu et al., 2003). The inter-syllable interval (ISI), i.e. the silent break between two adjacent syllables, amounts to 250 ms in P5 pups, resulting in a repetition rate of 4 Hz.
Certain features render pup calls attractive to a mother, such as the duration and the frequency of single syllables (Ehret & Haack, 1982). In terms of duration, single syllables between 25 and 270 ms proved attractive to mothers (Ehret & Haack, 1982; Ehret, 1992). Regarding frequency, for pup calls to be successfully distinguished from noise, their dominant frequency has to be above 40 kHz. 20 kHz calls in contrast are not attractive to mothers (Ehret et al., 1987). In particular, the sound energy of that attractive frequency has to be focused in one critical band of the ultrasonic range (Ehret, 2006). Critical bands define perceptual boundaries of the mouse and will be discussed later in the auditory cortex section. Among other factors, the ISI and the associated pup syllable repetition rate proved to be informative indicators of pup call attractiveness (Schiavo et al., 2020). This was shown by a study in which the ISIs of natural pup calls were systematically changed, showing that the naturally occurring ISIs of 150-200 ms triggered the strongest pup retrieval behavior in mothers. Besides, pup calls attenuate in a directional fashion due to their high frequency nature and thereby can remain not perceivable. Nevertheless, pup calls can be perceived by adults, even across distances of 100 cm between the nest and the pup (Haack & Ehret, 1983).

Since not only infants emit vocalizations, but also adults do so, it is crucial for adult mice to separate and categorize these calls perceptually, even though they overlap spectrally. This is of special importance since these different calls confer vastly different meanings, e.g. care for an isolated pup or react to a mating partner, and therefore prompt for the execution of particular behaviors. The study of Liu and colleagues

in 2003 investigates the difference of pup calls and mating calls to probe which features might inform an adult female about the sender's identity and purpose (Liu et al., 2003). They found pup calls are longer in duration, have lower frequencies and are emitted at a lower repetition rate than adult calls. Additionally, pup calls have a stronger stereotypic spectro-temporal shape as compared to the more variable adult counterparts, which can contain more frequency-modulated syllables. These call features overlap in parameters such as dominant frequency, duration and onset frequency modulation, but allow adults to perceptually generalize across calls from the same sender group and discriminate across sender groups. Given that mothers retrieve pups of different ages, and that the spectral content of pup calls was shown to vary with age (Liu et al., 2003), one can reason that mothers are able to generalize across variations of pup calls. The neural implications of this will be discussed in the next chapter.

1.3 NEURAL CIRCUITS DRIVING PARENTAL BEHAVIORS

What are the neural circuits governing parental behaviors? In general, we can divide circuits into those that promote parental behaviors on one hand and those that suppress them on the other (**Fig. 1.1**). Moreover, different animal groups treat offspring differentially, such that two main groups of animals can be distinguished based on their particular offspring directed behavior. First, the group of animals where parental behaviors are promoted is composed of mothers, fathers and co-caring females. Second, the group of animals where parental behaviors are suppressed is composed of foreign parents and naive virgins. These two groups of animals have opposing intentions, namely to promote the well-being of the offspring or, on the other hand, to physically avoid the offspring and do not care for the offspring or even actively deteriorate the well-being of the offspring. These two opposite behaviors are triggered in the brain by turning on circuits that promote parental behavior and turning off avoidance and aggressive behaviors and vice versa, both of which can be modulated by hormones. The next sections will first treat the influence of hormones onto parental behaviors. The above-mentioned animal groups with their behavioral implications as well as their neural circuits will follow.

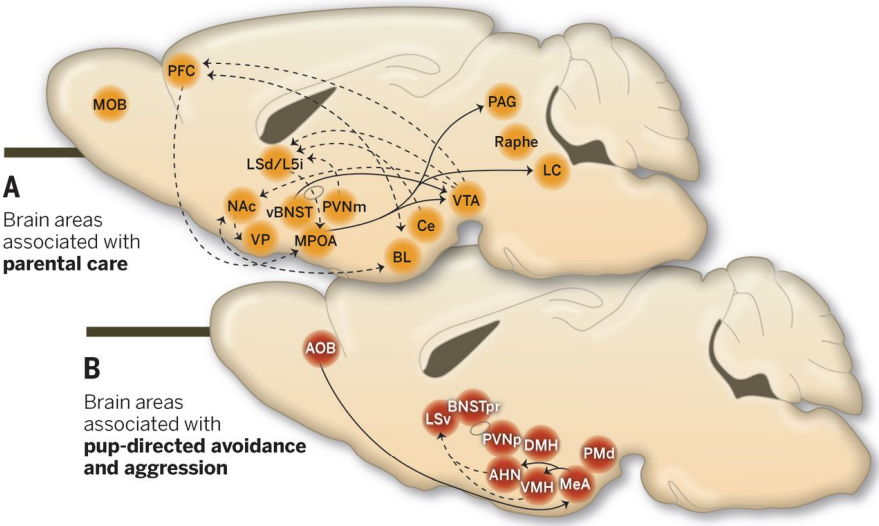

Figure 1.1 | Parental circuits in the brain Schematic depicting brain regions that are implicated in parental care (*above*) and in pup-directed avoidance and aggression (*bottom*). Brain regions referred to in the text include the medial pre-optic area (mPOA), ventral bed nuclei of stria terminalis (vnBST), ventral tegmental area (VTA), ventral pallidum (VP), nucleus accumbens (NAc), paraventricular nucleus (PVN), main olfactory bulb (MOB), accessory olfactory bulb (AOB), basolateral amygdala (BL) and the medial amgydala (MeA). Taken with kind permission from (Dulac et al., 2014).

1.3.1 *Hormonal and non-hormonal basis of parental behaviors*

Hormonal fluctuations strongly influence and stimulate parental behaviors (Terkel & Rosenblatt, 1968; Moltz et al., 1970). For instance, the hormones estradiol and prolactin as well as the neuropeptide oxytocin are key drivers for the onset of maternal behaviors after parturition (Rosenblatt, 1980; Kim & Strathearn, 2016). The neuropeptide oxytocin, which is widely known as the "cuddling hormone", can stimulate maternal behaviors, such as pup retrieval, pup grooming and licking in non-lactating animals (Numan et al., 2005; Marlin et al., 2015). Shortly before parturition, progesterone levels decline, while estradiol and prolactin levels increase (Moltz et al., 1970). Noteworthy, these hormonal changes are not required for maintaining maternal behaviors after parturition (Numan & Insel, 2003). It has been suggested however, that these hormonal changes cause some brain circuits to adapt in the long-term, thereby inducing engagement of circuits promoting parental behaviors (Bridges, 2015). Once these circuits are active, hormones are not essential anymore. This triggering role of hormones can be seen in multiple behavioral instances. For example, when injecting naive virgins with hormones in the form of blood plasma from a female that just gave birth, one can induce maternal behaviors in these recipient naive females (Terkel & Rosenblatt, 1968). Also in males, hormones modulate paternal behavior. In humans for example, decreasing levels of testosterone during fatherhood have been detected in males involved in infant care (Gettler et al., 2011).

Maternal behaviors can also be induced in the absence of gestation-dependent hormones. This is clearly shown by naive virgins that initially show dismissive behavior towards pups, but after a few days of exposure will tolerate them and eventually care for pups (Fleming & Rosenblatt, 1974). This process is referred to as sensitization. Animals that lack estradiol, a major hormonal driver of maternal behavior, can still display pup directed caring behaviors, supporting the experience-dependent component of this behavior (Rosenblatt, 1967). Further studies have shown that neither the experience of parturition nor that of suckling is needed to initiate maternal behaviors (Moltz et al., 1967). Also in the case of experienced virgins that are co-housed with a mother and her offspring, maternal behaviors are performed by the non-maternal mouse.

1.3.2 *Brain circuits governing parental behaviors*

1.3.2.1 *Circuits promoting parental behaviors*

One key player in promoting maternal behavior is the medial preoptic area (mPOA), located in the hypothalamus, which is tightly connected to the dopamine (DA) system (**Fig. 1.1**) (Numan & Stolzenberg, 2009). This area is rich in steroid receptors, and can thereby sense the course of pregnancy. It is also involved in the transition from offspring aver-

sion to offspring attraction and care (Rilling & Young, 2014). It is suggested mPOA neurons are activated upon infant stimulation, and both activate the DA system that would render pups rewarding and attractive, as well as inhibit antisocial circuits that would otherwise trigger aversion and rejection of offspring (Numan et al., 2005; Numan, 2007). Together with the ventral bed nuclei (vnBST), the mPOA was shown to initiate the onset and maintain maternal behavior in rodents (Numan & Numan, 1997). Immediate early gene studies have shown the involvement of these areas in parental behavior as well as the lateral septum (O'Connell & Hofmann, 2011). Additionally, the ventral tegmental area (VTA), which is part of the mesolimbic dopaminergic pathway, is involved in facilitating parental behavior (**Fig. 1.1**). In particular, it is part of the reward system, and pups can act as rewarding and reinforcing stimuli to adults (Hauser & Gandelman, 1985; Numan & Stolzenberg, 2009). Specifically, dopamine is released from the ventral striatum of maternal rats upon interaction with pups, which was suggested to enhance pup retrieval behavior in these animals (Hansen et al., 1991; Hansen et al., 1993). The VTA in turn innervates the nucleus accumbens (NAc, located in the forebrain, which can potentiate dopaminergic effects to depress inhibitory connections from NAc to the ventral pallidum (VP) (**Fig. 1.1**). This in turn reduces the expression of maternal behaviors (Numan & Stolzenberg, 2009). Such a disinhibitory effect on VP makes it more responsive to incoming pup-generated stimuli via the amygdala. These connections make up the VP attraction and reward system, promoting the attraction between a caring adult and the young. Additionally, the VP is connected to motor circuits and can thereby modulate pup-directed caring behaviors (Rilling & Young, 2014).

Small molecules are also able to modulate the aforementioned regions. For example, the neuropeptide oxytocin enhances the effect of dopamine release on the NAc. Neurons of the paraventricular nucleus (PVN) synthesize oxytocin centrally in the brain, which in turn can activate the mPOA, like estradiol and prolactin. Peripherally released oxytocin is important for parturition and lactation (Pedersen C.A. et al., 1982). Interestingly, estradiol was found to induce expression of oxytocin receptors in the mPOA, making it more responsive to oxytocin (Champagne et al., 2001). Additionally, mPOA connects to PVN neurons, and thereby potentially prompts more oxytocin release (Champagne et al., 2001; Rutherford et al., 2011).

1.3.2.2 *Circuits inhibiting parental behaviors*

Some of the aforementioned players, as well as the basolateral amygdala (BLA), carry out the converse side of parental care, namely pup avoidance and aggression (**Fig. 1.1**). Different neurons reside in the BLA: Those that represent positive valence by responding to pleasant stimuli, and those neurons that represent negative valence by responding to aversive stimuli (Tye, 2018). Therefore, negative valence BLA

neurons promote pup aversion behavior, found in NVs upon their first encounter with pups (Numan, 2007). The BLA projects to the NA and the VP, and can thereby relay sensory information about pups (Moltz et al., 1970; Brog et al., 1993; Maslowski-Cobuzzi & Napier, 1994). It is proposed that novel pup odors activate the described circuits in the amygdala, promoting fearfulness, avoidance and defensiveness in naive virgins (Numan, 2007). The medial amygdala gets part of its input from the accessory olfactory bulb, and this connection was shown to mediate suppression of maternal behaviors and promote initial avoidance in rats (**Fig. 1.1**) (Numan et al., 1993; Numan, 2007). Via such a mechanism, either the negative valence encoding or the positive valence encoding neurons could be activated, and in turn activate the downstream parental behaviors via their respective circuits. When the medial amygdala is lesioned, maternal behavior is facilitated and the initial avoidance response is abolished (Fleming et al., 1980). However, it still takes 2-3 days until full maternal behavior is displayed, indicating that other stimuli might be necessary to elicit it.

Among these aggressive behaviors is also infant-directed aggression, which is abundant in animals not involved in the rearing of offspring (Blaffer Hrdy, 1979). Aggression is again largely mediated by olfaction, because initial avoidance is absent when NVs olfactory sense is ablated (Fleming & Rosenblatt, 1974). Once male mice become fathers, this aggressive state can switch transiently into the parental promoting state, with pro-social circuits being active as described above (Vom Saal, 1985). A subset of galanin expressing neurons in the mPOA has been found to be active during parental care in both females and males, and upon ablation induced disruption of offspring care alongside with pup-directed aggression in males (Wu et al., 2014). These findings again emphasize the dual role of these circuits: Certain circuits are active during parenting and promote caring behaviors, but when inactive induce avoidance and aggressive behaviors. In contrast to this, when the mPOA is maternally triggered as described in the previous paragraph, these antisocially triggered connections from the BLA are inhibited.

These maternal circuits can be triggered through different sensory inputs that were already described in the previous sections. However, the initial triggers of pup retrieval behavior are pup calls. Many studies have investigated the plastic changes that occur in the auditory cortex (AC) of maternal animals. In the next section, the general architecture of the auditory system, the coding features at each hierarchical level and the plasticity associated with motherhood in the AC will be discussed.

1.4 THE AUDITORY SYSTEM

Audition is crucial for an animal's adaptability to its environment. Hearing can protect it from potential predators, lead it to potential mating partners or can assist greatly in species-specific communication. Intraspecies-specific communication can guide behavior and is of utmost importance in social animals.
Sound stimuli are first collected by the ears and then pass through the cochlea in the inner ear. Here, sound waves are transformed into electrical signals through mechanical movement of hair cells. These signals are conveyed via the spiral ganglion to the central nervous system, where they pass through several cochlear nuclei in the medulla to the inferior colliculus (IC) located in the midbrain. IC neurons project to neurons in the medial geniculate body (MGB) of the thalamus, and these in turn project to the AC. Each of these processing stations will be discussed separately in the following paragraphs (**Fig. 1.2**).

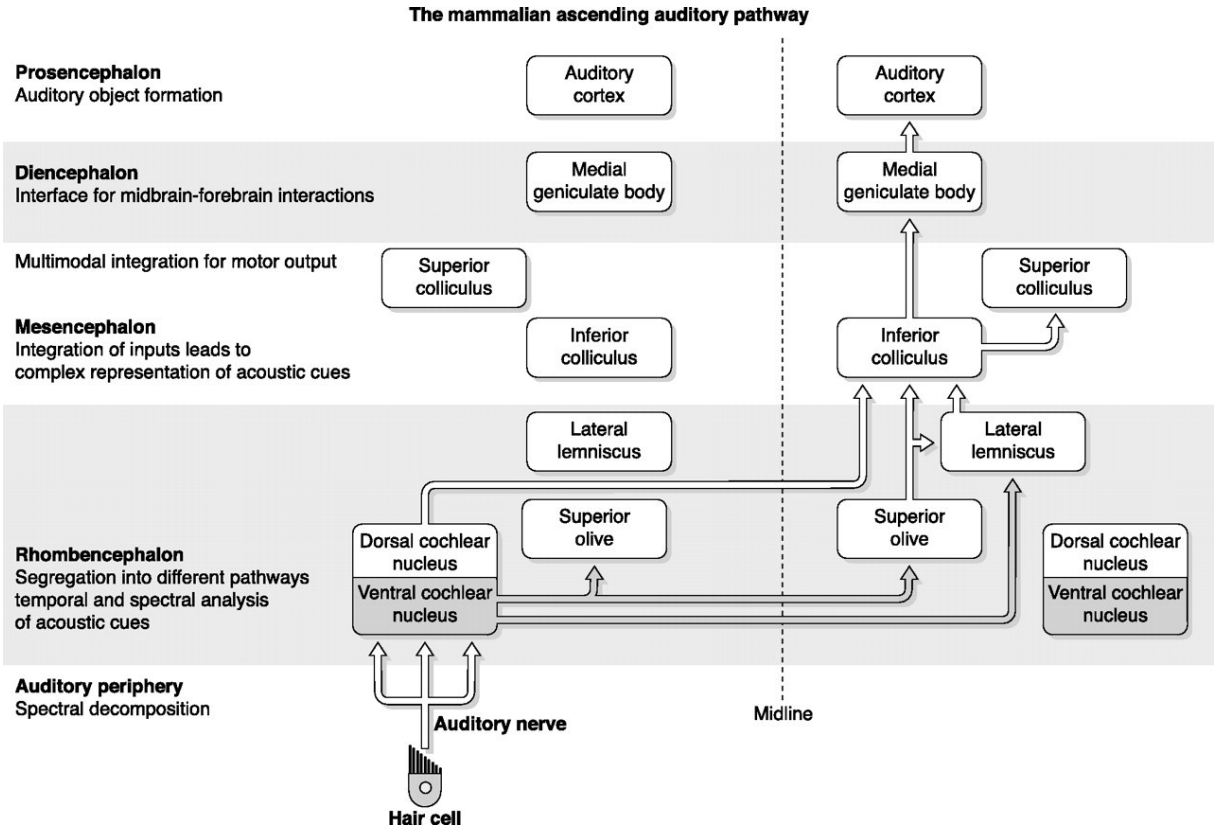

Figure 1.2 | The mammalian ascending auditory pathway. Schematic depicting the main ascending processing stations in the mammalian auditory system. Taken with kind permission from (Grothe et al., 2010).

1.4.1 *Subcortical auditory system*

The ears capture arriving sound waves, thereby causing the tympanum, a delicate membrane, to vibrate with the arriving sound wave. Small

bones convey and amplify these vibrations via the middle ear to the cochlea. The cochlea is the mammalian hearing organ of the inner ear. Liquid-filled cavities within the cochlea capture sound vibrations through sensory receptor cells, i.e. hair cells, sitting on the basilar membrane and forming the organ of Corti (Ruggero, 1992). Depending on the nature of the sound stimulus, the basilar membrane is deflected to different degrees and at different spatial locations, due to its varying mechanical properties (Patuzzi et al., 1984; Martin & Hudspeth, 1999). These mechanical deflections are transduced into electrical signals via so-called hair cells, which reside along the entire length of the basilar membrane. Because of this spatial tiling, a frequency-based compartmentalization of sound waves is observed (Ruggero, 1992). This organization is referred to as tonotopy.
Cochlear hair cells are innervated by primary sensory neurons of the spiral ganglion, where hair cell receptor potentials are converted into action potentials. In general the auditory system is geared to high temporal precision, in order to ensure the localization of sound sources(Corey & Hudspeth, 1979; Grothe et al., 2010; Heil & Peterson, 2015). A key feature of auditory temporal precision is called phase-locking, which describes the phenomenon where action potentials occur only at specific phase angles of the sound stimulus. Phase-locking is generated already at the level of the spiral ganglion, whose projections make up part of the VIII nerve, and can be additionally observed in the cochlear nuclei and the superior olivary complex (SOC). (Galambos & Davis, 1943; Peterson & Heil, 2020).
Axons in the auditory nerve terminate on cochlear nuclei in the brain stem in a tonotopic fashion inherited from the basilar membrane innervation. While neurons in the ventral cochlear nucleus (VCN) sharpen timing and spectral information of sounds (Golding et al., 1995; Wang & Manis, 2006; Oertel et al., 2011), dorsal cochlear nucleus (DCN) neurons integrate acoustic and somatosensory information (Wright & Ryugo, 1996; Shore, 2005). In terms of projection targets, the DCN projects predominantly directly to the IC, whereas the VCN projects predominantly indirectly to the IC via the SOC and to the lateral lemniscus (**Fig. 1.2**). Thus, four parallel ascending pathways, one to the DCN and one to the VCN per hemisphere, carry acoustic information from the nerve fibers.

The medial part of the SOC, the medial superior olivary complex (MSO), processes interaural time differences (ITD), generated through phase locking neural firing to the stimulus frequency. In the lateral superior olivary complex (LSO), level differences are compared between both binaural inputs (ILD) (Goldberg & Brown, 1969). Both cues, ITDs and ILDs, represent essential signals that inform an animal about the location of a sound source in azimuth. These signals are critical for animal behavior when for instance fleeing from a predator.

At the level of the IC, a lot of convergence occurs, making it an area with rather complex response properties. Different acoustic features

assigned to separate streams of information, such as streams of information about ILD, ITD and spectral composition of a sound converge here, cementing the IC as a high level processing station. (**Fig. 1.2**) (Beyerl, 1978; Brunso-Bechtold et al., 1981).

IC neurons mainly project to the ipsilateral MGB, which inherits its tonotopic organization. The view of the auditory thalamus, the MGB, as a simple relay station of sensory information has been challenged in the last decades. Nowadays, the MGB is seen more as an auditory-state integrative site that can bring auditory cues into meaningful context (Edeline, 2003). This notion is supported by the fact that more than 80% of synapses impinging on a thalamocortical cell carry information about the state of an animal (Winer & Schreiner, 2011). Cholinergic inputs to the MGB for instance, can modulate MGB activity according to wakefulness and arousal of the animal (Tebecis, 1972; Motts & Schofield, 2010). Additionally, multimodal input from visual and somatosensory areas reach the MGB (Morest, 1965). The final processing station in the auditory system is the auditory cortex, which will be discussed in the following section.

1.5 THE AUDITORY CORTEX

1.5.1 *Anatomy of the auditory cortex*

All described ascending pathways ultimately connect to the AC, which is characterized by a highly interconnected network of corticocortical projections (Kraus & Canlon, 2012). Based on differential immunolabeling of MGB neurons, the core and matrix theory describe two main information streams reaching the AC (Hashikawa et al., 1991; Jones, 1998). The first stream of information comes from the lemniscal pathway, which represents inputs from the core regions of MGB, bringing precise information about acoustic properties to the AC. The second stream comes from the non-lemniscal pathways, consisting of the matrix or belt regions of the MGB (MGD and MGM), which provide contextual information to the AC, ultimately binding these two inputs together into one representation of an auditory percept.

Inputs from the MGV terminate in layer 4 of the AC, and confer a tonotopic arrangement to the core AC areas (Smith et al., 2012). Moreover, non-auditory sensory inputs can also be found in the AC, among which are inputs from visual, somatosensory and olfactory cortices (Bizley & King, 2009; Budinger & Scheich, 2009). The AC is also contacted by higher order associative cortical areas, such as the caudal temporal cortex that itself is highly interconnected with the amygdala and additionally gets input from the visual cortex (Vaudano et al., 1991). Top down input to the AC deriving from the prefrontal cortex (PFC) gates AC responses during attention, whereby novel stimuli are rendered more salient (Fritz et al., 2007; Schönwiesner et al., 2007). Further, motor inputs interface with AC neurons and modulate their activity depending on the motion state an animal is in (Schneider et al., 2014). These diverse input sources highlight the integrative role of AC, rendering it much more than a simple auditory evaluation center. Section 1.5.3 elaborates on the effects of these diverse inputs on coding in the AC.

Anatomically, the AC can be divided into several subregions that vary depending on the species. The core regions of the rodent AC are the primary auditory cortex (A1) in the caudal part of the AC, the anterior auditory field (AAF), located rostrally, the ultrasonic field (UF) in the dorsorostral region, the dorsoposterior field (DP) and the second auditory field (A2) located ventrally to AAF and A1 (Stiebler et al., 1997). Both A1 and AAF are tonotopically organized, with A1 having an ascending tonotopy from low to high frequencies in the caudal to dorsorostral axis, and AAF showing a reversal of that tonotopy. The tonotopy in A1 and AAF is organized in so-called iso-frequency lines that are perpendicular to the frequency gradient. These lines are spatially clustered bands of neurons responding preferentially to the same frequency range. Iso-frequency bands in A1 range from 2 kHz, which is the lowest bound for hearing in mice, up to roughly 40 kHz (Stiebler et al., 1997). The observed tonotopy can be also described

as having a "salt and pepper"-like organization, since neighboring neurons do not necessarily respond to the same frequencies, but the tonotopic gradient is apparent at a coarser scale (Bandyopadhyay et al., 2010; Rothschild et al., 2010). The other areas do not show any obvious tonotopy. In A2, the tonotopy is fractured, and neurons were shown to have a broader frequency selectivity (Issa et al., 2014; Liu et al., 2019). This likely reflects the convergence of inputs from a variety of frequency ranges (Schreiner & Cynader, 1984; Schreiner & Winer, 2007). Higher frequencies than 40 kHz are more likely to be represented in the UF, which is a specialized area in the rodent AC and might contain neurons responsive to high frequency communication calls, such as pup or adult calls (Ehret, 1975; Liu et al., 2003).

1.5.2 *Function of the auditory cortex and feature representations*

Which features other than tonotopy in A1 and AAF are represented in the AC? Binaural interaction, for example, is represented in the AC by modules with neurons excited by inputs from both ears (EE), and modules that are excited by unilateral input and inhibited by contralateral input (EI) (Schreiner & Winer, 2007). These modules, i.e. EI and EE, alternate along one iso-frequency contour in cat AC (Imig & Brugge, 1978). The behavioral implications of EE and EI modules are not entirely clear, but neurally they are potentially implicated in auditory object formation and scene segregation (**Fig. 1.2**) (Cohen & Knudsen, 1999).

The concept of an auditory object refers to the idea that multiple senses are involved in representing this object; it is linked to prior experience, typically coming from one source and ultimately forming a perceptual entity (Griffiths & Warren, 2004; Nelken, 2004). Additionally, in cat, rat and bat, clustered intensity selective neurons tile along iso-frequency contours topographically (Suga & Manabe, 1982; Phillips et al., 1994; Polley et al., 2007). Some neurons in cat A1 respond differentially within one iso-frequency contour to different bandwidths, so that certain neurons prefer tones within a narrowband and others respond to a broader frequency range, which could be attributable to distortions in the tonotopic map of the cochlea (Imaizumi & Schreiner, 2007). In squirrel monkey and cat, even response latency is represented spatially organized in A1 (Mendelson et al., 1997; Cheung et al., 2001).
Another relevant feature is frequency modulation of tones. Popular stimuli to probe auditory frequency modulation responses are sweep tones that change their frequency gradually. Interestingly, the direction of such sweeps, i.e. from low to high frequencies for instance, is preferred by neurons that typically respond to low frequencies and vice versa. This organization of cortical direction selectivity was found in rat and squirrel monkey (Zhang et al., 2003; Godey et al., 2005). Additionally, different species have different species-specific features

that are mapped onto their ACs, likely because of their ethological relevance (Schreiner & Winer, 2007). For instance, in specialists such as in the moustached bat, essential parameters for echolocation are represented in their AC; in mice, their UF region is specifically dedicated to represent ultrasonic communication calls (Ehret, 1975; Suga, 1990).

Altogether, different features, such as tonotopy and binaural sound source localization, are found in the AC and reach the cortex in distinct processing streams. But also other features, and specific feature-based representations of communication calls, are generated *de novo* in the cortex (Sołyga & Barkat, 2019; Montes-Lourido et al., 2021). These emerging features can be the result of intersecting pre-existing maps that endow each AC neuron with mixed selectivity responsiveness.

1.5.3 *Coding in the auditory cortex*

1.5.3.1 *Single cell responses in the auditory cortex*

How do neurons in the AC represent sounds? A graphical depiction that is often utilized to profile the responses of AC neurons is the so-called frequency response area (FRA), which describes the neural response to a range of frequencies at different attenuation levels (**Fig. 1.3**). Thus, in a way, FRAs are descriptions of parts of the receptive fields of AC neurons. Graphically, these fields are depicted with increasing frequencies on the x-axis and attenuation levels on the y-axis. A classical FRA has a V-shaped form, meaning that the neuron has a decreasing frequency selectivity with increasing sound pressure levels (**Fig. 1.3**). Another way of depicting an AC neuron's response preference is the spectro-temporal receptive field (STRF) (**Fig. 1.3**). As the name implies, temporal information about a neuron's response is included next to its spectral preference, namely with which delay a neuron is excited or inhibited by a certain tone frequency. In a study by Rothschild and colleagues in 2010, roughly 30% of layer 2/3 neurons that passed a quality threshold were responsive to tones, of which 45% had the classical V-shaped FRA. While 25% of their neurons did not show any tone-driven responses, 33% did not show any clear preference toward a certain stimulus frequency (Rothschild et al., 2010). This study also highlights the "salt and pepper"-like tonotopic organization of the AC, as it reports a high degree of variability in tone-response profiles of neighboring single neurons.

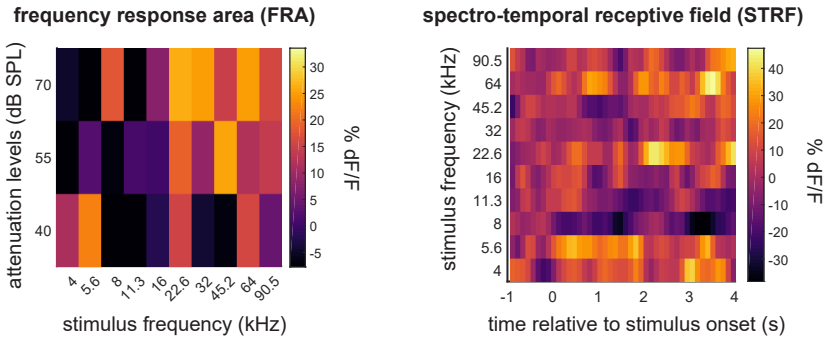

Figure 1.3 | Exemplary frequency response area and spectro-temporal receptive field of an auditory neuron. Responses of a mouse auditory neuron imaged with the two-photon microscope under anesthesia. *Left* plot shows the frequency response area (FRA) of a mouse auditory neuron to pure tone sequences of different frequencies at three different attenuation levels. Response magnitude is indicated by heat of plot. *Right* plot shows the spectro-temporal receptive field (STRF) of the same neuron to the same stimulus frequencies at 75 dB SPL.

Other hallmarks of AC single neurons responses are sparsity and unreliability (Hromadka et al., 2008; Tischbirek et al., 2019). As for the unreliability of neuronal responses, this refers to differential response types upon repeated exposure to the same stimulus (Bandyopadhyay et al., 2010). By sparsity, one refers to the fraction of neurons that respond to auditory stimulation. Depending on the method of neural recordings and the chosen response thresholds, varying numbers of responsive neurons are found for rodent AC (Hromadka et al., 2008; Rothschild et al., 2010; Tischbirek et al., 2019). However, when quantifying solely the fraction of highly responsive neurons, i.e. neurons responding with 20 or more spikes per second, only 5% of neurons fall into that category of responsive neurons (Hromadka et al., 2008). In general, the distribution of single neuron spike rates in AC follows a log-normal distribution, which is characterized by a long tail. This means that a few neurons fire the most (Hromadka et al., 2008). While the median spontaneous firings rate, that is the neuronal firing in absence of any auditory stimuli, is roughly 3 spikes per second, the median evoked neuronal firing is only slightly higher, at 5 to 7 spikes per second (Hromadka et al., 2008).

In macaque AC, a small number of neurons with temporally precise activity carried the vast majority of stimulus related information (Ince et al., 2013). Interestingly, in guinea pig AC, highly vocalization responsive neurons could be found in layer 2/3. These neurons had complex receptive fields, and responded to two out of eight call types only (Montes-Lourido et al., 2021). In contrast, neurons of the thalamorecipient layer 4 showed a much more distributed response across the neuronal population (Montes-Lourido et al., 2021). Similar to layer 4, the MGV did not show call selective responses. Instead, MGV neurons

responded throughout call duration to all calls (Montes-Lourido et al., 2021). Moreover, the preferred stimulus of an AC cell can be complex, as shown in a study from Wang and colleagues in 2020, in which some AC neurons responded well to a complex tone that consists out of several frequency bands, but would not respond to any of the individual frequencies (Wang et al., 2020).
The AC was also shown to prefer novel over expected sounds (Yaron et al., 2012; Polterovich et al., 2018). This feature becomes apparent when presenting stimulus sequences to an animal that have many repetitive and a few novel stimuli in a so-called oddball paradigm. In this paradigm, AC responses increase once the surprising deviant tone is perceived (Polterovich et al., 2018). Generally, a highly heterogeneous spectrum of neural responses can be measured in the AC (Kanold et al., 2014; Li et al., 2017; Tischbirek et al., 2019).

As mentioned above, next to sound driven activity, some of the AC activity is modulated by other sensory modalities. For instance, substantial input from visual areas modulate mouse AC responses, so that 20% of AC neurons respond to visual stimulation via hyperpolarization (Budinger et al., 2006; Sharma et al., 2021). This potentially assists in sensory neural processing. In contrast to visual cortex, AC neurons are suppressed at the onset of and during movement, as a result of local inhibitory neuron activity (Polack et al., 2013; Schneider et al., 2014; Zhou et al., 2014). Inputs from secondary motor areas project to the AC and modulate its activity. This demonstrates a synaptic mechanism by which corollary discharge, a copy of motor signals, suppresses self-generated sounds, such as during grooming, head orienting or general locomotion (Schneider et al., 2014).

1.5.3.2 *Population coding in the auditory cortex*

Intriguingly, the population of AC neurons can capture and represent auditory stimuli much more robustly than single neurons (Bathellier et al., 2012). This feature became apparent when presenting a mouse with different auditory cues, and correlating population response vectors of one auditory stimulus with another. These single trial correlations were grouped via hierarchical clustering, which showed the existence of discrete switches between response modes to groups of sounds across the entire population (Bathellier et al., 2012). A response mode describes a consistent neural activity pattern that is elicited upon sound stimulation. The resulting response modes that can be visualized are much less numerous than the initial number of stimuli utilized, which implies that the AC responds by discretizing a continuum of auditory stimuli. In another study, from Bao and colleagues in 2013, rats were raised while being exposed to naturalistic sounds, resulting in improved stimulus processing and a selective representation of sounds in the neural population (Bao et al., 2013). More neurons participated in representing these sounds, whereas fewer neurons responded to single complex sounds, indicating yet again the capacity

for generalization of AC neural populations to more variations of complex sounds, leading to discretization (Bao et al., 2013). Altogether, these findings support the notion of nonlinear transitions of local response modes in the AC. These are performed to potentially improve the representation efficiency of biologically meaningful categories.

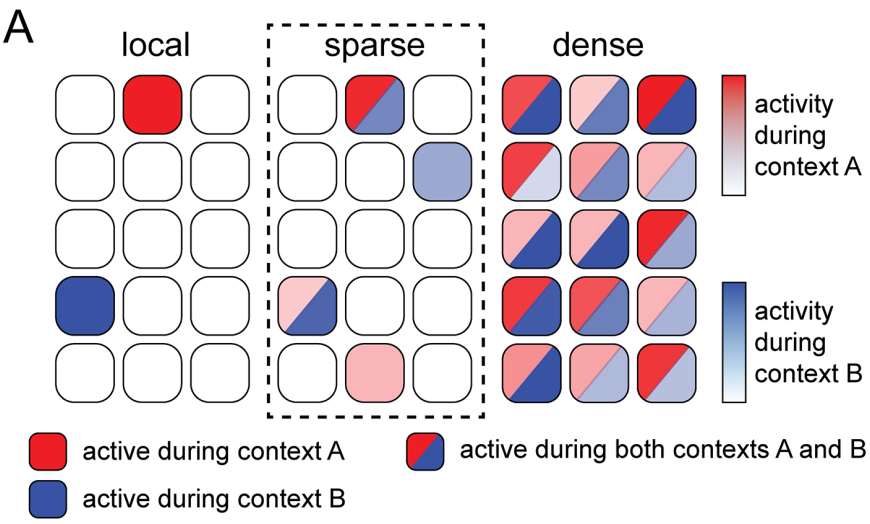

Figure 1.4 | Types of population codes. Schematic showing the three main types of population code. A local code with a few neurons responding solely to one context on the left side. In contrast, a dense or distributed code on the right side with all neurons participating in representing multiple stimuli at the same time. In the center, the sparse code with a few neurons representing multiple stimuli at the same time. Reproduced from *https://doi.org/10.1371/journal.pcbi.1006908.g001 (CC-BY)*.

In neural coding theory, there are three broad categories of neural codes that represent information about a context or a stimulus (**Fig. 1.4**) (Beyeler et al., 2019). A local code, on one extreme, has few responding neurons that each respond specifically to one stimulus, with little capacity for generalizing across stimulus features. On the other extreme, a dense code contains many responding neurons that each can contribute differentially to different stimuli, allowing great generalization at the expense of large cross talk among single neurons, besides the associated high energetic costs. The presumed optimum of this scenario lies in the middle: A sparse code (**Fig. 1.4**) (Barlow, 1972; Beyeler et al., 2019). In such a scenario, a minimum of neurons is active, and these neurons harbor mixed selectivity responses such as in the dense code, but with much less cross talk. For all these three scenarios, different amounts of neurons represent a stimulus, or in other words, differential fractions of neurons would allow for confident decoding of a stimulus. As it was shown in a few studies about the auditory cortex, a few sparsely active neurons typically contribute disproportionally to the decoding of a stimulus (Centanni et al., 2013; Garcia-Lazaro et al., 2013; Ince et al., 2013). Mixed-selectivity neurons were found in cortical areas where neurons often had heterogeneous response patterns and responded to different task-related variables

simultaneously (Rigotti et al., 2013). These neurons are thought to have a computational advantage, leading to a richer population representation via different linear combinations (**Fig. 1.4**) (Rigotti et al., 2013; Panzeri et al., 2015). This is because sparse coding allows an increased number of neuronal activity patterns to be discriminated efficiently from an energetic standpoint (Rolls & Treves, 1990). Evidence from modelling and experimental studies shows that the coding in the AC might approach this described optimum by employing a set of filters to extract behaviorally relevant sound features, and represent this by efficient and precise spiking (Rokem et al., 2006; Smith & Lewicki, 2006; Hromadka et al., 2008). How this is implemented under natural conditions in awake freely moving animals is still poorly described.

1.6 PLASTICITY IN THE AUDITORY SYSTEM

Plastic changes in the auditory system occur either during early postnatal development or, to a lesser extent, later in the adult animal. Plasticity in the adult animal occurs as animals need the ability to constantly adapt their behavior to an ever-changing environment. The following paragraphs will discuss early and late plasticity, with a focus on the latter. As the central theme of this study is the investigation of associated neural changes during the transition into motherhood, the last section will be devoted to that plasticity regime in particular.

1.6.1 *Early life plasticity*

An example that illustrates early life plasticity in the auditory system is the sound source localization system in barn owls. Barn owls are nocturnal animals that rely heavily on their sense of audition to hunt prey, and therefore have an extremely precise ability to locate a sound source (Knudsen et al., 1979). When one ear of a young barn owl is occluded, these animals initially misorient, but then over time learn to adapt and recover accurate orienting (Knudsen, 1983; Knudsen, 1999). This effect is due to an initial misalignment of visual and spatial auditory inputs in the SC that slowly recovers over time (King et al., 1988).
Similarly, when vision is obstructed by raising barn owls with specific prism glasses, accurate orienting behavior toward a sound source can be recovered after weeks (Knudsen & Knudsen, 1989; Knudsen & Brain, 1991). What happens on the neural circuit level is that that newly formed connections from the central to the external nucleus of the IC represent the novel ITDs. At the same time, original inputs are suppressed by inhibition (Zheng & Knudsen, 1999). The persistence of these original connections likely allows these young animals to later switch back to the original setting when prisms are removed. Conversely, this plasticity does not occur in adult animals that did not experience a prism displacement period during their juvenile stage, and therefore are not able to later switch back to that configuration (Brainard & Knudsen, 1995; Knudsen, 1999). It is speculated that these pre-existing experiences leave a permanent trace in the brain in the form of existing but silent connections that can be reactivated later (Knudsen, 1994). This shift in the ability to adapt neural circuits is referred to as the critical period (Hubel & Wiesel, 1970; Katz & Shatz, 1996). However, it is noteworthy that the IC of birds, such as barn owls, is not a homologue of the IC in mammals, and these mechanisms of early life plasticity might be different in the mammalian subcortical auditory system.

In mice, early plasticity also occurs in their sensory systems. Classic deprivation studies during early life can alter the processing of certain stimuli (Gordon & Stryker, 1996; Hübener & Bonhoeffer, 2010). For

instance, in the visual system, monocular deprivation – the closure of one eye – causes 'cortical blindness', in which cells in the visual cortex do not receive sufficient connections early on in development to ensure normal visual acuity (Gordon & Stryker, 1996; Huang et al., 1999). In particular, a shift in ocular dominance plasticity occurs associated with an increased input connectivity to the open eye (Frenkel & Bear, 2004). Similarly, monaural deprivation - occlusion of one ear - causes tonotopic maps to be distorted and hinders binaural integration (Popescu & Polley, 2010). In the mouse auditory system, critical periods shape neuronal circuits to facilitate the representation of relevant features, such as the expansion of certain frequencies (Berardi et al., 2000). Interestingly, different sound features are affected in different ways, such that critical periods for pure tone and frequency-modulated tones are sequentially organized and independent of each other (Nakamura et al., 2020). Therefore, critical periods in the mouse auditory system also depend on the type of stimulus (Barkat et al., 2011; Bhumika et al., 2020). Finally, dendritic spine maturation in thalamocortical connections was shown to occur during the critical period of pure tone plasticity in mice, representing a means by which critical periods can be influenced (Barkat et al., 2011).

1.6.2 *Adult plasticity*

Plasticity can also occur outside of the aforementioned critical periods in the adult AC of rodents, which is assumed essential for performing and learning relevant behaviors (Gold & Bajo, 2014). A prominent concept of plasticity in the adult auditory system is map expansion. It refers to a, sometimes transient, expansion of the tonotopic map in the AC to enhance the representation of a momentarily relevant frequency (Schreiner & Winer, 2007). This was observed in a tone discrimination task in rats, in which certain frequencies were coupled to a reward upon correct identification of the target frequency (Reed et al., 2011). This behavioral learning was accompanied by transient auditory map expansion for that specific target frequency. However, this map expansion is not necessary for improved performance. Most likely, a renormalization process after learning brings initial map expansions back to original values (Schreiner & Polley, 2014). During the aforementioned critical period, passive exposure to sound stimuli can result in auditory cortical map expansion in rodents (Zhang et al., 2001). Nevertheless, such map expansion does not follow passive exposure to sounds in adults. Albeit, simultaneous nucleus basalis stimulation - a cholinergic input to the AC - can have similar effects even in adult animals (Reed et al., 2011; Chun et al., 2013). Cholinergic inputs onto the AC can be activated under natural conditions when an animal finds itself in a state of heightened attention, which in turn can dramatically affect neural coding in the AC (Polley et al., 2006; Schreiner & Polley, 2014). Under heightened attentional states, when animals for instance attend to a task-relevant frequency, a change in

STRF shape with increased excitation at the target frequency is induced (Fritz et al., 2003). The effects on cortical coding include reduced response latencies and increased behavioral sensitivity (Fritz et al., 2007). Processes like long-term synaptic potentiation or depression are usually confined to the critical period (Crair & Malenka, 1995), but simultaneous stimulation of the nucleus basalis for example can restore this plasticity in the adult (Blundon et al., 2011). Cholinergic drive can therefore be a gating mechanism for plasticity in the adult stage (Schreiner & Polley, 2014). Next to attention, also motivation can influence map expansion, such that the degree of expansion can also depend on how motivated an animal was (Rutkowski & Weinberger, 2005). Moreover, when stimulating dopaminergic input to the AC - mimicking reward and increasing motivation levels in animals - sound sequence discrimination is improved (Kudoh & Shibuki, 2006).

To date, exactly which processes contribute to the change in plasticity of the AC when animals enter the adult stage is still a matter of debate (Schreiner & Polley, 2014; Blundon et al., 2017). Dendritic spines represent a highly promising site of plasticity (Engert & Bonhoeffer, 1999; Hofer et al., 2009). In addition, extra neural processes, like perineuronal nets of extracellular matrix proteins, can affect the susceptibility of AC circuits to experience-dependent plasticity, such as map expansion. Through wrapping of cortical neurons in juveniles, neurite motility of neurons is reduced and thus the ability to form new synapses and new projections is diminished (Friauf, 2000; Pizzorusso et al., 2002).

It has been suggested that not only the increasing amount of inhibition with age, but inhibition in general further dampens the ability to plastically adjust cortical circuits (De Villers-Sidani & Merzenich, 2011). With time, sound-evoked inhibition becomes more prominent, thereby sharpening AC neurons' responses (Chang et al., 2005), while becoming more balanced with excitation over time (Dorrn et al., 2010). In addition, inhibition is reduced upon nucleus basalis stimulation, causing enhanced excitation in AC neurons (Froemke et al., 2007). Interestingly, chronic exposure to diffuse sounds reduces inhibitory tone, thereby allowing map expansion. Due to a reduction in inhibition, the adult AC becomes more prone to a cortical excitation inhibition imbalance that occurs in aging, autism or for instance schizophrenia (Gogolla et al., 2009; Fung et al., 2010; Takesian et al., 2012; Zhou & Merzenich, 2012).

1.6.2.1 *Motherhood associated plasticity in the auditory cortex*

One of the most dramatic events in the life of an animal is the transition into motherhood. Primiparous mothers are confronted with a completely new set of stimuli, which in turn trigger a particular set of behaviors. Primiparous, as opposed to second or third–time mothers, show long-lasting memories for pup caring (Fleming et al.,

1999; Scanlan et al., 2006). In mice, an essential sensory input are the calls emitted by isolated pups that want to be retrieved back to the nest and be taken care of (Sewell, 1970). These pup calls are the initial trigger of this pup retrieval behavior, and are represented in the AC of mice upon playback (Liu & Schreiner, 2007; Galindo-Leon et al., 2009).

Plasticity in the auditory system therefore plays a central role during the transition into motherhood, particularly when caring for the offspring as in pup retrieval. In particular, neural responses to pup calls become stronger and more abundant after parturition in mothers (Liu & Schreiner, 2007; Cohen & Mizrahi, 2015; Marlin et al., 2015). However, this increase in neural responsiveness to pup calls is not limited to mothers, but also the aforementioned experienced virgins (EVs) show elevated levels of neural activity, although not as strongly as those for mothers (Cohen et al., 2011). As discussed in the previous section, pure tone exposure can elicit auditory map expansions under certain circumstances, but this is not the type of plasticity that occurs during motherhood. In fact, the frequency range that is occupied by pup calls is not enlarged, whereas the separability between pup and adult vocalizations is improved (Shepard et al., 2015). Specifically, a larger fraction of pyramidal neurons responded to pup calls in mothers compared to naive virgins (NVs). Additionally, the spontaneous firing rate was lower in pup call responding neurons of mothers than those of NVs, which increased the signal-to-noise ratio in those neurons (Shepard et al., 2015).

When comparing pup and adult call elicited spike trains of AC neurons, it was observed that there was a higher dissimilarity between spike trains between adult call and pup call evoked spiked trains in mothers compared to NVs, measured by using van Rossum spike train metrics (van Rossum, 2001; Shepard et al., 2015). This spike train dissimilarity could be the source for better discrimination of these two semantically different call types, despite their high spectral similarity. These findings further substantiate the notion of the AC coding for higher order sound features (Nelken, 2008; Bizley & Cohen, 2013; Elyada & Mizrahi, 2015). Noteworthy, the general responsiveness to pure tones seems unaffected in mothers compared to NVs (Lin et al., 2013).

The spectro-temporal structure of a pup call is essential in eliciting strong time-locked responses in the AC of maternal animals (Schiavo et al., 2020). When artificially modifying the inter-syllable-interval (ISI) between single pup call syllables, and stimulating mothers and NVs with those, it was shown that animals not only behaviorally preferred natural pup calls over modified ones, but also neurons in the AC responded preferentially to the natural pup calls (Schiavo et al., 2020). Mechanistically, this is carried out by an initially extremely narrow excitatory response to the natural pup call in NVs, and concomitant untuned inhibition. The narrow excitation is widened in mothers, so that these animals respond also to mild variations in ISIs, resulting in

the neural responses being generalized across a broader range of pup calls.

In order to improve the detectability of high frequency pup calls, inhibitory neurons were found to respond particularly to frequency bands lateral to pup calls, thus enhancing the contrast between behaviorally irrelevant low frequencies and behaviorally relevant high frequencies (Galindo-Leon et al., 2009; Lin et al., 2013). This contrast enhancement also manifests itself behaviorally in mothers and EVs. In particular, mice prefer a natural pup call over a behaviorally irrelevant 20 kHz pure tone in a two-alternative choice test (Lin et al., 2013). In contrast to elevated levels of excitation, feedforward inhibition was found to be generally reduced upon parental experience, resulting in a disinhibition and thus increased excitability of pyramidal neurons (Cohen & Mizrahi, 2015; Marlin et al., 2015).
As already mentioned before in the adult plasticity section, the synaptic excitation-inhibition (EI) current balance is a critical regulatory factor that influences plasticity. In the seminal study from Marlin and colleagues in 2015, the EI balance in the AC of mothers and EVs was well established, but not in NVs (Marlin et al., 2015). Interestingly, the neuropeptide oxytocin (OT) has exactly this effect of balancing excitation and inhibition in AC neurons of mothers and EVs, providing a mechanism by which the AC can boost spike timing precision for pup calls (Marlin et al., 2015). Oxytocin was found to accelerate the onset of maternal behavior, and is implicated in the development of maternal behavior in EVs (Marlin et al., 2015; Carcea et al., 2019). Interestingly, oxytocin positive neurons in the PVN fired during shepherding events and during pup call play-backs. These responses preceded the pup call evoked responses in the AC of freely moving mice (Carcea et al., 2019). Whether these AC circuit changes are innate and hard-wired or experience-dependent is unclear.
Nevertheless, auditory signals are only part of the multisensory context triggering maternal behavior: Among others, also olfactory cues from the pups contribute substantially to this behavior (Lévy et al., 2004; Lévy & Keller, 2009). Most importantly, a study from Cohen and colleagues in 2011 demonstrated that pup odors also synergistically enhance pup call-evoked responses of A1 neurons in mothers and experienced virgins, which improves the detection of pup calls in these animal groups. Another sensory modality that influences AC responses is tactile information, in rodents largely received via whiskers. In a social context, it was shown in rats that whisker contact enhances A1 responses to USVs of conspecifics (Rao et al., 2014). Whether the same holds true for maternal mice retrieving vocalizing pups remains unclear.

Next to these plastic changes on a single cell level, there is also experimental evidence of changes at the population level. One way to assess neural responses at this level is to quantify noise correlations. Noise correlations measure the trial-to-trial variability of single neurons within a population to co-vary in absence of any stimulus. Generally,

it is assumed that neurons with high noise correlations are synaptically connected to each other (Ko et al., 2011). While single cell response properties were comparable between mothers and NVs, pairwise noise correlations and population noise correlations were substantially increases in mothers compared to NVs (Rothschild et al., 2013). The type of plasticity regime in place in maternal animals remains a topic of debate. While some argue that parturition and maternal care represent sensory enrichment (Rothschild et al., 2013), others argue in favor of experience-dependent plasticity (Lau et al., 2019). It thus remains to be solved, to which degree maternal circuits are innate, and therefore hard-wired.

1.7 METHODS OF PROBING NEURAL ACTIVITY *in vivo*

In this study, I chose to utilize one-photon optical recordings to measure neural activity in the AC of awake behaving mice. Optical imaging measures neural activity through fluorescent indicators, which can either report neural activity indirectly through calcium and glutamate, or directly through voltage (Chen et al., 2013; Lin & Schnitzer, 2016). Both glutamate and voltage indicators need a very high (> 300 Hz) sampling rate that is not achieved with our approach. Additionally, glutamate and voltage imaging have not yet been successfully used in one-photon microscopy, which will be used in this study. Thus, I chose to use calcium imaging. Imaging calcium dynamics in neurons serves as a proxy for neural activity. It is a proxy since neural activity is accompanied by fluctuations in cellular calcium levels, such that they rise in an electrically stimulated cell (Baker et al., 1971; Borst & Sakmann, 1998). Through changes in fluorescence that occur upon binding of calcium, indicators report the fluctuations in cellular calcium that occur upon electrical activity and by this, neural activity is measurable. Calcium imaging also allows to record from specific cell types, and to track the same neurons across days of imaging (Peron et al., 2015; Sheintuch et al., 2017). Since calcium indicators are constantly expressed in neurons, once introduced - either via transduction with a viral vector or via genetically modified mouse lines - the refinding of the same neurons over the course of several days is possible. This can be achieved based on matching of the cell 'footprints', i.e. their x and y-coordinates in an imaging field of view (Sheintuch et al., 2017; Giovannucci et al., 2019). The advent of protein based calcium indicators, such as GCaMPs, allows access to neural activity with high sensitivity (Chen et al., 2013). However, as advantageous as calcium imaging can be for studying neural responses across time, this advantage also comes at the expense of temporal precision and signal to noise ratio (Chen et al., 2013). The signal to noise ratio of a calcium indicator describes the relation between the amplitude of the calcium signal during neural activity and the background signal in absence of neural activity. Current versions of GCaMP have optimized this ratio, such that fluorescence in absence of neural activity is minimal and is enhanced during neural activity (Chen et al., 2013; Dana et al., 2019). Calcium indicators bind calcium nonlinearly, and generally have a faster rise than decay time, which can be resolved with sufficiently high sampling frequencies (Tian et al., 2009). Depending on the optical system used, one can image hundreds of neurons simultaneously (Sofroniew et al., 2016). And just as a single character of the alphabet is not enough to form a meaningful word, there is increasing evidence that sensory inputs are coded by orchestrated activity patterns across thousands of neurons, rather than by the sum of many single neuron responses (Niessing & Friedrich, 2010).

The conventional microscopy method for calcium imaging is two-photon microscopy (Denk et al., 1990; Svoboda et al., 1997). Two-photon microscopes have superior imaging quality compared to one-

photon microscopes, owing to their deeper tissue penetration, efficient light detection, reduced light scattering and reduced photo-bleaching (Svoboda et al., 1997). These features are achieved due to the two-photon effect, which describes the lower probability of two photons exciting a fluorophore simultaneously than one single photon at a time. Achieving this two-photon effect requires higher light intensities, provided by excitation through pulsed lasers. Pulsed lasers, in combination with the high wavelengths used to penetrate tissue, result in a high resolution of the fluorescent specimen with little light scattering around the focus point.

As useful as two-photon microscopes are for probing neural circuits in vivo, animals have to be head-restrained and until very recently could not freely move while being imaged. Miniaturized two-photon microscopes were developed almost a decade ago (Helmchen et al., 2013). However, their practical application remains challenging and it is still in its early stages (Zong et al., 2017).
In addition, it becomes increasingly clear that anesthesia severely affects various neuronal response properties, including overall activity levels, stimulus-specific tuning of neurons or synchrony within neuronal populations (Gaese et al., 2001; Goltstein et al., 2015; Geissler et al., 2016). In addition, the currently more standard head-fixed but awake preparation would not allow us to study AC activity in a natural setting. The goals of this study are to both investigate how natural stimuli are represented in the AC of freely behaving mice, and to understand how the neural coding of auditory stimuli changes with hormone- or experience-dependent plasticity. Therefore, miniscope imaging is an appealing methodological approach and was the recording method of choice.

The revolutionizing invention of one-photon fluorescence miniaturized microscopes, (miniscopes for short) allows for calcium imaging in freely moving mice under naturalistic conditions (Ghosh et al., 2011). The miniscope utilized in this study weighs roughly 2 grams and is equipped with blue excitation light, i.e. a blue light-emitting diode (LED), to excite green fluorescent particles such as GCaMPs and records the emitted fluorescence with a simple complementary metal-oxide-semiconductor (CMOS) sensor. Compared to two-photon microscopy, miniscope imaging has an inferior spatial resolution. Movement artifacts can also be found in head-fixed preparations. Nevertheless, these artifacts can be more pronounced in freely moving imaging. Fortunately, computational advances in analyzing miniscope data have helped in discerning single one-photon sources, and correcting for motion artifacts (Lu et al., 2018).

2

MATERIALS & METHODS

2.1 ANIMALS

Wild type CBA/CaJ female mice were bred in-house at the MPI of Neurobiology and kept in standard type II cages (530 cm^2) of two same sex individuals. Cages were equipped with little red plastic houses, serving as a shelter for mice, wooden chips as bedding as well as enrichment and nesting material made from wooden shavings. Mice were fed *ad libitum* with standardized pelleted food and had constant water supply.

In this study, a total of 16 female mice were used. 12 mice were used for the main experiments, whereby 4 animals were assigned to each experimental group: Mothers (MTs), experienced virgins (EVs) and naive virgins (NVs). MTs were mated with a male at the age of ~12 weeks, roughly 2 weeks after cranial window implantation. Males were removed from the cage once females were visibly pregnant. Afterwards another female was placed into the mother's cage. EVs were placed into a cage of a pregnant MT, 2-3 days prior to the first miniscope imaging session. NVs remained in the same cage with one additional female throughout the entire course of experiments, and were never co-housed with neither a pregnant female nor pups. For pup retrieval experiments, alien pups at P2-P5 that did not belong to the home cage of the tested animal were used.
The other four adult animals were used for the comparison of tone-evoked responses between the two-photon microscope and the one-photon microscope.

2.2 BEHAVIORAL ARENA

The 46 cm x 35 cm behavioral arena had 20 cm high walls and was equipped with a hydrophobic 3 mm thick black ground cover of ethylene-vinyl-acetate (Cellofoam, soniEVA) with soundproofing features (**Fig. 3.1**). The walls were covered by a black 50 mm thick soundproof material (Cellofoam, soniRESIST) that absorbs sounds and is ideally suited for minimizing background noise. The ground was covered by the same mouse bedding material used for mouse cages. 10 cm away from the short arena side an additional separating wall was integrated into the arena. This wall was 14 cm high and separated the designated nest area from the rest of the arena. This nest area covered roughly 350 cm^2. Nesting material that mice also encountered in their home cages was placed behind the separating wall to facilitate the nest delineation for mice. Mice could freely move from the nest to the open arena compartments. A cable fixture above the arena reduced

the amount of weight that miniscope cables exert on the mouse's head. Next to the miniscope, a behavioral camera was installed above the arena to capture mouse behavior throughout the entire experiment. Additionally, the arena, equipped with 2-4 ultrasound sensitive microphones (Avisoft, CM16/CMPA, Avisoft UltraSoundGate 416H), allowed the recording of the full auditory scene during experiments. In order to stimulate animals with auditory cues, a loudspeaker was placed on the opposite side of the nest. To further reduce noise contamination, the entire arena together with the described additional devices, was placed inside a 120 cm x 100 cm x 100 cm soundproof chamber.

2.3 AUDITORY STIMULI

We used a set of pure and natural tones for stimulation presented with ultrasonic speakers (Tucker-Davies Technologies, ES1 electrostatic speakers, ED1 speaker driver). Pure tone pulses ranged from 4 kHz to 90.5 kHz with half octave steps between stimuli, adding up to a total of 10 pure tone stimuli, each lasting 80 ms. Natural calls included two pup USVs (1.9 s duration), two pup WCs (1.7 s - 2.1 s), each recorded from two different individuals, and one mating call (1.1 s) recorded from a male. Next to these five natural calls, we created four synthetic versions of these natural calls. Synthetic versions consisted of an 80 ms pulse of a multi-band frequency (6.1, 12.2, 18.3 kHz) sound resembling a WC, a train of these pulses lasting 1.7 sec, a train of 80 ms pure tones at 64 kHz mimicking a USV, as well as a train of behaviorally non relevant 22.6 kHz pure tones. Artificial stimuli were generated with Adobe Audition. Noise levels of pre-recorded natural calls were reduced with the same software. We employed two sound stimulation protocols that will be described in the following paragraphs.

The first sound stimulation protocol was used for awake miniscope sessions, in which auditory stimuli were delivered via an electrostatic free field speaker located centrally at the opposite side of the nest (Tucker-Davis Technologies, ES1 electrostatic speaker, ED1 speaker driver) with 5 pseudo-random repeats each, at an inter-stimulus interval of 4 sec. All stimuli were calibrated so that 70 dB sound pressure level (SPL) were measured 20 cm away from the speaker. Each of the artificial stimuli had a 5 ms cosine fade in and fade out at a distance of ca 10 cm from the mouse ears.

The second sound stimulation protocol was used during the comparison of miniscope and two-photon responses under anesthesia. Here, we used a modified version of the stimulus protocol mentioned above, consisting out of five pure tone pulse stimuli ranging from 4 to 64 kHz at full octave steps between stimuli, pure tone sequences at the same frequencies at two attenuation levels, 40 and 70 dB SPL, one pup USV, one pup WC and additionally a 80 ms white noise burst, as well

as a 2 sec long train of 80 ms white noise bursts.

2.4 VIRUS INJECTION AND CRANIAL WINDOW IMPLANTATION

Cranial windows over the left auditory cortex were implanted on 12 adult female CBA/CaJ mice (10 weeks old at the first surgery). Mice were anesthetized by intraperitoneal injection of a mixture of 0.05 mg/kg Fentanyl (Hexal), 5 mg/kg Midazolam (Ratiopharm), and 0.5 mg/kg Medetomidine (Sedin®, Vetpharma). Anesthesia was supported by an analgesic (Carprofen, Rimadyl®, Zoetis Deutschland GmbH, 4 mg/kg, subcutaneous injection) which was administered immediately before surgery and for two days after surgery. During the surgery, mice were kept constantly at 38 ºC body temperature and the corneas were protected from drying by applying eye cream. After disinfection of the skin, a rostrocaudal incision was made starting behind the eyes and ending at the height of the ears. Skin was removed above the midline and the left side of the skull. To prevent liquid leakage, bone sutures were carefully cauterized with heat and the exposed skull was covered with a thin layer of cyanoacrylate glue (Histoacryl). A metal headbar (0.3 x 1.4 cm) was fixed onto the skull along the rostrocaudal axis with dental cement.

We performed a circular craniotomy (3 mm diameter) with a biopsy punch after removing part of the temporalis muscle, exposing the tempoparietal suture that served as a landmark. By help of this landmark the craniotomy was centered over the auditory fields in the left hemisphere. Virus was injected at 3–4 sites into the auditory fields, using a mixture of AAV2/1.Syn.Flex.GCaMP6s.WPRE (PennU, LOT CS0385) and AAV2/9.CamKII0.4.Cre.SV40 (PennU, LOT V3807TI-R-DL). The GCaMP6s virus served as a functional marker that indirectly reported neural activity, while the second virus expressed Cre under the CamKII promotor. The latter sparsened the expression of the GCaMP6s virus, meaning only a few excitatory neurons will express the calcium indicator. The GCaMP6s virus was diluted to reach a final titer of ~1.8^{12} genome copies/ml, while the CamKII virus was diluted to reach a final titer of ~1.5^{10} genome copies/ml. The virus solution was injected using glass pipettes (tip diameter, 10–40 µm) and a pressure micro-injection system, at 200–450 µm below the cortical surface (150–250 nl/injection site, pressure injected with 15-20 injection puffs at 6-30 psi for 10-300 ms at a repetition rate of 0.2 Hz). After the injections, the craniotomy was closed using a glass cover slip (3 mm diameter) and Histoacryl. After the histoacryl was completely cured, super-glue was used to fixate the cover slip to the bone. After 2 weeks of viral expression, an imaging check was performed and the lens for miniscope imaging was implanted. Dental cement was used to close all exposed skull areas and to further fixate the window and headbar. Animals slowly woke up after injecting the subcutaneously 1.2 mg/kg Naloxone (Ratiopharm), 0.5 mg/kg Flumazenil (B. Braun Melsungen

AG), and 2.5 mg/kg Atipamezole (Veyx-Pharma GmbH) post-surgery, while kept under experimenter's surveillance below a warming red light.

2.5 LENS IMPLANTATION

In the second surgical intervention, mice were subjected to a light isoflurane anesthesia (0.5-1.5%), since only the imaging lens was implanted on top of the already inserted cranial window, and the mouse just needed to be static for this procedure. Mice were subcutaneously injected with a sedative (0.5 mg/kg bodyweight chlorprothixene) 30 min prior to the procedure in order to stabilize the isoflurane anesthesia. We performed the lens implantation under constant visual inspection through a micromanipulator-controlled miniscope positioned above the cranial window to ensure a good placement of the lens. Similarl to the first surgical intervention, mice were kept at a constant body temperature and the corneas were protected from drying. After a region of interest with well-transduced neurons was located, we lowered the miniscope together with the attached lens right above the chosen area. The lens is screwed into a plastic focus adjustment ring, which is lowered until it gently touches the sides of the cranial window implant. Once the lens is in place, I applied a thin layer of super glue and after curing, sealed the entire implant with dental cement to ensure a tight connection between the existing cranial window and the miniscope lens. Afterwards, the miniscope was retracted and lens implants were covered with a plastic protection cap until the next field-of-view inspection. The mouse was placed in a separate cage to allow for slow and undisturbed waking from the anesthesia before placing it back into its home cage.

2.6 HABITUATION

After the miniscope lens was implanted, we started habituating animals to the experimenter as well as to the arena and the miniscope for seven consecutive days. On the first day of habituation, mice were handled two times by carefully placing the mouse on the experimenter's hand and allowing the mouse to freely move from the hand back into the cage. This procedure was repeated three times on the next day, while additionally holding on to the animal's headbar for a few seconds while the mouse sat on the experimenter's hand. On the third day, animals were again handled three times, while the animal was allowed to walk freely on a flat running wheel without holding on to its headbar during the last period of habituation. On the following day, animals were once more handled three times; the animal was now shortly held by its headbar for a few seconds while the animal ran on the running wheel. During the two last habituation periods of the same day, the animals were placed into the experimental arena

and allowed to freely explore the arena for ten minutes at a time. On day five, animals were handled three times as usual, and slowly habituated to placing the miniscope dummy on its implant while they were moving on the running wheel. During the last two periods of habituation, animals were again placed into the arena for 10 min each, while now carrying the dummy miniscope on their heads. During the three habituation periods of the following day, the dummy miniscope was carefully placed on the animal's head while on the running wheel, and animals were allowed to explore the arena again for 10 min with the head-mounted dummy scope. On the last day of habituation, animals were trained again three times, while now carrying the real miniscope on their heads. This habituation protocol was adjusted, in case an animal needed more habituation to the miniscope.

2.7 MINISCOPE IMAGING

A 2.2 g miniaturized fluorescence microscope (miniscope) with a surface lens was used (Doric, Snap-in fluorescence body model S). The miniscope is connected to a 488 nm wavelength LED that served to illuminate the brain and excite GCaMP6s. A CMOS sensor collected emitted green light between 500 and 550 nm wavelength and transmitted signals to a data acquisition board. We used an objective lens with an NA of 0.5 and obtained FOVs at 630 x 630 pixels, corresponding to a FOV size of 970 x 970 µm and a pixel size of 1.5 μm^2. To avoid twisting of the cables that are connected to the miniscope on a freely moving mouse, a pigtailed rotary joint was inserted between the LED light source and the miniscope. Single miniscope recordings were triggered through the Doric Controller software. Frame pulses were recorded and synchronized with the microphones, speaker and the behavioral camera frames through a custom-written LabVIEW program.

We performed awake miniscope imaging sessions every two days on well-habituated mice over the course of 4 weeks. The interval length between miniscope sessions varied for individual cases, as these experiments depended on the presence of newborn pups (see animal section for details). While the first session was a baseline or pre-birth time point for mothers (MTs) and experienced virgins (EVs), the following sessions occurred while pups were in the home cage of these experimental groups. In the baseline time point, MTs were pregnant, but did not give birth yet, and EVs were co-housed with a pregnant MT. Starting from the following session, pups were born. NVs never encountered pups in their home cage, solely during pup retrieval experiments.

On each experimental day, mice underwent three repetitions of awake miniscope imaging. Before starting experiments, mouse cages were placed in the miniscope setup room, allowing animals to slowly adjust to the new room. Each repetition started with a 2 min phase

of spontaneous activity (SA), in which animals could freely explore the already familiar arena, while we recorded neural activity from their left auditory cortex (AC). Right after the SA phase, followed the pup retrieval (PR) phase, in which we placed consecutively 4 to 6 alien pups into the arena. The animals were given 6 to 8 min to retrieve all pups back to the nest, before the experiment ended and pups were removed from the arena. These blocks of SA followed by PR were repeated three times on a given day. Additionally, animals were confronted with playbacks of a set of pure tones, pre-recorded natural calls and synthetic variants of calls. This passive stimulation (PS) phase followed either the second or the third SA phase of a given day, before animals retrieved pups during PR phases.

2.8 TWO-PHOTON IMAGING

Two-photon imaging was performed only under a light isoflurane anesthesia (0.8-1.2% isoflurane in O_2). Mice were subcutaneously sedated 30 min prior to initiation of isoflurane anesthesia with 0.5 mg/kg chlorprothixene. After 30 min, mice were placed inside a soundproof chamber (22 x 10 cm), which was flooded with isoflurane. After falling asleep, mice were fixated into a stereotactic head bar holder inside the soundproof chamber and kept at 37°C with a feedback controlled heated mat below the mouse throughout the experiment. Imaging was carried out with mice head-fixed in this soundproof chamber. Pedal reflexes by toe pinching front and back paws were tested to ensure adequate anesthesia.

A custom-built two-photon microscope was used to acquire the images. The microscope was driven by a Ti:Sapphire laser combined with a DeepSee pre-chirp unit (Spectra Physics MaiTai eHP, <100 fs pulse width, 80 MHz repetition rate), set to an excitation wavelength of 940 nm. Laser power was controlled with a half-wave plate in combination with a polarizing beam splitter, and adjusted to a power between 7-35 mW as measured with a photodiode after the objective (Olympus, 4x, 0.28 NA). We placed a beam dump in the focal plane between the scan and tube lenses to block the laser beam at the scan turning points. Photons from the objective were divided by a beam splitter (FF560 dichroic) and directed into two separate photomultiplier tubes (PMT, Hamamatsu R6357) with green (525/50-25 nm) and red (607/70-25 nm) band pass emission filters respectively. Data were digitized with a high-speed digitizer (NI-5761, National Instruments, 500 MHz), which was combined with a field-programmable gate array (FPGA) to translate the PMT signal into pixels.

Two-photon imaging was performed twice for those animals undergoing awake miniscope sessions: The first time when the FOV for implanting the miniscope lens was chosen, and the second time after the awake miniscope sessions. The same FOV was imaged at a depth

of ~120 µm with a sampling frequency of 17 Hz. Every two frames were averaged. The imaging FOV size was 1140 x 1490 µm, with a single pixel size of 0.9 μm^2.

2.9 PERFUSION

All experimental animals were sacrificed by transcardial perfusion after the final imaging step. Immediately after the imaging session ended, mice were deeply anesthetized by an intraperitoneal injection of 1.5-2 times the dose of fentanyl based anesthesia that was used for cranial window surgeries (see section 2.4). After ~20 min, the entire absence of the pedal pain reflex was tested to ensure extremely deep anesthesia. Once animals showed no signs of pain reaction, they were fixated on a perfusion stage and after disinfection of the fur, the skin over the xiphoid process was carefully cut, to open up the intraperitoneal cavity. The diaphragm was cut, and the cut and opened chest flap was fixated to the side, uncovering the lungs. The heart was carefully held with forceps, while cutting a small incision into the right ventricle and inserting a 25 G needle attached to a peristaltic pump into the left ventricle. Over the course of the next 15 min, a cold saline solution containing lidocaine (0.1%) and heparin (1 U/ml) was used to slowly flush the cardiovascular system of the mouse. After all blood was washed out and the lungs turned white, we switched the saline solution to a 4% paraformaldehyde (PFA) solution, which fixated the tissue for the next 15 min. After 15 mL of both solutions rinsed the system successively, the brain was removed carefully and stored at 4°C. Before brains were cut and stained, we transferred them into a solution of 30% sucrose in phosphate buffered saline (PBS) at 4°C until they sank.

2.10 IMMUNOHISTOCHEMISTRY

Brains were cut into 50 µm thick coronal sections with a microtome (Thermo Fisher Scientific) and washed three times for 10 min in PBS. Slices were placed overnight into 300 µL blocking buffer (PBS containing 10% goat serum, 3% Triton X-100). After removing the blocking buffer, slices were stained with 300 µL of the primary antibody solution (blocking buffer containing 1:2000 rabbit anti-calbindin from Swant, 1:2500 mouse anti-GFP from Fitzgerald) overnight. Next, sections were washed again three times for 10 min in PBS and placed overnight into 300 µL of the secondary antibody solution (blocking buffer containing 1:200 Alexa 647 rabbit and 1:500 Alexa 488 mouse). After washing the sections again three times for 10 min in PBS, they were stained in red Nissl (1:100) for 20 min, washed again twice in PBS, subjected to DAPI (1:1000) staining, washed again for three times and afterwards mounted on slides for fluorescence microscopy (Axio Imager 2, Zeiss, 10x objective).

2.11 DATA ANALYSIS

2.11.1 *Miniscope source extraction*

Miniscope data was registered and source extracted using MIN1PIPE (Lu et al., 2018). The inputs to this function were the imaging data, the framerate, and the size of a structural element. The MIN1PIPE algorithm (Lu et al., 2018) consists of three main modules: (1) The neural enhancing module, (2) the movement correction module and (3) the seeds-cleansed neural signal extraction module.

With help from the neural enhancing module, the background, i.e. everything except neuronal cell bodies, is estimated and removed. Then, the background and the foreground are separated via anisotropic diffusion denoising (Perona & Malik, 1990). Additionally the first step encompasses a morphological opening operation (Serra & Vincent, 1992), in which anything smaller than the pre-defined structural element size (corresponding to half the size of a neuron) was considered noise and was removed with the background.

In the second step, frames were movement corrected. Movement correction was performed in a hierarchical manner, such that first stable sections were registered and then unstable sections were incorporated into the registered stack of the stable sections. In particular, stable and non-stable sections were identified by using the Kanade-Lucas-Tomasi tracker (Shi & Tomasi, 1994) During this hierarchical procedure, as in initial step, small translational displacements within stable sections were registered with the fast Lucas-Kanade tracker (Lucas & Kanade, 1981). Large deformations were corrected by diffeomorphic Log Demons registration (Vercauteren et al., 2009). Secondly, all registered stable sections were registered to each other. For this, sectional images were extracted for each registered stable section, and these sectional images in turn passed through the Kanade-Lucas-Tomasi and the Log Demons algorithm. Lastly, non-stable sections were incorporated into the registered stable sections on a frame by frame basis.

In the last module of MIN1PIPE, neural signals, i.e. sources, were extracted. First, a set of potential regions-of-interest (ROIs) was identified, also containing false positive sources. Second, this over-complete set of ROIs was passed through a cleansing process to reduce this initial set of ROIs to the true set of ROIs. The cleansing process consisted out of three main stages: First, a two-component Gaussian mixture model detected ROIs with large fluctuations, thereby eliminating false-positive ROIs. Second, a previously trained recurrent neuronal network was applied to further reduced the fraction of false-positive ROIs, based on their abnormal background fluctuations. To avoid having multiple ROIs for the same neuronal source, ROI time traces were compared and merged upon high temporal similarity. Third, an iterative process that optimized the spatial and temporal footprints of single ROIs was carried out, similar to what is proposed in constrained non-negative

matrix factorization, short CNMF, an alternative source extraction method (Zhou et al., 2018).

I temporally down sampled recordings to 10 Hz. The outputs of MIN1PIPE were registered and background subtracted sources, namely single neurons, spanning the duration of the recording. It is noteworthy that MIN1PIPE can only detect active neurons, since the source identification relies on strong temporal fluctuations in calcium.

2.11.2 *Miniscope cell matching*

In order to match the single neurons obtained from MIN1PIPE, we used the cell matching function from the open source toolbox CaImAn (Giovannucci et al., 2019). This function relies on computing distance matrices between cells from two recordings. When cells pass a certain threshold criterion, they are considered the same cells. This is done for each pair of recordings, so that for each experiment the identity of neurons also found in other experiments is known for data from the same mouse.

2.11.3 *Behavioral annotations*

All behavioral videos recorded along with each experiment underwent manual inspection, and different behaviors were annotated with the open source software Boris (Friard & Gamba, 2016). In total, ten event types were annotated, one of which tracks the timing of pup placement by the experimenter, while the remaining nine event types were concerned with the behavior of the adult mouse. Five of these behavioral events are pup caring unrelated events, such as eating, digging, grooming rearing and sitting. The other four event types are linked to pup caring, namely retrieving nesting material, sitting on a pup outside the nest, contacting a pup outside the nest and retrieving a pup. We defined specific criteria for each event type: For pup placements, we annotated the video frame in which a pup was placed into the arena and added information about the pup identity. For pup-unrelated event types, the onset was defines as physical contact with a food pellet for eating, start of digging with the forepaws in the bedding for digging, physical contact of the forepaws with the face for grooming, physical contact with a wall for rearing and halting for at least two seconds for sitting. In each case, the offset of the behavior was defined as the time point in which the mouse refrained from that particular behavior. All pup related event types were additionally annotated with information about pup identity, just as for pup placements. Similarly as for non-pup related events, the onset for pup-related events each started with physical contact of the adult mouse with the pup. In particular, the nose of the adult mouse had to physically contact the respective pup. For the event types sitting on pup and contacting pup

outside the nest, the offsets were defined as physical abandonment of the pup. The category of pup retrievals was further subdivided into successful and unsuccessful retrievals. The offset for successful retrievals was identified by dropping the pup into the nest area, whereas unsuccessful retrieval offsets were annotated when the mouse dropped the pup outside the nest. Moreover, pup contact outside the nest was further classified into gentle or aggressive contact. These annotations were later imported into Matlab, synchronized with the other experimental measurements and analyzed by custom written Matlab scripts.

2.11.4 *Pup retrieval latency calculation*

To quantify the temporal delay between the placement of a pup into the arena and its subsequent retrieval by an animal, we measured retrieval latencies for single pup placements. If a pup was retrieved successfully, the temporal delay between placement and retrieval was noted. However, if a pup was not retrieved, we added a punishment delay for that specific pup. Specifically, we calculated the potential retrieval time animals had to retrieve that pup, which was the temporal window from placement until the end of the experiment. This value was assigned as the retrieval latency for that specific pup.

2.11.5 *USV detection*

USVs emitted by pups and adults were detected by the open source software DeepSqueak, version 2.1.2 (Coffey et al., 2019). Audio files were imported into DeepSqueak, and the default detection neural network for mouse calls was employed to detect single USV syllables. Full audio files were analyzed in 30 sec chunks. The user-defined parameters included an allowed overlap between single syllables of 0.008 sec, with a high frequency cut off above 120 kHz and a low frequency cut off below 30 kHz. We did not include a scoring threshold. DeepSqueak segmented the audio file into smaller chunks and based on sonograms, i.e. a spectrotemporal depiction, object contours were detected by help of a faster-regional convolutional neural network (faster-RCNN). The faster-RCNN was previously trained with hundreds of mouse vocalizations and used to isolated thousands of mouse USVs, which again served as input to re-train this network, thereby building a network with high recall and robustness (Coffey et al., 2019). A so called region proposal network further segmented the object contours into proposed areas of interest. These areas of interest in turn were passed to the aforementioned classification network that detects mouse USVs, which eliminated false positive syllables and kept true mouse USVs. Each audio recording was manually checked after DeepSqueek detection, and remaining false positives were removed. Based on the identification of single syllable contours, the software detected on- and offsets, the dominant frequency, the loudness and

the frequency modulation for each syllable. This output was imported into Matlab and further analyzed with custom written Matlab scripts. Spectrograms of exemplary calls were imported into AvisoftSAS Lab Pro (Avisoft) and exported to plot for display.

2.11.6 *USV discrimination*

In order to discriminate pup from adult USVs, we utilized the DeepSqueak functionality to train a supervised network for classification after manual labeling. For this purpose, 6773 adult and 6614 pup calls were fed into training, and the classification network was validated on unseen USV data (adult calls n = 4229, pup calls n = 4524). Data used for training came from recordings in which solely adult mice or pups were in the arena. Pup calls used for training the network matched the employed distribution of pup ages in our experiments. For adult recordings, we used the SA phases of our experiments with each experimental group being represented. The pup USVs were recorded separately in the same behavioral arena. Here, only pups were placed into the arena outside a retrieval context. We employed this network to classify the sender identity (adult or pup) of DeepSqueak detected USVs in PR experiments.

2.11.7 *Support vector machine (SVM) analysis*

In order to decode events such as pup calls, pup call frequency, pup call power, pup call ISI, adult calls, pup retrieval events, location and speed, we used linear SVMs with 5-fold cross-validation to validate decoding accuracies. Decoders were trained on a single trial basis with all neurons at hand. Binary classifiers included the ones for pup calls, adult calls and pup retrievals. The other ones were multiclass classifiers: Pup call frequency and power had six categories. Pup call ISI had twenty, speed had five and location had twenty-five label categories. The label categories for pup call features (frequency, power, ISI) and mouse speed were chosen based on the distributions from all pup calls for these three decoders. For decoding mouse location, the arena was split into a 5x5 matrix, resulting in a total of twenty-five label categories. All decoders were compared to control decoders trained with n=5 shuffles of the label vector.

For decoding pup call presence after training in one trial and predicting pup call presence in another trial, we used matched cells(see above). Since not all trials had equal amounts of pup calls in them, we took 95% of the data per trial, from the trial that had lower pup call occurrences and took matched number of frames for the no pup call category. This was performed for all pairwise comparisons. Similarly, we used 5-fold cross-validation to validate decoding accuracies, and included a regularization parameter of lambda = 0.01 for L1 lasso reg-

ularization. The decoders were compared to control decoders trained with n=5 shuffles of the label vectors.

2.11.8 *Statistics*

Throughout this study, we reported medians across single trials for single animals in thin lines, as well as medians across all animals of an experimental group in thick lines. This was true for all time courses across sessions. To test for significance across experimental groups, a non-parametric Wilcoxon rank sum was used to compare single trials for each time point. We corrected for multiple comparisons via the Bonferroni correction.

When comparing response ratios to pup calls and random events, we first pooled all the data of single cell and event response ratios. We did this for three cell response types: Random neurons (RN), high weight neurons (HW) and high response ratio neurons (RR). The latter category was chosen based on the 90^{th} percentile of response ratios to pup calls of a given trial. Ultimately, these response ratio vectors were statistically compared by an one-way ANOVA and corrected for multiple comparisons.

3

RESULTS

How are behaviorally relevant communication calls represented in the auditory cortex of mice that are engaged in a natural behavior? In this study, we addressed this question by employing head-mounted miniscopes, which allow imaging of neural activity in freely moving mice. In particular, we focused on identifying and characterizing the neural code for pup calls in mouse auditory cortex (AC). During imaging, the pup retrieval (PR) behavior of these mice was interrogated and followed along six non-consecutive days of pup exposure. Furthermore, we aimed to shed light on the plasticity occurring in the AC over time and compare this across different pup exposure regimes. These regimes were examined in the form of three experimental groups, namely mothers (MTs), experienced virgins (EVs) and naive virgins (NVs).

To address these questions, we first investigated behavioral performance across days for each experimental group. Next, we separated pup from adult calls and described their evolution across time. Based on the aforementioned behavioral and audio descriptions, we quantified the neural activity measured in our task. Finally, we analyzed the features of the population code that underlies the encoding of pup calls across time in the AC.

3.1 PUP RETRIEVAL BEHAVIOR

To study the representation of communication calls in mice, I employed PR as a natural behavioral paradigm. In this paradigm, just as during PR, newborn pups emit USVs (Ehret, 1975), which elicit retrieval behavior in MTs but also in EVs, virgin females that learned to care for pups (**Fig 3.1A**). This behavior is usually not observed on the first session in mice that do not have any prior experience with pups, termed NVs (**Fig. 3.1A**).

I performed six consecutive sessions of PR in a behavioral arena, while closely monitoring mouse and pup behavior with a camera from above (**Fig. 3.1B,C**). These six sessions started with a pre-birth time point at which mothers were already pregnant, followed by five sessions after parturition (**Fig. 3.1C**). While EVs were already co-housed with a pregnant female in the pre-birth session, this session marked the first encounter between NVs and pups in the task. Throughout the entire time, NVs were co-housed with another naive female, without pups being present in their home cage. The behavioral arena used for our experiments was rectangular, with a separating wall that delineated the nest area (**Fig. 3.1B**). In order to facilitate adoption of this area as the nest, I placed nesting material into that area and always placed the first pup in each trial into the nest. Thereby, familiar nesting material as well as the pup odors guided mice into the nest and marked it as a safe shelter.

Pup retrieval experiments were repeated three times in a given session, and always followed a preceding spontaneous activity (SA) phase, in which animals explored the arena in the absence of pups (**Fig. 3.1B**). We used three measures to quantify behavioral performance during the task, all based on pup retrievals, which we defined as the times when an adult mouse picked up a pup and brought it within the predefined nest area. The first measure was the percentage of total pups that were successfully retrieved into the nest. The second measure quantified the temporal latency by which each pup was contacted for subsequent retrieval after being placed into the arena. The last measure quantified the fraction of successful retrievals. In unsuccessful retrievals, mice picked pups up but did not bring them to the delineated nest area, and instead released them elsewhere in the arena.

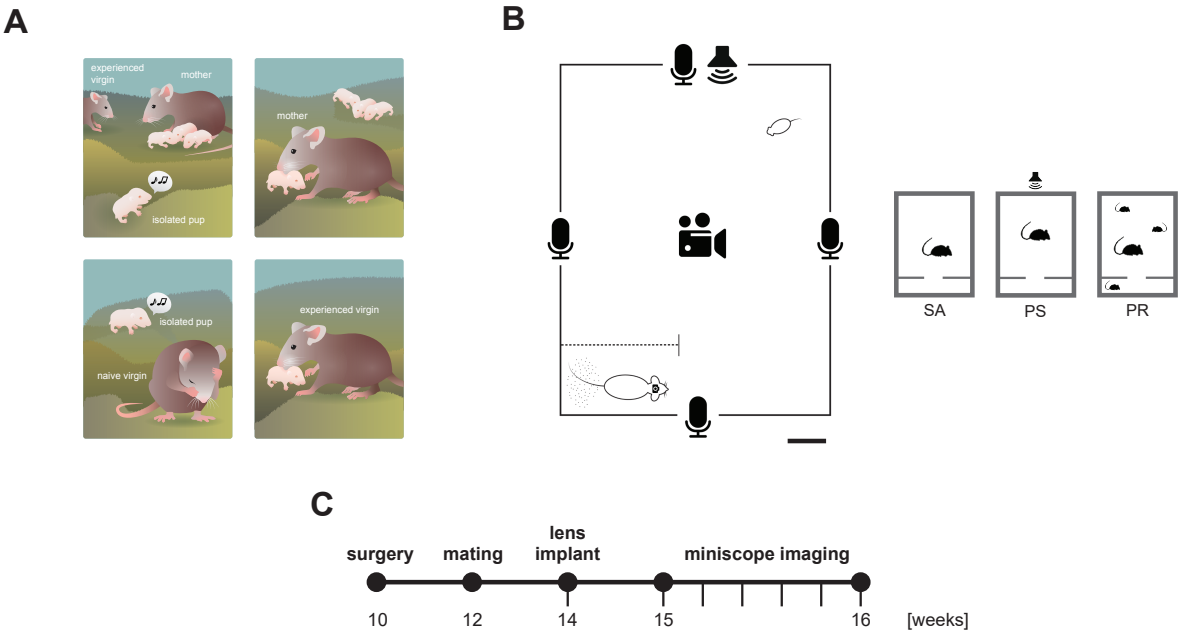

Figure 3.1 | Experimental paradigm of pup retrieval. A *Left*: Schematic of pup retrieval in the three groups: Mothers (MTs) naturally care for pups (*upper left*). When an isolated pup emits ultrasonic vocalizations (USVs) to signal distress, MTs react to the pup USVs by approaching the calling pup and retrieving it to the nest (*upper right*). In contrast, naive virgins (NVs), that never encountered pups before, will not retrieve isolated pups in a pup retrieval task (*lower left*). When co-housing virgins with a mother and its litter, the former will retrieve pups and is therefore defined as an experienced virgin (EV) (*lower right*). **B** *Left*: Outline of behavioral arena, with icons indicating the microphones, camera and speaker locations, scale bar = 5 cm. *Right*: Schematics of the three experiment types: spontaneous activity (SA), passive stimulation (PS) and pup retrieval (PR). **C** Experimental timeline with vertical bars indicating imaging time points, mouse age is indicated in weeks.

We found that, on average, all groups increased their behavioral performance across sessions (**Fig. 3.2A**). MTs started with the highest performance, with one MT already retrieving all of the placed pups in the pre-birth session. EVs reached the 100 % performance state few sessions after, but with two out of four EVs not retrieving any pup during two sessions (**Fig. 3.2A**). NVs started with the lowest performance on the baseline session and then steadily increased their performance, reaching a peak on session four (**Fig. 3.2A,B**). The temporal latencies with which pups were retrieved to the nest comprised the time delay between placement of the pup into the arena and successful retrieval to the nest. After three sessions, EVs and NVs reached comparable retrieval latencies as MTs, which already retrieved pups with short latencies during the pre-birth session (**Fig. 3.2B**). The fraction of successful retrievals increased steeply in EVs and NVs, even after one

session (**Fig. 3.2C**). This indicated that once the sequence of PR was performed successfully, mice rarely failed in retrieving a pup to the nest. In contrast to the initial high fraction of unsuccessful retrievals in EVs and NVs, MTs showed a high fraction of successful retrievals also in the pre-birth session (**Fig. 3.2C**).

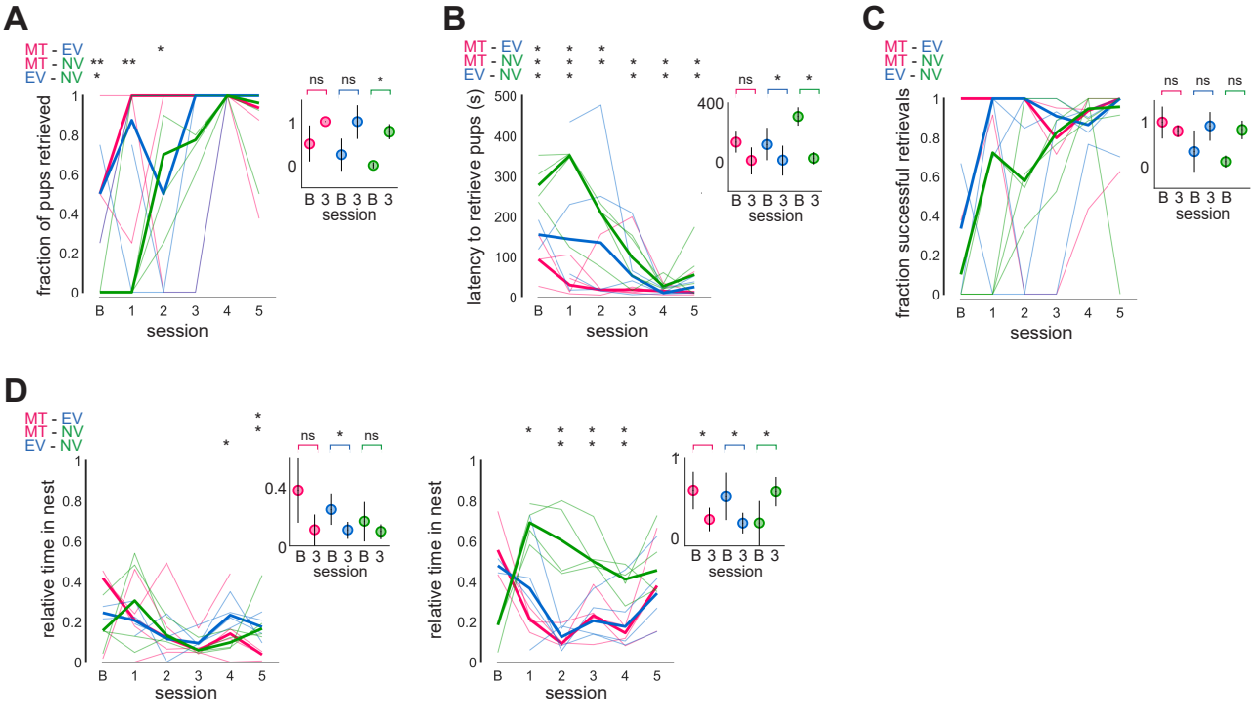

Figure 3.2 | Repeated pup retrieval in mothers, experienced and naive virgins. A Behavioral performance plotted as percentage of total pups retrieved. Thin lines represent single animal medians across trials, thick lines the median across animals. Stars indicate significant differences between groups, $p<0.05$, Wilcoxon rank sum test corrected for multiple comparisons. *Inset*: Comparison between baseline and session three per group, star indicates $p<0.05$, Wilcoxon rank sum test, magenta = mothers, blue = EVs, green = NVs. **B** Latency to retrieve pups over sessions, stars indicate significant between group differences, $p<0.05$, Wilcoxon rank sum test corrected for multiple comparisons, *Inset*: Comparison between baseline and session three per group, star $p<0.05$, Wilcoxon rank sum test. **C** Fraction of successful retrievals over sessions. **D** Relative time mice spent in the nest versus outside the nest. *Left*: During SA phases. *Right*: during PR phases.

The nest is a very prominent location within the arena, as it is the key location where pup caring, like warming the pups, occurs, but it can also be seen as a retreat area for mice. Therefore, I quantified the relative amount per trial mice spent inside the nest. MTs and EVs initially spent a lot of time together with retrieved pups in the nest, whereas NVs were found more often outside the nest (**Fig. 3.2D**). For this quan-

tification, I calculated the relative time mice spent in the nest during PR, and compared this to SA experiments. In PR experiments, MTs and EVs did indeed spend more time in the nest during the pre-birth session than NVs did (**Fig. 3.2D**). This initial preference of MTs and EVs for the nest over the rest of the arena decreased in the following sessions and ultimately increased again. Interestingly, this U-shape of the temporal profile was inverted for NVs, which initially spent most of the time outside the nest (**Fig. 3.2D**). During sessions two and three, NVs spent most time in the nest, which is also supported by the observation that once a pup was retrieved by a NV, the respective NV took more time to leave the nest and search for new pups. In contrast, MTs and EVs tended to switch faster into a search mode after having retrieved a pup. This observation is supported by time courses of total distance travelled in an experiment (**Fig. 3.3A**). While mice of all groups covered the same distance during SA experiments, increased distances were covered in later PR sessions compared to baseline PR sessions (**Fig. 3.3A**). In sessions two and three, MTs and EVs covered significantly more distance during PR than NVs did (**Fig. 3.3A**). Only in sessions three or later, NVs covered similar amounts of distance during PR, indicating that these animals were more strongly involved in PR than in earlier sessions, and more often left the nest to search for new pups.

We then looked more closely at the retrieval events, in particular how fast adult animals retrieved pups by use of speed profiles (**Fig. 3.3B**). Typically, mice came to a halt when contacting the pup, thereby triggering the onset of the retrieval phase. After finding a good grip on the pup, mice retrieved pups into the nest. These three episodes, i.e. approach, holding pup and retrieval, were part of the speed profiles. We found that MTs were the quickest in approaching isolated pups and retrieving them to the nest (**Fig. 3.3B,C**). Each group showed an increase in approach and retrieval speed across sessions. However, MTs and EVs reached a marked speed increase already in sessions one and two, respectively. NVs reached a higher speed only in session three, with their maximal speed measured in the last session. Interestingly, MTs showed a more pronounced asymmetry between approaching and retrieving speeds, so that the retrieval speeds in session three and four are much faster than the respective approach speeds (**Fig. 3.3C**). This asymmetry was not detected in either of the other groups, and set MTs apart from EVs. Moreover, we found that the time needed to adequately grip the pup was elevated in NVs compared to EVs and MTs (**Fig. 3.3D**). This indicated their decreased pup handling abilities compared to MTs and EVs. Overall, this alteration in speed profiles between groups highlights, next to the other retrieval performance measures, both the successive improvement of pup retrieval behavior across sessions in all groups and resolves more detailed differences between MTs and EVs.

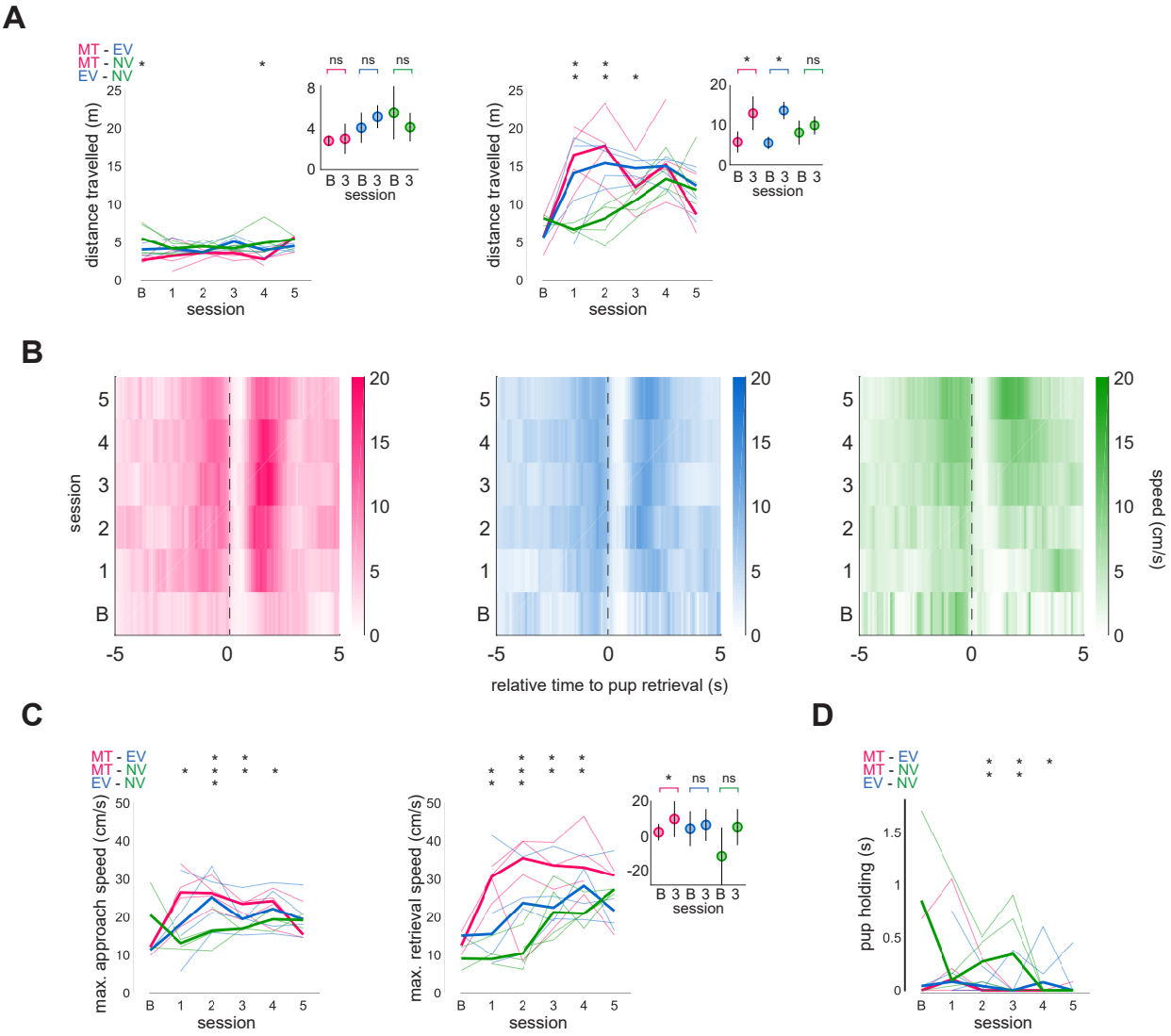

Figure 3.3 | Speed profiles of pup retrieval. A Time courses of median distance mice travelled during SA (*Left*) and PR (*Right*). Stars indicate significant differences between groups, $p<0.05$, Wilcoxon rank sum test corrected for multiple comparisons. *Inset*: Within group comparisons of distance travelled between baseline session and session three, $p<0.05$, Wilcoxon rank sum test. **B** Heat maps indicating average speed of mouse in cm/s before and after a pup retrieval event, dotted line marks the onset of retrieval, *Left*: MTs, *Middle*: EVs, *Right*: NVs for each session. **C** *Left*: Time course of maximal approach speed, stars indicate significant differences between groups, $p<0.05$, Wilcoxon rank sum test corrected for multiple comparisons. *Right*: Maximal retrieval speed. *Inset*: Within group comparisons of speed differences between retrieval and approach speeds, $p<0.05$, Wilcoxon rank sum test. **D** Pup holding durations per group over sessions, $p<0.05$, Wilcoxon rank sum test corrected for multiple comparisons.

Altogether, we find that all experimental groups increased their retrieval performance across sessions and, interestingly, pup exposure in the task alone is sufficient for inducing stable retrieval behavior in NVs. A marked difference among groups could be measured in the baseline session, where MTs outperformed both EVs and NVs in their temporal latency to retrieve pups. The initial preference of the nest area in MTs and EVs compared to NVs, might reflect their increased pup caring behaviors, learned during their co-housing with their own or foreign pups respectively. Furthermore, speed profiles revealed yet undescribed differences among groups, highlighting faster approach and retrieval speeds in MTs and EVs compared to NVs. Additionally, the increased retrieval compared to approach speeds in MTs, were not found in both other groups and underlined a strong pup retrieval efficacy in MTs. Concisely, these behavioral measures reflected distinct group differences that were indicative of the respective pup exposure regimes.

3.2 PUP RETRIEVAL ASSOCIATED VOCALIZATIONS

It is well known that PR behavior in mice is initially triggered by USVs emitted by isolated pups. These USVs direct a mouse's attention towards the isolated pup, guiding it to that pup and leading to a retrieval behavior (**Fig. 3.1A**). The experimental setup is equipped with ultrasound sensitive microphones to capture those communication calls and the entire auditory scenery during experimental sessions (**Fig. 3.1B**). Until now, it was unknown whether adult females would vocalize in a pup retrieval context.

Interestingly, we came across these adult calls when analyzing the audio recordings of SA experiments, in which only adults explored the behavioral arena (**Fig. 3.4A**). More specifically, we detected increasing amounts of adult USVs during SAs from the pre-birth session onwards, with MTs eliciting many USVs in session one, right after birth of their own pups, shortly followed by EVs and finally NVs (**Fig. 3.4B**).

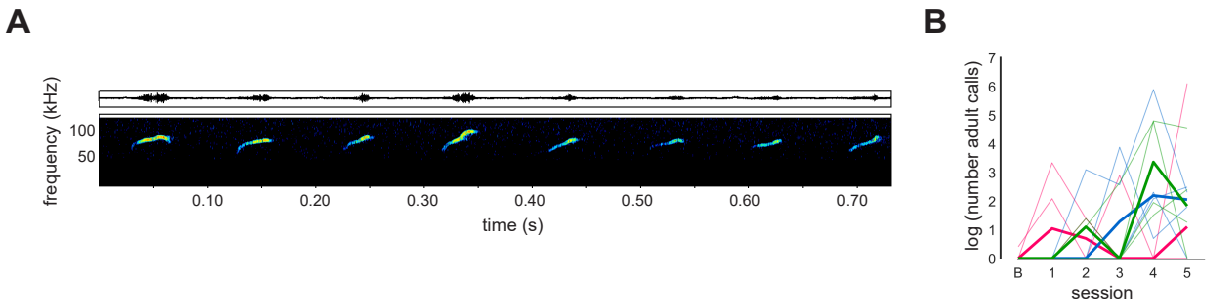

Figure 3.4 | Adult vocalizations. A Exemplary spectrogram of adult vocalizations during SA. **B** Quantification of adult call numbers measured in absence of pups during SA phases, thin lines represent single animal medians across trials, thick lines represent medians across animals, magenta = MTs, blue = EVs, green = NVs.

Since it was unknown whether adult females would vocalize in a PR context, we could not discern whether a syllable recorded during PR originated from an adult or a pup (**Fig. 3.5A**). To elucidate this, we first compared USV features from audio recordings in which only pups were present in the arena to the SA recordings in which only adults were in the arena. When comparing pup and adult calls, it became apparent that the two populations of USVs overlapped in frequency, power, and duration, and are thus not easily separable from each other (**Fig. 3.5B**). For instance, the population of pup calls had a low (75 kHz) and a high frequency subpopulation (around 105 kHz), whereas adult USVs had one main population slightly above the low frequency pup subpopulation. Pup calls lasted up to 100 ms, which was in stark contrast to the much shorter maximal duration of 40 ms measured for adult calls (**Fig. 3.5B**). Within each call category, we observed a

large variety of call shapes, displayed in a t-SNE plot (**Fig. 3.5C**). Since we wanted to test whether both animal groups vocalized in a pup retrieval context, we had to find a way to separate both call categories. As both USV populations overlapped in parameter space, applying a simple threshold for certain parameters such as frequency or call duration does not separate both populations from each other. Therefore, the overlapping USV populations prompted us to employ a more complex classification approach to distinguish both call types. This approach consisted of training a supervised call classification network, a part of the DeepSqueak (Coffey et al., 2019) software package, with over 10,000 pup and adult calls (**Fig. 3.5D**). After having trained the classification network, we tested its performance and generalizability on previously unseen call data. Both training and testing of this network was performed on audio recordings containing exclusively pup or adult calls. Despite the large variety of call shapes within each category and the overlap in simple call parameters, this classification network allowed us to confidently identify pup and adult calls (**Fig. 3.5C,D**).

Next, we applied this classification network to the audio recordings of PR experiments. To our surprise, we found that during pup retrieval not only pups emitted USVs, but also adult mice vocalized (**Fig. 3.5E**). Subsequently, we obtained time series for both call categories during PR experiments (**Fig. 3.5E**). Despite the increasing proportion of adult USVs in SAs across time, the number of adult calls emitted per second was elevated on average in all PR experiments compared to all SAs, irrespective of the experimental group (**Fig. 3.4B, 3.6A**). Interestingly, the amount of adult calls per PR session went up across sessions, similarly to the aforementioned increase in adult calls during SA experiments (**Fig. 3.4B, 3.6A**). While adult animals vocalized more across sessions, the amount of emitted pup calls remained constant (**Fig. 3.6B,C**). Additionally, the trained network allowed us to track call parameters across PR sessions. Finally, we found a high degree of variability across individuals of one group (**Fig. 3.6D,E**).

Collectively, we first found that adult animals emitted USVs during SA. This finding prompted us to compare both pup and adult call categories. Both call categories overlapped and as we wanted to test whether adults also called during PR, we employed a neural network to separate these call types. By help of this network we demonstrated that adults indeed called during PR. Interestingly, the amount of adult calls detected increased over repeated PRs in all groups, whereas in the first session MTs emitted the most, followed by EVs and NVs. The enhanced occurrence of adult calls in SAs after the pre-birth time points suggests that these calls emerge increasingly with more pup exposure in our task. The meaning of adult calls in this context is currently unknown and will be a subject of the discussion.

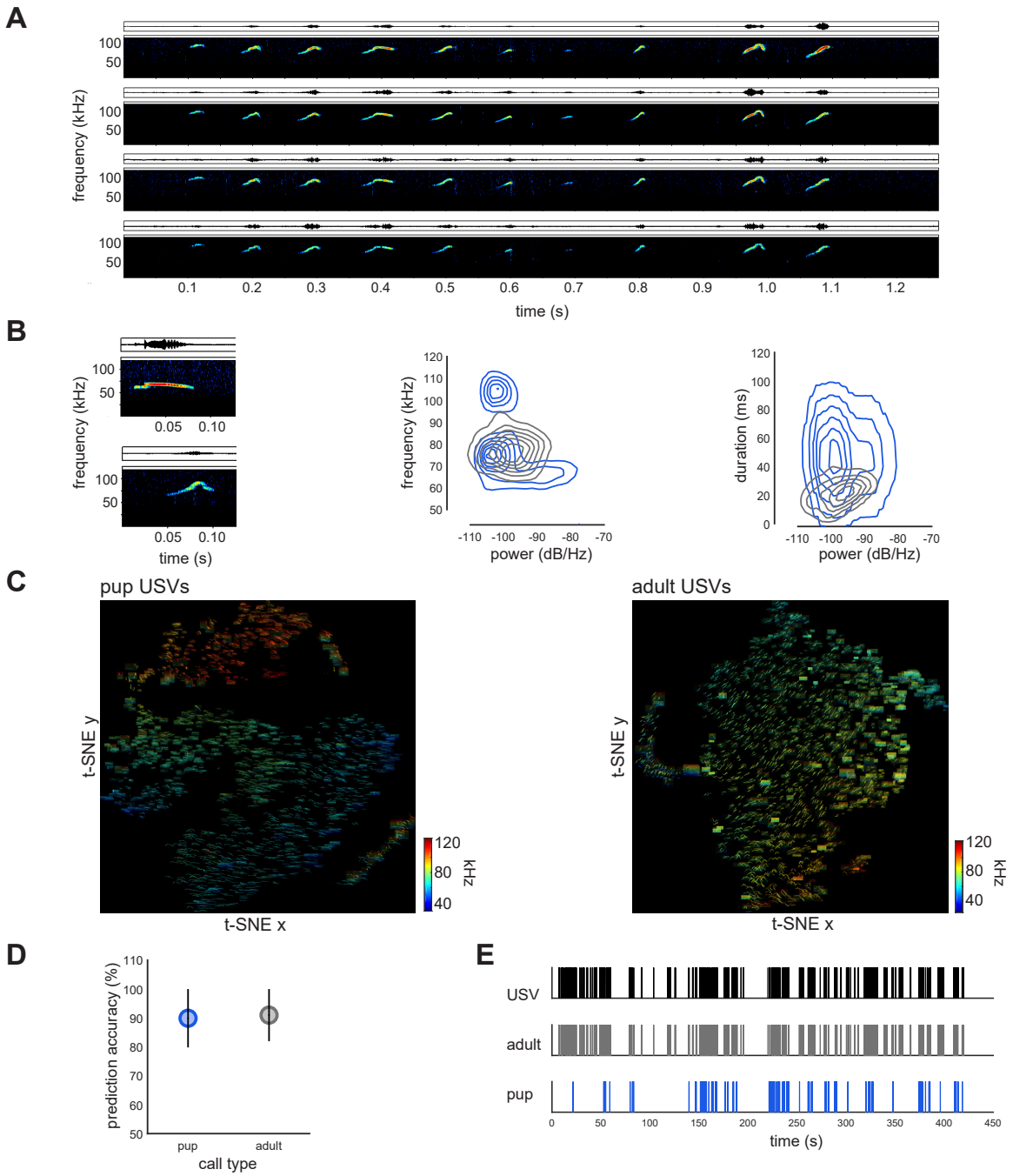

Figure 3.5 | Separation of pup and adult vocalizations. A Audio recordings with four microphone array during exemplary PR with raw audio signal shown above each spectrogram. **B** *Left*: spectrogram of an exemplary syllable from a pup (*top*) and an adult (*bottom*). *Middle*: Kernel density estimates of the population of calls plotted according to their duration and power, *Right*: The same population plotted according to their principal frequency and power, pup calls n=11,138, adult calls n=11,002. **C** T-SNE embedding of pup (*left*) and adult (*right*) USVs used for training separated according to contour shape, the color of each contour indicates frequency. **D** Prediction accuracy for testing classification network on unseen call data (pup calls n=4,524, adult calls n=4,229) based on training of a neural net. **E** All call instances in an exemplary pup retrieval experiment shown in black.

Classified adult calls (grey) and pup calls (blue) are shown below.

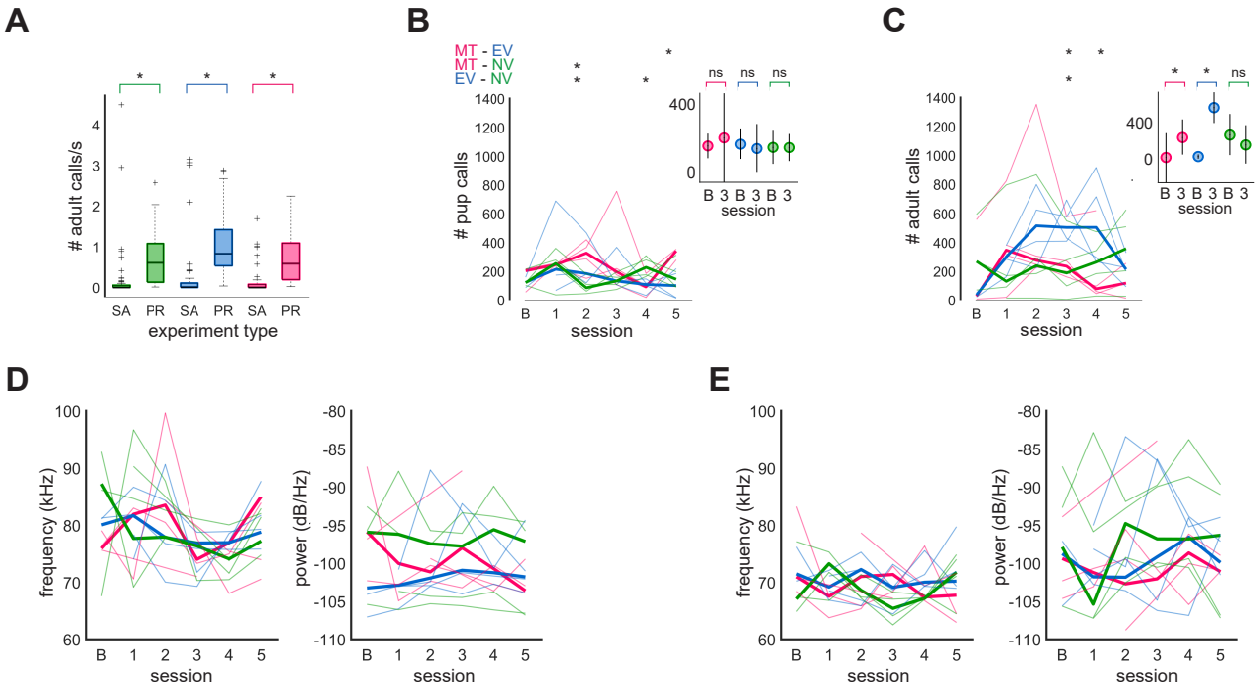

Figure 3.6 | Pup and adult vocalizations during pup retrieval. A Boxplot showing all adult and pup call quantifications during SA and PR phases for the three experimental groups, $p<0.05$, Wilcoxon rank sum test. **B** Number of pup calls quantified in SA experiments, thin lines are single animal medians, thick lines medias across animals per group, *Inset*: Within group statistical comparison between baseline and session three, $p<0.05$, Wilcoxon rank sum test. **C** Number of adult calls quantified in pup retrieval experiments, thin lines represent single animal medians across trials, thick lines the median across animals. *Inset*: Within group statistical comparison between baseline and session three, $p<0.05$, Wilcoxon rank sum test. **D-E** Evolution of call parameters across sessions for adult (**D**) and pup calls (**E**), *Left*: Dominant frequency, *Right*: Loudness of calls.

3.3 NEURAL ACTIVITY IN THE AC DURING PASSIVE SOUND STIMULATION

With the one-photon miniscopes I was able to record Ca^{2+} signals as an indirect proxy for neural activity from hundreds of neurons in the AC simultaneously (**Fig. 3.7A,B,C**). To first verify that also with a miniscope, we can record sound-evoked activity in the AC of mice that is similar to the same recordings obtained with a two-photon microscope, I presented mice with classic sound stimuli under head-fixation and isoflurane anesthesia (**Fig. 3.8**). In this setting, mice were anesthetized with isoflurane and head-fixed below either a custom-built two-photon microscope, or a one-photon miniscope, while sound stimuli were presented (**Fig. 3.8A**). The auditory stimuli used consisted of five 80 ms long pure tone pulses, ranging from 4 to 64 kHz at one-octave steps, a 80 ms long white noise pure tone, and several sequences (**Fig. 3.8B**). The pure tone sequences extended over 2 s, containing ten equally spaced 80 ms pure tone pulses at both 40 and 70 dB SPL (**Fig. 3.8B**). Lastly, two natural stimuli were part of the stimulus set: a pup WC and a pup USV (**Fig. 3.8B**). Stimuli were repeated 16 times per session.

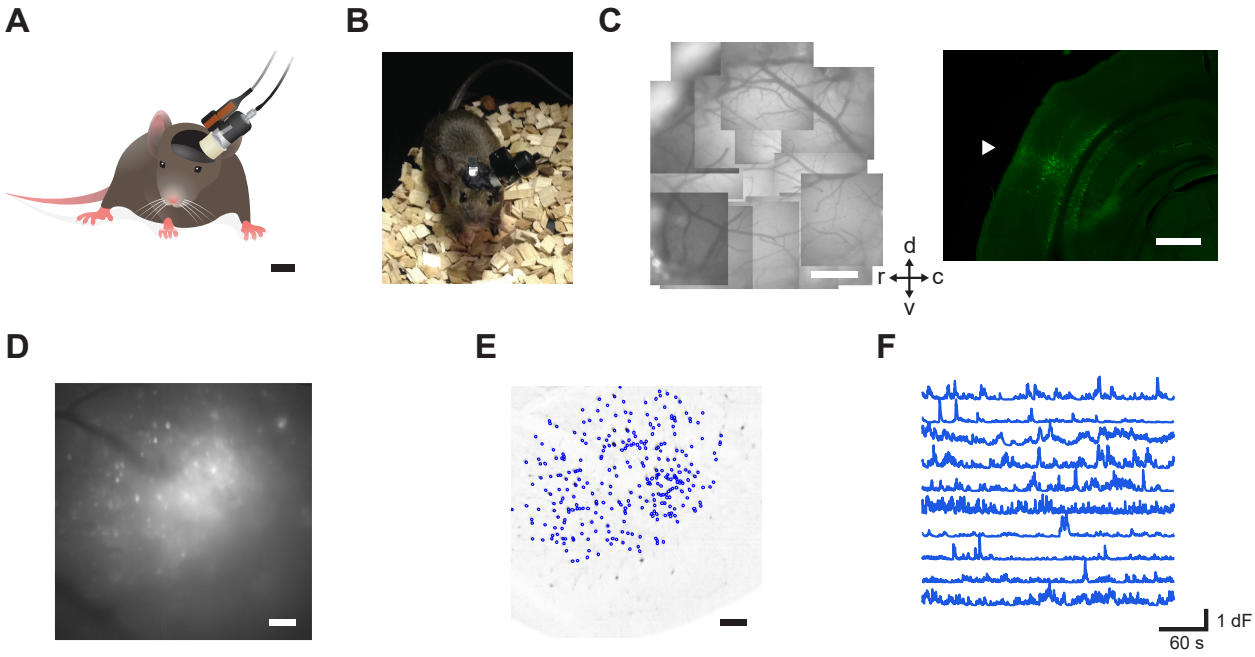

Figure 3.7 | Miniscope imaging of the auditory cortex in freely behaving mice. A Schematic of a mouse carrying a head-mounted miniscope in its left auditory cortex scale bar = 1 cm. **B** Photograph taken of a mouse carrying the head mounted dummy microscope on top of the left AC, while freely moving in the behavioral arena. **C** (*Left*): Exemplary stitched miniscope frames recorded from the surface of the cranial window right before lens implantation, displaying the blood vessel pattern, scale bar = 500 µm. Arrowheads indicate anatomical directions, rostral (r), caudal (c), ventral (v), dorsal (d). (*Right*): Immunohistochemical staining of GCaMP

neurons in the AC, indicated by white arrowhead, scale bar = 500 µm. **D** Exemplary miniscope raw field of Cre-GCaMP6s transduced CaMKII$^+$ neurons in the auditory cortex (same example as in C), scale bar=100 µm. **E** Neuronal footprints from the same field-of-view from D, obtained with MIN1PIPE (Lu et al., 2018). **F** Traces of 10 exemplary neurons from the same field-of-view shown in D and E.

I was able to revisit the same region of GCaMP6s-transduced excitatory cells under both the two-photon microscope and the miniscope (**Fig. 3.8C**). Each animal was imaged three times on one day with two-photon microscopy and two days later with the miniscope. These experiments revealed that we can record sound-evoked activity from the same neurons under both two-photon and miniscope microscopy (**Fig. 3.8D**). In particular, in two exemplary neurons shown, the neural activity to a pup WC was consistent on average across the three sessions on a given day (**Fig. 3.8D**).

After having confirmed that AC responses to sound stimulation were also measurable with the miniscope, I performed experiments on freely behaving animals carrying a miniscope. In general, we observed that AC activity in the awake compared to the anesthetized mouse was elevated, with tone-evoked responses having higher amplitudes than under anesthesia (**Fig. S1**). During experiments on freely behaving animals, mice listened passively to the play back of auditory stimuli on each session, in so called passive stimulation (PS) experiments (**Fig. 3.1B**). In those PS experiments, auditory stimuli, similar to the ones mentioned above, were played back while the animal was allowed to freely navigate in the behavioral arena. Passive stimulation with auditory stimuli generally enhanced neural activity in AC, compared to experiments without any sound stimulation (**Fig. S2**).

To determine whether clear tone-evoked responses can be measured in the AC of freely moving mice, we isolated single cell responses to auditory stimuli during PS (**Fig. 3.9**). Some single neurons showed clear tone-evoked responses to longer (> 1 s) stimuli, such as pure tone sequences. However, the population of neurons showed rather weak responses (**Fig. 3.9**). In these awake PS experiments, only five repetitions of sound stimuli could be played back, to avoid photo-bleaching. Therefore, playbacks of pre-recorded pup calls showed an increase in responses in some neurons, while most of the population did not show clear tone-evoked responses (**Fig. 3.10**). These data confirmed earlier reports that AC cells show tone-evoked responses to auditory stimuli in awake mice (Hromadka et al., 2008; Tischbirek et al., 2019).

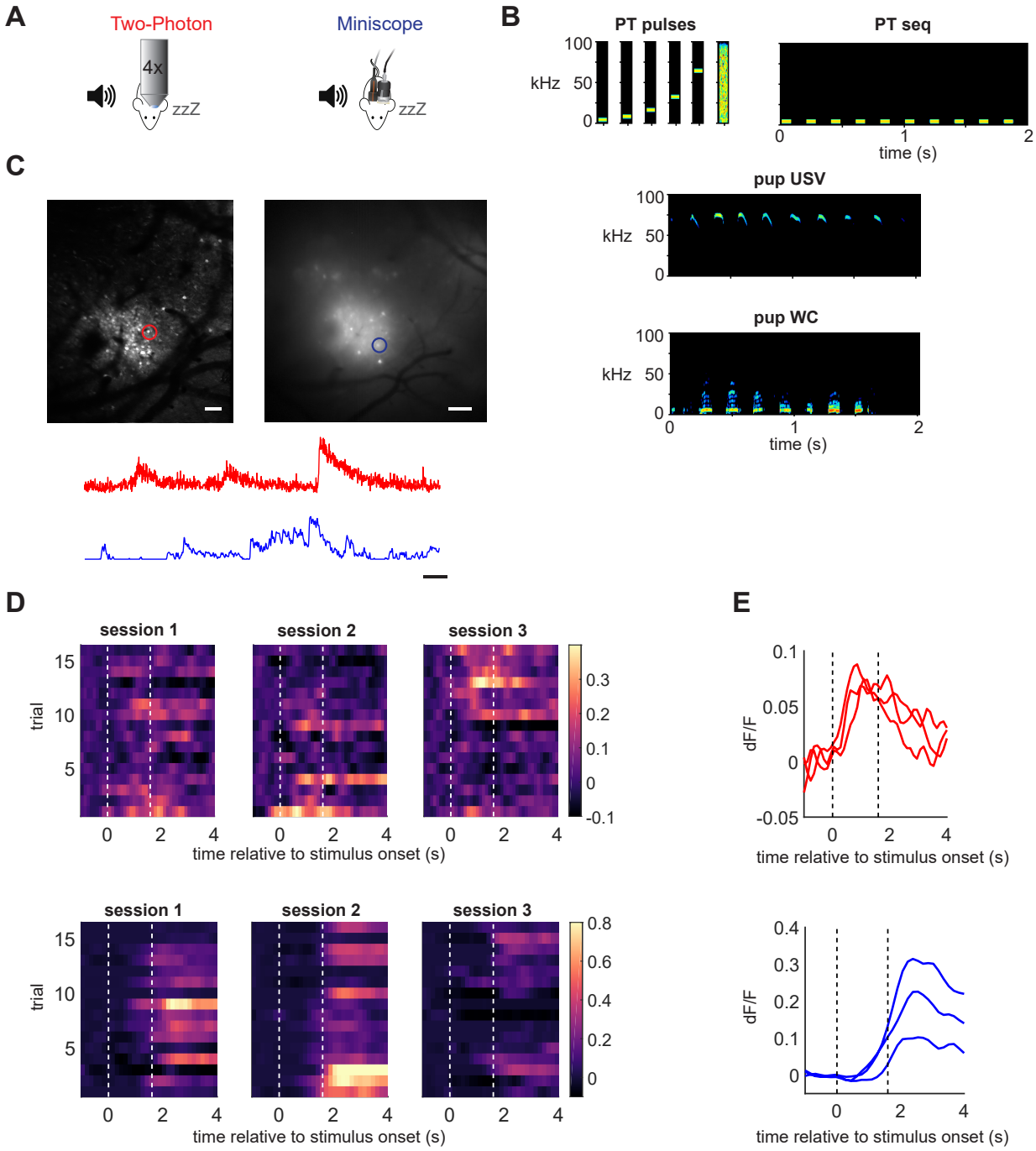

Figure 3.8 | Tone-evoked responses of AC under anesthesia with 2p and 1p microscopy. A Schematic of head-fixed imaging of the left AC of isoflurane-anesthetized mice with two-photon (*left*) and miniscope microscopy (*right*) in response to auditory stimuli. **B** Spectrograms of auditory stimuli, consisting out of six 80 ms long pure tone pulses at five different frequencies and a white noise stimulus covering frequencies from 2 to 100 kHz (each stimulus presented at 70 dB SPL). Additionally, each pure tone sequence was presented in pure tone sequences of ten pulses covering 2 s at two intensities (40 and 70 dB SPL). The white noise stimulus was also presented in a sequence, but at 70 dB SPL only. Lastly, two natural calls,

namely a typical pup USV and a pup WC were part of the stimulus set. **C** (*Top*): Exemplary field-of-views of Cre-GCaMP6s transduced CaMKII^{+} neurons in same mouse under the two-photon microscope (*left*) and the miniscope (*right*). (*Bottom*): One exemplary calcium trace is shown for each imaging method, scale bar = 10 s. **D** (*Top*): Exemplary traces of a single neuron imaged by two-photon microscopy. Each heat map indicates the trial-wise dF/F calcium trace to a pup WC in one session. (*Bottom*): Same depiction as above but for an exemplary neuron imaged with the miniscope. **E** (*Top*): Activity trace for the same exemplary neuron as in **D** to a pup WC, averaged across the 16 trials per session, imaged with the two-photon microscope. (*Bottom*): Same structure as above, but for the miniscope imaged neuron.

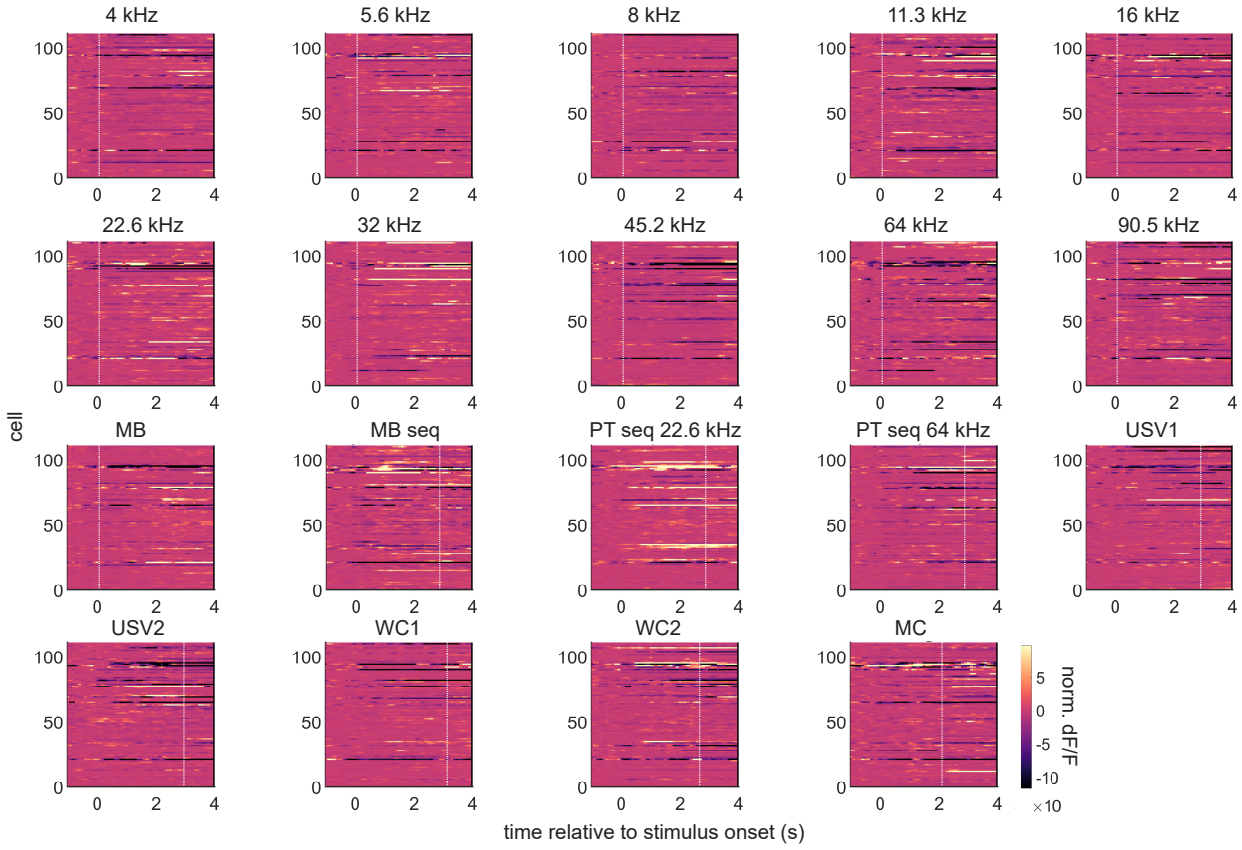

Figure 3.9 | Exemplary tone-evoked responses to passive stimulation in freely moving mice. Exemplary baseline subtracted single cell responses relative to stimulus onset, averaged across five repetitions per stimulus, heat indicates response amplitude. Stimulus identity indicated above each heat map. The first two rows show responses to 80 ms pure tones (PTs), below are responses to multi-band (MB), MB sequence (MB seq), PT sequence (PT seq) and natural stimuli like USVs, WCs and a mating call (MC).

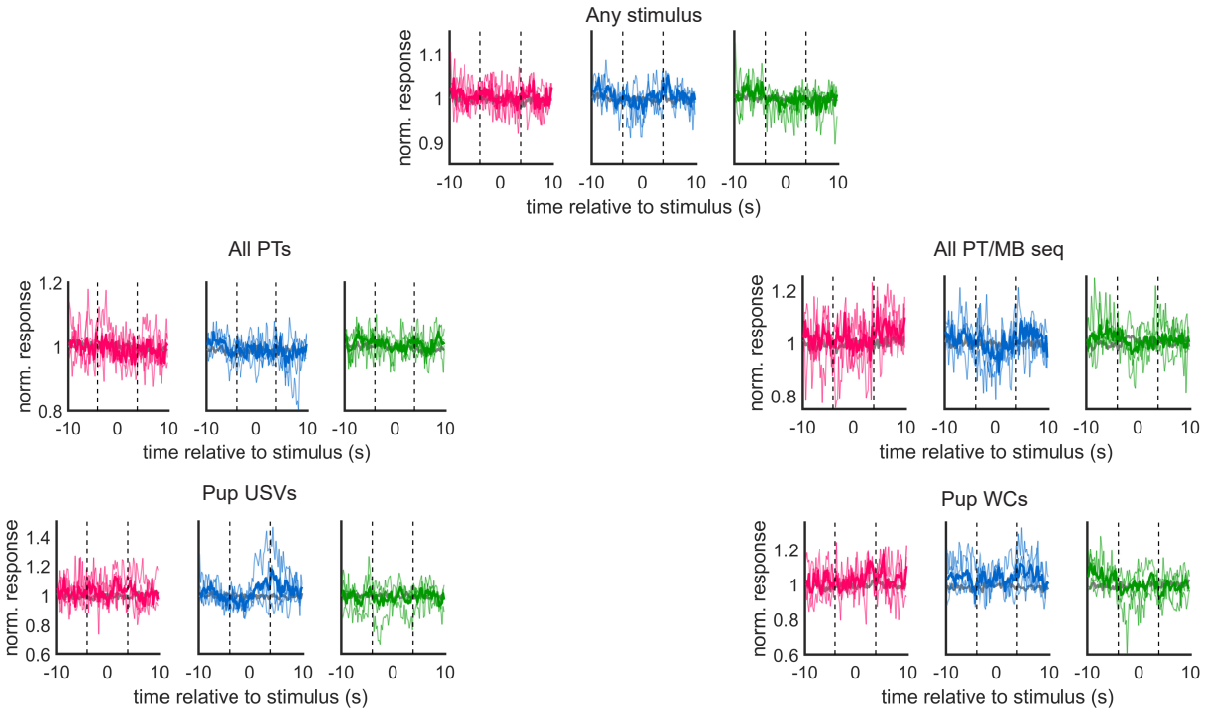

Figure 3.10 | Population tone-evoked responses to passive stimulation in freely moving mice. Thick lines represent overall medians across all trials per experimental group, thin lines represent single animal medians relative to stimulus onset. Responses to stimuli were either all pooled or grouped into all PTs, all PT and MB seq, pup USVs or pup WCs.

3.4 NEURAL ACTIVITY DURING PUP RETRIEVAL

During experiments in which mice were freely moving, I additionally recorded Ca^{2+} signals as an indirect proxy for neural activity in the left AC of adult females carrying head-mounted miniscopes (**Fig. 3.7A,B,C**). Using a surface lens, I gained optical access to a roughly 900 x 900 µm field of view in the upper layer 2/3 of AC, without the need of physically penetrating the brain tissue with a GRIN lens (**Fig. 3.7D**). Images were motion corrected and source extracted using the MIN1PIPE pipeline (Lu et al., 2018) (**Fig. 3.7E,F**).

After investigating neural responses in the AC in freely behaving mice, I will describe AC responses during the behavioral paradigm of pup retrieval, the core experiment in this thesis. As already shown before in the behavioral section of this study, isolated mouse pups emit calls that serve as an alarm signal and cause the retrieval of the calling pup to the nest (**Fig. 3.1A, 3.5**). AC responses to these pup calls in particular will be the focus of the following sections.

In order to visualize and inspect the responses of the imaged AC population in the context of the behavior, we generated so-called ethograms. Ethograms contained the audio information about pup and adult calls, as well as behavioral annotations and locomotive information about mouse position and mouse speed aligned to the neural activity (**Fig. 3.11A**). The position of the mouse was obtained from the videos by employing DeepLabCut (Mathis et al., 2018), a software that utilizes neural networks to track several body parts of an individual across multiple video frames. Behavioral events were manually annotated, including on- and offsets for pup related events, such as pup retrievals, pup contacts and nest material retrieval. Non-pup related events comprised grooming, rearing, digging, eating and pup placements by the experimenter. We aligned the activity of all neurons to these events after sorting them by employing the rastermap algorithm, which sorts neurons based on correlations among them (Stringer et al., 2019). Thereby, neurons with more similar Ca^{2+} responses were arranged next to each other in the neural activity subplot of the ethogram (**Fig. 3.11A**).

Since we were interested in the neural activity to pup calls, we first inspected single cell responses aligned to the onset of pup USVs (**Fig. 3.11B**). While a few cells showed responses to some pup calls in a trial, the majority of single neuron traces did not show classically defined sound-evoked responses, as measured in head-fixed preparations (**Fig. 3.8**). As clear single cell responses to pup calls were scarce, similar to PS experiments with sound stimuli (**Fig. 3.9**), we analyzed the median neural activity during these calls across all neurons per animal (**Fig. 3.12A**).

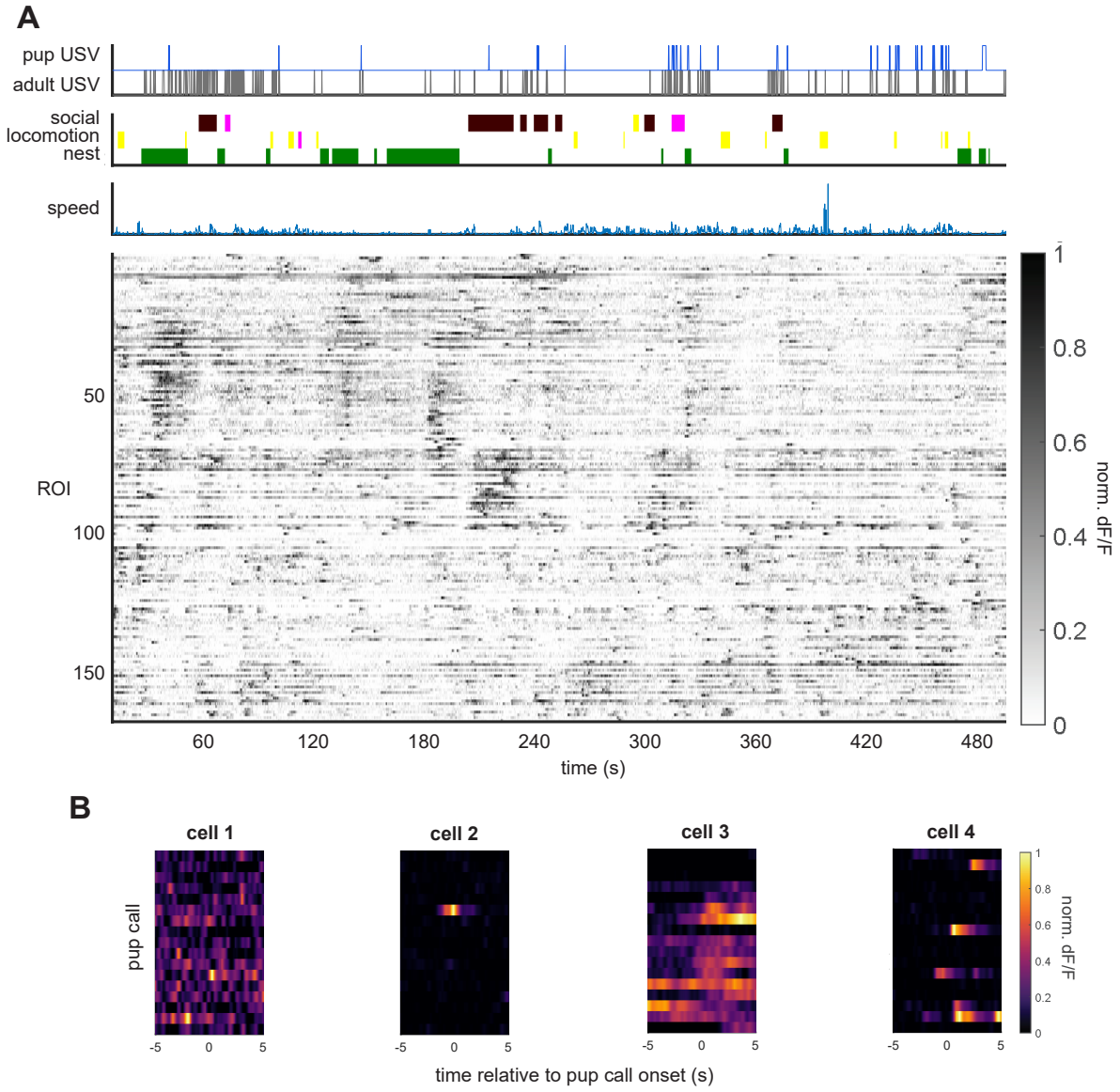

Figure 3.11 | Auditory cortex activity during pup retrieval. A Ethogram of one exemplary pup retrieval experiment with behavioral annotations aligned to neural activity. Upper panel shows the occurrence of adult calls (row 1) and pup calls (row 2). The second panel shows behavioral annotations with social events in the upper row (successful retrieval events in pink, unsuccessful retrievals in black) and locomotion events in the middle row (rearing in yellow, digging in cyan, grooming in pink and sitting in dark red) and mouse localization inside the nest in green. The third panel shows normalized mouse speed. The main panel at the bottom shows the activity of single neurons as delta F/F. Neurons are sorted by the rastermap algorithm (Stringer et al., 2019). **B** Normalized Ca^{2+} traces from four exemplary neurons aligned to multiple pup calls emitted during one pup retrieval trial.

We did this for all experimental groups, and compared pup call triggered medians (PCTMs) to random time point triggered averages on a per experiment basis. In these PCTMs, we did not find a clear time-locked response modulation at the onset of single pup calls (**Fig. 3.12A**). Nevertheless, the general activity levels around pup call onsets were elevated compared to control traces in all groups. In MTs and EVs, a slow ramping up of activity that preceded the onset of the pup call was observed, whereas a much smaller, increase in median activity around pup calls was found in NVs (**Fig. 3.12A**). However, especially for NVs, there is a great variability across single animals, which is slightly more restricted in MTs, and most consistent in EVs. Event triggered medians such as the PCTMs are a coarse measure of neural activity, and can only reflect meaningful profiles when strong global events occurred during the time of interrogation. In other words, many single cells would have to follow the same trend in order to be reflected in these median profiles. This is consistent with previous reports indicating that neural responses in the AC are sparse and unreliable, even in clearly trial structured experiments (Hromadka et al., 2008; Tischbirek et al., 2019).

In addition to pup call related activity, we were interested whether self-emitted vocalizations from the adult animals were reflected in the neural activity of the same animals. To test this, we also computed adult call triggered medians, i.e. responses to self-emitted vocalizations, but could not find significant responses, except for MTs during PR experiments (**Fig. S3**). It was reported that neural activity in the AC is generally suppressed during self-vocalization (Schneider et al., 2014), however, this suppression could be not measured in our recordings.

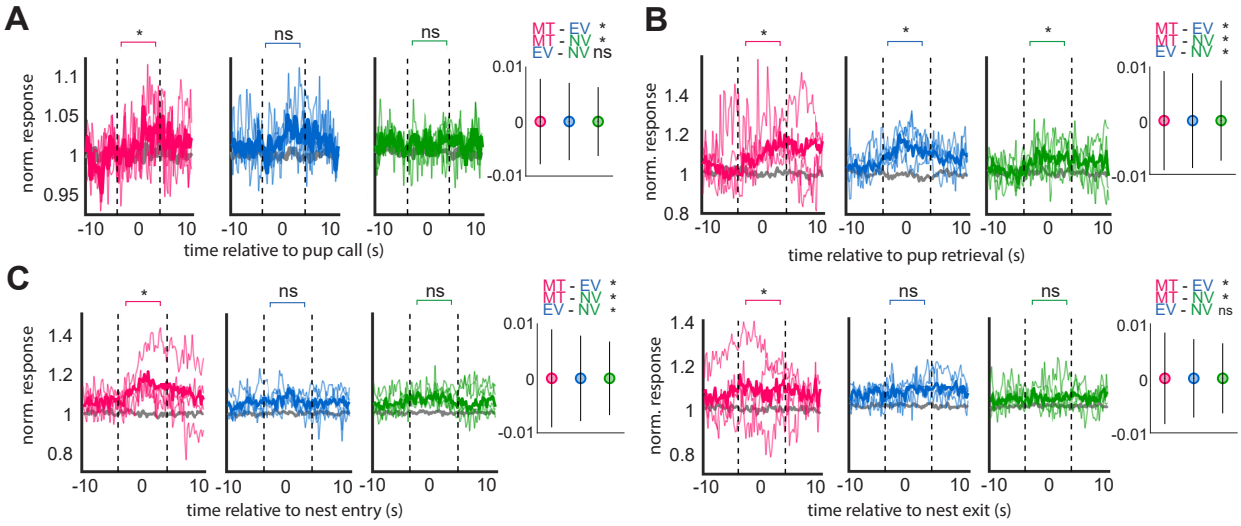

Figure 3.12 | Event triggered neural activity during pup retrieval. A Pup call triggered medians across all neurons, animals and sessions per

group in thick lines; thin lines represent medians across all sessions per animal. Dotted lines indicate time window for the statistical comparison between before and after call onset, with a one-way ANOVA, $p<0.05$. *Inset*: Statistical comparison between groups for the response deltas between to pup calls, one-way ANOVA corrected for multiple comparisons, $p<0.05$, dots represent medians and error bars represent MADs. **B** Same as in E, but for pup retrieval triggered medians. **C** Same as in A and B, but for nest entries (*left*) and nest exits (*right*).

Visual inspection of the ethograms showed periods of strong neural activity, during which different groups of neurons seemed to be co-active (**Fig. 3.11A**). To further analyze this observation, we again computed event triggered medians, this time for pup retrieval events (**Fig. 3.12B**). Compellingly, pup retrieval triggered medians in all groups showed a clear time-locked increase in neural activity upon onset of pup retrieval (**Fig. 3.12B**). This was most strongly pronounced in MTs and EVs (**Fig. 3.12B**). Since generally neural activity seemed to change upon nest entry and nest exit, as can been seen in the exemplary ethogram, we quantified triggered medians for those event types as well (**Fig. 3.11D,3.12C**). Interestingly, medians upon nest entry showed significantly increased levels of neural activity in MTs, whereas exiting the nest caused neural activity to be reduced (**Fig. 3.12C**). EVs and NVs showed the same trends, however less pronounced than for MTs (**Fig. 3.12C**).

Taken together, these data show that during pup retrieval, AC activity is highly correlated around retrieval events, with increased neural activity levels at the onset of retrievals and most prominently in MTs and EVs. Moreover, median neural activity is elevated during pup call periods on average, but without abundant single cell onset responses. Besides pup retrieval episodes, other non-auditory events such as nest entries and nest exits displayed a specific neural signature in the AC, particularly in MTs.

3.5 NEURAL CODE FOR PUP CALLS

Since single AC neurons showed activity that is not directly or consistently associated with pup call occurrences, we employed a population-based approach to uncover pup call representations in the AC. With this approach, we interrogated the joint activity of all neurons, and thereby probed whether information about pup calls was encoded at the level of the entire neural population. These population-wide interactions can for instance be embodied in certain types of correlations among neurons (Stefanini et al., 2020). Additionally, population analyses can help to reveal, which type of code the population utilizes to represent a certain stimulus. In fact, two extremes can be distinguished theoretically, which are a local code on one hand and a distributed code on the other hand. While a few neurons contribute strongly to decoding in a local code, all neurons participate to a certain amount to decoding a stimulus or event in the case of a distributed code, as described in the introduction (**Fig. 1.4**).

Here, by employing support vector machines (SVMs), all imaged neurons from one field of view were taken into account, and the representation of pup calls was quantified at this level. First, we tested whether the presence of a pup call was encoded in the population of AC neurons we recorded from. Accordingly, we trained a binary SVM on a trial-by-trial basis to quantify how well pup call presence could be decoded based on the neural activity alone (**Fig. 3.13A**). It is noteworthy that trials of the same animal can contain slightly different sets of neurons used for the trial-wise decoders. This can be either due to small changes in the field of view per trial, as miniscopes were removed after an experiment, or due to some neurons being differentially active in different PR trials. Strikingly, our SVM analysis revealed confident decoding of pup call presence across all experimental sessions based on population activity in each group (**Fig. 3.13A**), despite the lack of abundant single cell responses (**Fig. 3.11B**). When inspecting single animal decoding accuracies per session and fitting a linear regression line, we found that EVs showed a steady increase of decoding accuracies across sessions. Such increases were not found in MTs and NVs (**Fig. 3.13B**). In these groups, no consistent change of deccoding accuracies was detected across sessions. It also became apparent that a certain fraction of pup USVs was not captured by the decoder (**Fig. 3.13C**). Often, these missed USVs were single isolated syllables outside a train of USVs. Nevertheless, some trains of pup USVs remained undetected, though to a lesser extent.

Next, we aimed to investigate whether single neurons from a given trial contributed equally or differentially to the decoding of pup calls in that same trial. This would provide us with a better characterization of the code with respect to its type as discussed above (**Fig. 1.4**).

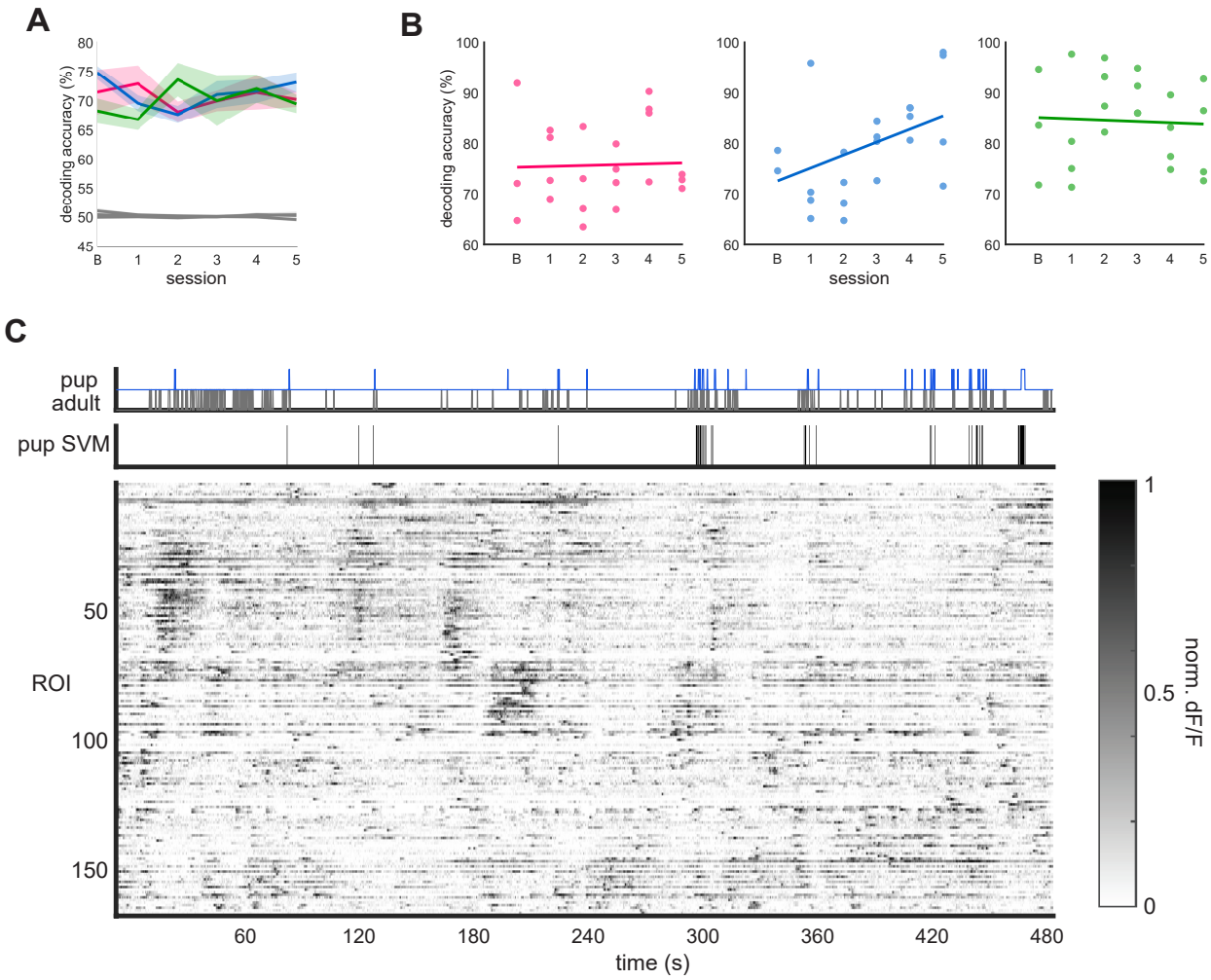

Figure 3.13 | Pup call decoding based on neural activity during pup retrieval. A Pup call decoding accuracies averaged across animals per group over experimental sessions, shading indicates SEM across animals. **B** Pup call decoding accuracies across sessions, dots indicate averaged trials per mouse, shading of dots indicates session. Linear regression fit for each experimental group, MTs: R^2=0.005, F-statistic p-value=0.89, EVs: R^2=0.622, p-value=0.06, NVs: R^2=0.013, p-value=0.83. **C** Ethogram of exemplary pup retrieval experiment with behavioral annotations aligned to neural activity. Upper panel shows the occurrence of pup calls (row 1) and adult calls (row 2). The second panel shows binary SVM results for pup call classification events. The main panel at the bottom shows single neurons, color-coded according to their activity. Neurons are sorted based on the rastermap algorithm (Stringer et al., 2019).

We therefore analyzed the neuronal weights that were assigned to single neurons. Each decoder assigns weights to all of the provided single neurons, thereby indicating to what degree the activity of that neuron contributed to the overall decoding of pup calls. To explore group-wide trends, we compared overall distributions of absolute neuronal weights from all classifiers per group irrespective of session.

Remarkably, neurons of MTs were assigned the strongest weights, significantly higher than those of EVs or NVs (**Fig. 3.14A**). Neurons from EVs had also increased weights, even though not statistically different from NVs. In general, neuronal weight distributions showed that a small fraction of neurons was assigned large weights, whereas the majority of neurons had low weights (**Fig. 3.14A**). This distribution argues in favor of a rather local or sparse code as introduced above, with a few strongly contributing neurons.

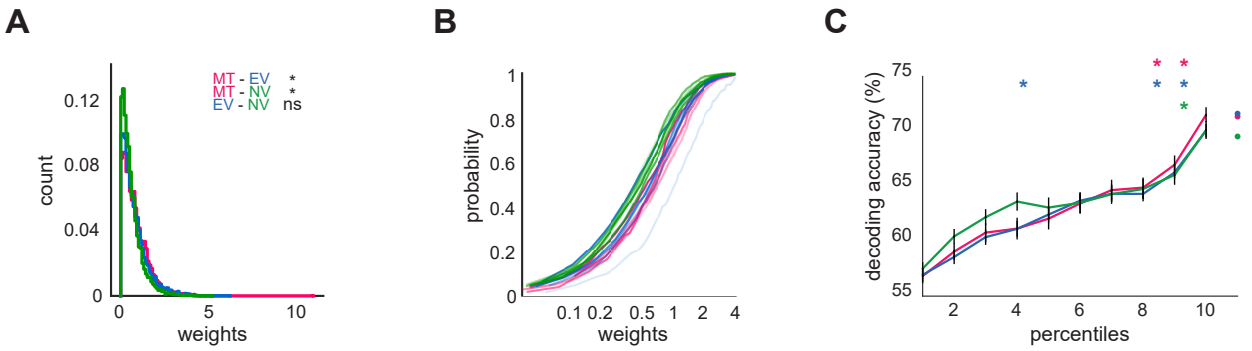

Figure 3.14 | Pup call decoding weights. A Cumulative distributions of classification weights per session for each group. **B** Probability normalized neuronal weight distributions from pup decoding for all animals per group, across group statistics by Wilcoxon rank sum test with correction for multiple comparisons, $p<0.05$. **C** Pup call decoding accuracy change upon inclusion of increasing neuronal percentiles, sorted in ascending order according to their assigned weights. Dots at the end indicate decoding with entire neuronal population at once, stars above line indicates significant difference between consecutive decoding deltas per group, Wilcoxon rank sum test, $p<0.05$.

To investigate whether the assignment of weights would stay constant or would change across sessions, we probed the distribution of these weights across experimental sessions. While no consistent change could be detected for EVs and NVs, MTs shifted their distributions slightly across time towards lower weights (**Fig. 3.14B**). This decrease of neuronal weights can be interpreted as a de-sparsification of the neural code, such that over time the information about pup call presence gets distributed across more cells.

To support the findings outlined above, we performed a progressive neuron addition analysis. In this approach, we again classified pup calls, but now based on increasing numbers of neurons. To do so, we first sorted neurons according to their assigned weights and split them into ten percentiles. Subsequently, we decoded pup calls in single trials with increasing numbers of neurons ordered by their weights (**Fig. 3.14C**). Confirming what we described based on the weight distri-

butions, we found that the highest percentile of neurons contributed a disproportionally large increase of decoding performance in all groups (**Fig. 3.14C**). In contrast, the majority of percentile steps prior to the last one increased the performance steadily but not as drastically as the last step (**Fig. 3.14C**).

We then wondered, how these high weight neurons responded to pup calls. To address this question, we first isolated a set of neurons that would be classically defined as responsive neurons. For this, we quantified response ratios to pup call onset between the periods before and after pup call onset. A neuron with a clear time-locked response to that stimulus would have a high response ratio and referred to as a RR neuron. Next, we compared the response ratio of the 90^{th} percentile of high weight (HW) neurons to the 90^{th} percentile of RR neurons and as a control to random neurons (RN) as well (**Fig. 3.15A**). This analysis revealed a clear and significant difference in response ratios to pup calls between HW and RR neurons for all groups (**Fig. 3.15A left**). Response ratios to random events were still significantly different between HW and RR cells; however, these differences were smaller than response ratios to pup calls. Since we compared the response ratios between neuron types that included all single cell responses to each pup call, we had a very large dataset, thus even small differences in response ratio between HW and RN were sometimes significant. Nevertheless, the actual response ratio differences between neuron types were prominent and showed that HW neurons were not classically responding to pup calls (**Fig. 3.15A**).

The single cell responses of high weight neurons supported this finding, as they did not show preferential activity to pup calls, visualized by aligning the Ca^{2+} responses to single pup call onsets (**Fig. 3.15B**). In fact, those traces were indistinguishable from traces aligned to random events. However, some HW neurons showed less noisy activity than some RN did (**Fig. 3.15B**). We further examined whether the population of HW neurons overlapped with the RR neurons per trial (**Fig. 3.15B**). Notably, almost no overlap was found between these two categories, meaning that high weight neurons were not classically responsive neurons (**Fig. 3.15C**). Altogether, this suggested that our decoder did not assign high weights to some neurons because of their high response ratio to a pup call, but because of some yet unknown feature.

We also wanted to assess how exclusive the pup call code is, namely whether the same neurons used to encode pup calls participated in other aspects of pup retrieval behavior (**Fig. 3.16**). To test this, we first trained classifiers on other behavioral events including pup call parameters such as the main frequency, the loudness, the ISI, but also non pup call related events (adult calls, pup retrievals, nest localization, speed and location) (**Fig. 3.16A**). Strikingly, all these event types could be decoded based on neural activity in the AC in all groups (**Fig. 3.16A**). The results of the overlap analysis showed that high weight

neurons of different event types were largely non-overlapping. Only parameters that concerned pup calls and pup call features shared some of the same high weight neurons (**Fig. 3.16B**).

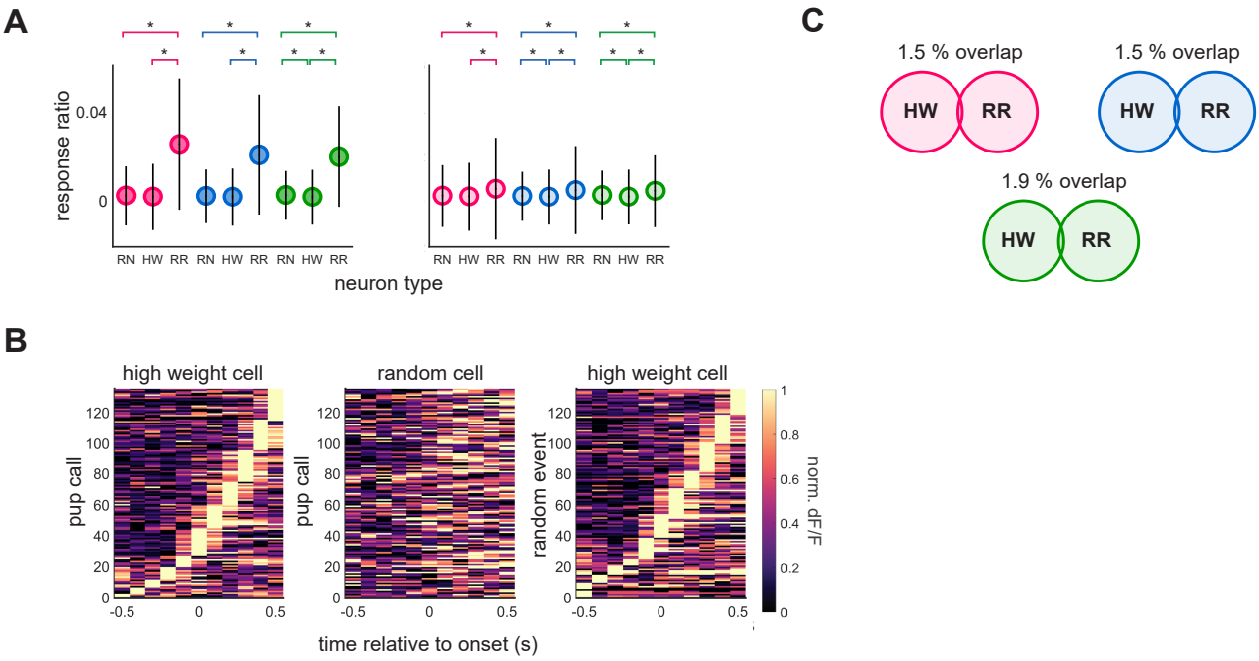

Figure 3.15 | High weight neurons for decoding pup calls. A Dot plot showing median response ratios of randomly selected (RN), high weight (HW) and high response ratio (RR) neurons to pup calls (*left*) and random events (*right*) during pup retrieval. Error bars indicate standard deviation. Statistical comparison between response ratios of different neuron types per group with one-way ANOVA, $p<0.05$, corrected for multiple comparisons. **B** *Left*: Exemplary traces of a single high weight neuron aligned to pup call events. *Middle*: Random, non-high weight neuron aligned to the same calls. *Right*: Traces of the same neuron to random events within a pup retrieval trial. **C** Venn diagrams indicating the percentage of overlap between high weight and high response ratio neurons.

To assess how dynamic the code is, we asked whether single neurons from one PR trial can be used to decode pup calls from a different trial. To this end, we employed a matching algorithm that allowed training a classifier on one trial and using this to predict the decoding accuracy on another trial, using the same set of neurons (**Fig. 3.17A**). Specifically, we trained a decoder on matched cells from one PR trial, and tested how well this classifier could predict pup call presence on a separate trial.

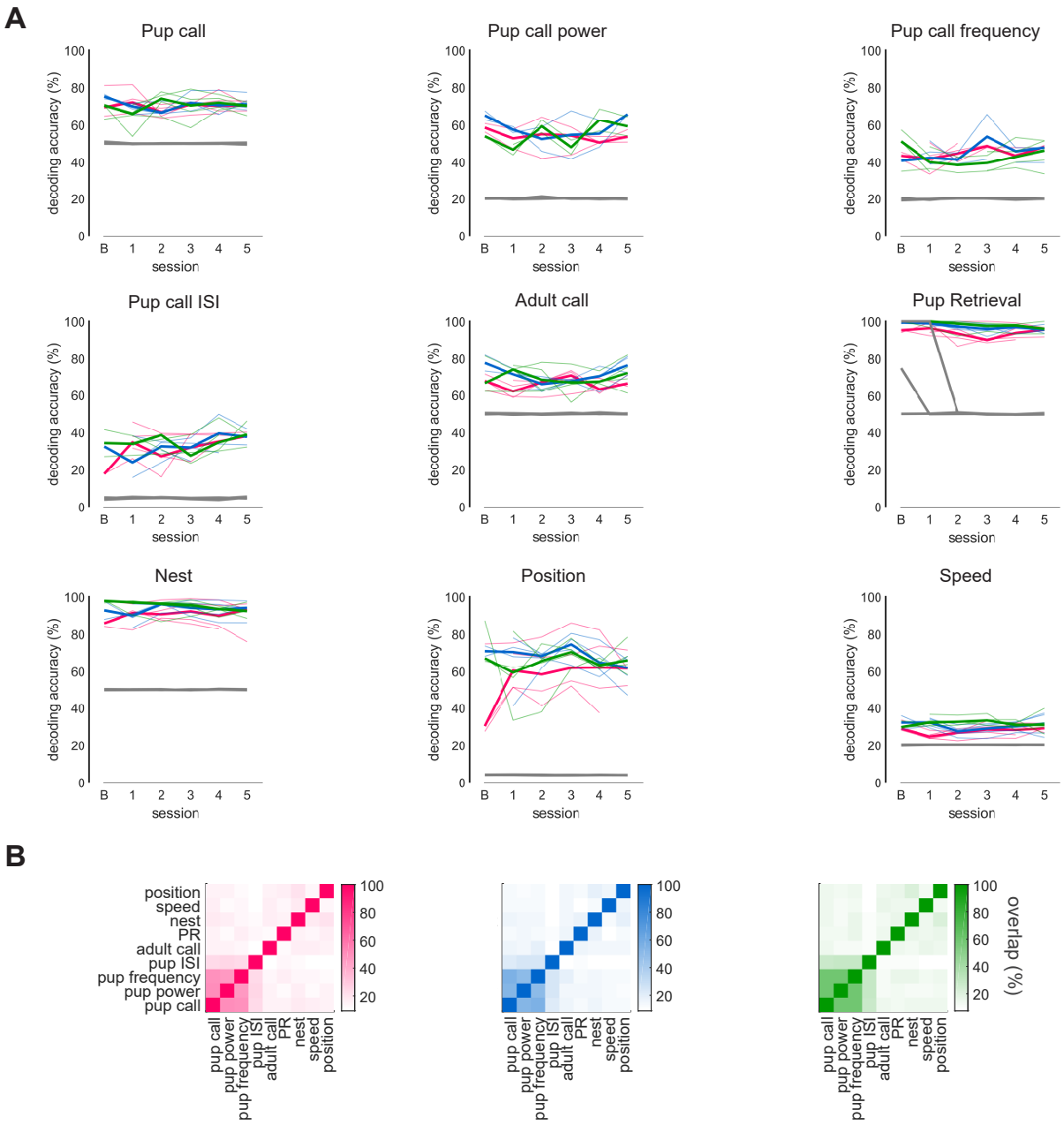

Figure 3.16 | Decoding of behavioral events during pup retrieval. A Decoding accuracies for different event types during pup retrieval across sessions per experimental group. Thick lines represent group medians across all trials per mouse and session, colored lines show decoding accuracies from correctly labeled sessions, grey lines show decoding accuracies for shuffled controls. Thin lines represent single animal medians. The shuffled controls were all at chance level for their respective classifier (i.e. depending on the number of categories). **B** Percentage overlap of the 90th percentile of weights between same cell for different classifiers.

An exemplary decoding accuracy heatmap shows that very few pairwise combinations yielded accuracies around 60%, while the majority of predictions remained at chance level (**Fig. 3.17A, S**). These higher

accuracies were sometimes found in temporally close PR trials (**Fig. S4**). To test whether a particular temporal structure underlies this, we followed decoding accuracies around different trial intervals (**Fig. 3.17B**). Therefore, the accuracy when training the classifier on one PR trial and using this classifier to predict the accuracy on a proceeding trial was investigated (**Fig. 3.17B, S4**). This analysis revealed that a decoder trained on one PR trial can in a few cases generalize to a processing trial. However, the accuracies for the majority of comparisons dropped back to chance level (**Fig. 3.17B, S4**). Altogether, this analysis revealed that the pup call code was dynamic even at the level of single trials. Moreover, it showed that no systematic change of accuracies occurred across time; rather a constant amount of fluctuating dynamics characterized this neural code.

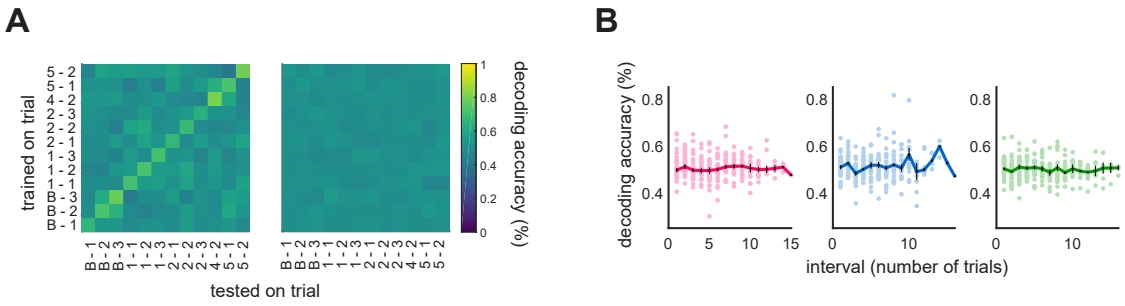

Figure 3.17 | Dynamic pup call encoding across sessions. A Example decoding accuracy from mouse EV4 for pup call presence on matched neurons, with pup retrieval session used for training the classifier on the y-axis and tested pup retrieval session of the x-axis. **B** Decoding accuracies after training on a PR trial and predicting pup calls on a separate proceeding PR trial, sorted by interval between trials. Dots indicate single trial comparisons; thick lines represent medians across single animals with error bars as SEM.

Finally, we aimed to link PR behavior to AC activity. In addition to following pup call decoding accuracies across sessions per animal (**Fig. 3.13B**), we now tested how this decoding accuracy related to behavioral performance per animal (**Fig. 3.18**). To investigate this, we used [1 - the retrieval latencies] as a readout for behavioral performance (**Fig. 3.2B**). While the behavioral performance of MTs was already high from the baseline session onwards, EVs and NVs steadily improved their behavioral performances (**Fig. 3.2B**). Despite MTs high behavioral performance throughout the time course, not all sessions showed equal pup call decoding accuracies (**Fig. 3.18**). Therefore, both variables were uncorrelated in MTs. On the other hand, the increase in EV behavioral performance (**Fig. 3.2B**), was accompanied by increasing pup call decoding accuracies over time (**Fig. 3.18**). In NVs, a weaker positive relationship between both parameters could be measured. This potentially reflects the different pup exposures EVs and

NVs underwent, and therefore might indicate a differential learning mechanisms employed by both groups.

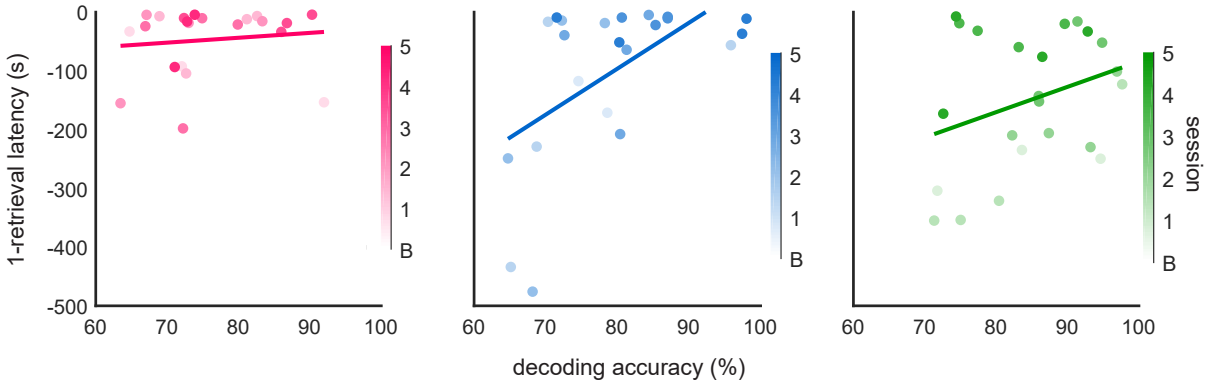

Figure 3.18 | Linking decoding of pup calls to behavioral performance. Behavioral performance, displayed as [1-pup retrieval latency (s)], plotted against pup call decoding accuracy per session for each mouse. Different shadings of single dots indicate session, with lighter dots representing earlier sessions, linear regression fits and F-statistic p-values: MTs: R2=0.014, p-value=0.6 EVs: R2=0.315, p-value=0.007, NVs R2=0.102, p-value=0.1.

3.6 SUMMARY

In this study, we have followed pup retrieval behavior along with neural activity in the AC of mothers, experienced and naive female mice in a natural setting. First, we used two-photon microscopy to verify that we are able to measure classic sound-evoked AC responses with a one-photon miniscope.(**Fig. 3.8, 3.9, 3.10**). Second, we found that the three experimental groups showed different behavioral profiles across PR experiments, with MTs generally performing more efficient and skillful pup retrieval. EVs, mice that were co-housed with a mother and her litter, showed slightly reduced PR ability, marked by lower retrieval latencies and lower retrieval speeds in the first sessions, as well as more unsuccessful retrievals in the first session. NVs started off with the poorest performance, yet increased this performance steadily across the experiment. Nevertheless, all groups performed PR behavior stably after a few sessions.

Additionally, we found that not only pups vocalize during pup retrieval, but surprisingly also adults emit a substantial amount of calls. Over time, adults called more, while the amount of emitted pup calls remained constant. After computationally disentangling these vocalizations by employing a neural network that separates the call types, we proceeded to analyze pup call related neural activity in the AC. To our surprise, only a few neurons showed pup call-elicited activity, while the majority of neurons was modulated more strongly by the more global pup retrieval events. MTs in particular showed a clear signature of neural activity in response to pup retrieval onsets. This indicated that behaviorally relevant events, such as retrievals in this task, are well represented in the AC.

Since single neuron responses to pup calls were scarce, we employed neural population analyses to test whether information about pup calls was present at the network level. Indeed, pup calls were encoded in the population of AC neurons in all experimental groups. After characterizing the underlying neural code, we ascertained that it was sparse, with few neurons carrying considerable decoding capacity. This sparse code evolved slightly across time in MTs, with a shift towards a less sparse and marginally denser code. Neurons that were assigned high weights were not those with high response ratios, and therefore did not show typical time-locked onset responses to pup calls, rendering the underlying detected pup call features unknown. Moreover, the neurons that carried information about pup calls were largely non-overlapping with neurons carrying information about other non-pup call related events during the same trial. Additionally, the underlying neural code was dynamic, i.e. training a classifier on one pup retrieval trial and using it to predict another trial yielded variable decoding accuracies, indicating that the neural code might rearrange over time without a consistent trend.

Lastly, when relating these decoding accuracies to behavioral performance, it became apparent that in EVs increasing behavioral performances were accompanied by elevated pup call decoding accuracies, suggesting that the AC network of EVs might get better at detecting pup calls over time and potentially allowed these animals to more efficiently react to vocalizing pups.

3.6.1 *Synthesis*

The findings described above let us conclude that:

1. The pup exposure regime dictates the time course with which mice learned to efficiently retrieve pups. The group of EVs closely followed the behavioral profiles of MTs. However, detailed measures, such as retrieval latency and retrieval speed, distinguished both groups. NVs, in contrast, showed different behavioral profiles that were most pronounced in the relative time they spent in the nest, the pup holding time, the speed profiles and the distance covered during PR experiments.

2. EVs have an advantage in retrieving pups over NVs, which has to be attributable to their co-housing with a mother and its offspring. Which factors of this co-housing might be beneficial to the improved pup retrieval behavior will be covered in the discussion.

3. These behavioral differences were partly reflected in neural responses that were measured during pup retrieval. The strongest PCTMs detected in MTs, weaker PCTMs in EVs, and weakest in NVs, are reflective of how well mice of these groups retrieved pups. From these data, we can conclude that more neurons in MTs compared to EVs and NVs responded to pup calls during retrieval. The thereby enhanced neural activity to pup calls in MTs might allow these animals to more quickly and efficiently react to calling isolated pups.

4. From the strong PCTMs to pup retrieval events in all experimental groups, we can infer that relevant behavioral events are represented in auditory cortical activity, even though these events themselves are not purely auditory. In particular, this pertains to more global event types, such as movement and speed, which were well represented in AC activity. Recent literature confirms this finding for other cortical areas, such as visual cortex, in which ongoing activity reflects running and whisking (Stringer et al., 2019).

5. At the population level, pup call presence was decoded successfully from all experimental groups. From this, we can deduce that pup calls are represented in the AC of all mice, but that classic sound-evoked responses to each pup syllable are not the way in

which these calls might be represented. When connecting these findings to the behavioral results, it became apparent that different pup exposure regimes differently affected neuronal weights that were assigned to AC neurons, when decoding pup calls. Namely, a few MT neurons had the strongest assigned weights, speaking for a sparse representation of pup calls. A sparse neural code is an efficient way to represent relevant stimuli in the cortex and might explain the superior behavioral performance of MTs in their representation of pup calls in the AC. Interestingly, we found a significant relationship between pup call decoding and behavioral performance in EVs. This data suggests that an improved pup call decoding ability in EVs might have contributed to more efficient pup retrieval behavior over time.

Altogether, here we have shown that mice that are actively engaged in a pup retrieval task represent pup calls and other relevant event types in their AC differentially depending on the underlying pup exposure regime.

4

DISCUSSION

In this work, I have established a novel experimental setup in which female mice, retrieved pups into a nest while carrying a head-mounted miniscope, which imaged neuronal populations in their AC throughout the task. At the same time, ultrasonic microphones captured mouse communication calls, and a camera above the arena kept track of the behavior. This setup allowed me to track AC neurons across repeated PR sessions and to compare those dynamics across different experimental groups representing distinct learning regimes, namely mothers (MTs), experienced virgins (EVs) and naive virgins (NVs). I have shown five key results in this study:

1. MTs, EVs and NVs all retrieved pups efficiently to the nest. Noteworthy, single groups showed distinct behavioral performance time courses. MTs displayed the most proficient PR behavior, whereas EVs and NVs followed in that order (**Fig. 3.2, 3.3**).

2. Next to pup USVs, adult females likewise emitted USVs during PR. Both call categories could be separated by training a supervised classification network. Overall, the amount of adult USVs during PR exceeded that in the absence of pups, i.e. during SA experiments, and generally ramped up after the baseline session in all groups (**Fig. 3.6**).

3. While clear pup call responsive single neurons were scarce, a strong activity modulation was observed for more globally occurring events, such as pup retrievals and nest entries or exits. The event triggered medians across single neurons for PRs and nest entries and exits were most pronounced in MTs (**Fig. 3.12**).

4. Although single cell responses to pup calls were sparse, the population of neurons carried information about pup calls throughout sessions and in all groups (**Fig. 3.13**).

5. The neural code representing pup calls in the awake AC can be described as sparse and dynamic. It is sparse, as a small fraction of neurons is much more informative about pup call presence than the majority of other neurons. In addition, the sparse population code rearranges between single trials, so that a decoder trained on matched cells from two different trials varied between confident and chance level decoding for different comparisons and did not stay constant, even though pups were retrieved in most trials (**Fig. 3.14, 3.17**).

4.1 DISTINCT BEHAVIORAL PROFILES FOR DIFFERENT PUP EXPOSURE REGIMES

PR behavior is a ubiquitous offspring-directed caring behavior that has been described in multiple species, such as prairie voles, marmosets and even reptiles like the American alligator (Kushlan, 1973; Nunes et al., 2001; Olazábal & Young, 2006). Until now, several studies have highlighted the presence of PR behavior in female mice (Sewell, 1970; Ehret, 2005; Cohen et al., 2011; Lin et al., 2013; Schiavo et al., 2020; Tasaka et al., 2020). In these studies, researchers have typically examined the PR behavior of MTs and compared it to either EVs or NVs on a single time point. While one study examined all three groups simultaneously in one session (Marlin et al., 2015), another one followed PR behavior of EVs and NVs across three days (Schiavo et al., 2020). Common quantification metrics were the percentage of animals that retrieved pups (Koch & Ehret, 1989; Ehret & Buckenmaier, 1994), the percentage of pups that was retrieved (Wu et al., 2014), or the temporal latency by which pups were retrieved (Cohen et al., 2011). In this study, we combined these measures, and additionally quantified the fraction of successful retrievals, the relative time animals spent inside the nest, and examined speed profiles with which mice approached and retrieved pups (**Fig. 3.2, 3.3B**). By integrating these measures, we revealed differences among groups that were previously undescribed.

4.1.1 *Maternal behavior in naive virgins*

While a previous study showed that repeated pup exposure elicits PR behavior in NVs, which are animals that have not been exposed to pups before (Okabe et al., 2017), a detailed comparison of the PR ability across subsequent days was lacking. In line with the study from Okabe and colleagues in 2017, our NVs also displayed an incremental increase in PR behavior across sessions (Okabe et al., 2017). Nevertheless, our pup exposure regime included three brief, maximally 6 min long, PR trials on each of six consecutive sessions. While in the study from Okabe and colleagues, NVs were tested on two separate days only. They made use of two exposure types, and subdivided NVs into two groups. The low exposure group was exposed to pups for 20 min, and four days later re-exposed to pups for 20 min. The high exposure group was exposed six times to pups on the same day in the pre-exposure session, and four days later re-exposed to pups. The latter retrieved pups faster than the former, but the exact evolution of this behavior was not followed in detail. In our study, I probed the evolution of PR behavior in greater detail, and could find that NVs retrieved more than half of the isolated pups after three consecutive sessions (**Fig. 3.2A**).
This so called alloparenting behavior in NVs is caused by pup sensitization, in which constant exposure to pups triggers the onset of maternal behaviors, such as PR in NVs (Champagne et al., 2001). It

is hypothesized that an endocrinal state similar to that of MTs in their late pregnancy triggers these behaviors (Bridges, 1996, 2015). This notion is further supported by hormone-induced triggering of maternal behaviors in NVs (Rosenblatt, 1994; Bridges, 1996). These hormones, such as estradiol, in turn increase OT receptor levels in the brain, thereby activating mPOA, a region that was shown to be highly involved in governing maternal behaviors (Shahrokh et al., 2010). This activation also most likely induces PR behavior, which is a main characteristic of maternal behavior in rodents (Pedersen et al., 1994; Champagne et al., 2001). Additionally, we found that NVs showed inverted profiles across time for the relative time they spent in the nest during PR compared to EVs and MTs (**Fig. 3.2D**). They did not spend as much time inside the nest in the baseline session compared to following sessions. MTs and EVs in contrast initially spent a larger amount of time in the nest, which then decreased in the following sessions (**Fig. 3.2D**). This, alongside the differences in pup retrieval performances, is another piece of evidence supporting the differential effect the pup exposure regime has on the different groups. One potential explanation is that NVs might initially be overwhelmed by the novel situation and have not yet acquired the skills and or activated the circuits needed to care for pups and display maternal behaviors in general. This notion is supported by NVs running around the arena, occasionally sniffing isolated pups but otherwise ignoring them. Additionally, data on the distance animals covered during PR experiments showed that NVs only covered as much distance as MTs and EVs in later sessions (**Fig. 3.3A**). Nevertheless, some NVs started retrieving pups in the first session after baseline, and spent a lot of time with the retrieved pups in the nest. This indicates that there was an additional inter-individual discrepancy within the group of NVs, with some NVs reacting faster to pups than others.

4.1.2 *Maternal behavior in experienced virgins*

In our experiments, EVs were co-housed with MTs a few days prior to MTs parturition. EVs outperformed NVs on the baseline time point with respect to the percentage of pups retrieved, as well as the retrieval latency (**Fig. 3.2A,B,**). In fact, NVs did not retrieve pups during baseline, whereas some EVs retrieved pups at that time, even though they had not encountered pups yet. Since the baseline session represents the first encounter with pups in the task, EVs might have an advantage over NVs due to their co-housing with a pregnant MT. This co-housing is potentially associated with pheromone induced facilitation of pup caring behavior. Pheromones are short-lived volatile molecules that are secreted together with body fluids, such as urine, saliva or tears, and can serve as alarm signals or be implicated in mating behaviors of mice by reducing male aggression (Brechbühl et al., 2013; Cavaliere et al., 2020). Also, mouse pups can secrete pheromones in their tears, whereby mating behaviors are generally reduced and the pup can gain

the full maternal attention (Tirindelli, 2021). Such pheromones could be also present in the body fluids of the co-housed MT, and thereby promote an early onset of maternal behavior that precedes the onset of such behaviors in NVs. Besides pheromones, EVs could have an additional advantage over NV PR behavior in sessions following the baseline session, due to observational learning from the co-housed MT (Carcea & Froemke, 2019). When monitoring the cages of co-housed EVs and MTs, the group of Robert Froemke could identify periods in which the respective MT 'shepherded' the co-housed EV to pups that were accidentally dragged outside the nest by the MT (Carcea et al., 2019). During these shepherding events, MTs dragged EVs to the isolated pup, and retrieved it in front of the EV (Carcea et al., 2019). I did not monitor the home cages of our experimental animals between sessions, and therefore did not measure whether or to which extent this "shepherding" might have occurred and could have influenced EV behavior in the task.

Another important aspect in the group of EVs was the relatively high variability across single animals (**Fig. 3.2A**). Two EVs did not retrieve a single pup in two different sessions, even though they retrieved pups in a previous session (**Fig. 3.2A**). I can only speculate why these animals stopped retrieving in those sessions. One factor could be that the co-housed MT was stressed and transmitted this state through pheromones or distinct stress-specific behaviors to the respective EV. Another contributing factor could be a potential stress state of the respective EV in the task. Stress could have impacted the animals during the experiments, as all experiments were performed with awake behaving animals. Even though I aimed to standardize all procedures, certain unnoticed factors might have contributed to EVs refraining from retrieving pups. Lastly, it is reported that there is in general some degree of variability in PR behavior of different groups, which is why certain studies excluded animals from their cohort when they did not retrieve in some sessions or already retrieved during baseline (Marlin et al., 2015; Carcea et al., 2019). I did not exclude animals based on these criteria, and therefore potentially increased the quantified amount of behavioral variability among our animals per group.

4.1.3 *Distinct speed profiles with inverse nest preference across groups*

Since all experimental groups eventually retrieved all isolated pups, we differentiated between groups at a finer resolution. Speed profiles served as a good indicator of how well pups were retrieved by the different groups (**Fig. 3.3B**). These profiles consisted of, speed that mice had when performing pup retrieval in a 10 sec period. The speed profiles reflect the ease and efficiency with which pups were retrieved. Depending on the animal group, roughly one second before retrieval onset (physical contact with pup) animals accelerated and came to a halt when trying to get a good grip on the pup. For MTs and EVs, this

fast approach was already observed in session one, while only session three in NVs showed comparable approach speeds. The amount of time animals needed to find sufficient grip to pick up a pup is comparable between MTs and EVs (**Fig. 3.3D**). NVs, in contrast, needed more time in the beginning to initiate the retrieval phase after picking up the pup. These differences among the groups especially set apart the NVs from both MTs and EVs, revealing NVs comparably inferior PR abilities. This might be on one hand due to the aforementioned lack of hormonal or pheromonal priming these animals received, and on the other hand, the lack of experience with pups in their home cage. As mentioned before, I did not monitor home cages in detail, but EVs could have theoretically performed PR in their home cage aside from PR in the experiment, and therefore have a further advantage over NVs. EVs would thereby be able to fine tune PR, and thus resemble MTs more closely in their speed profiles.

Another interesting feature was that MTs show a pronounced asymmetry in approach and retrieval speed in sessions one to four (**Fig. 3.3C**). In particular, MTs retrieved pups to the nest with an even faster speed than they approached pups. Presumably, this reflects the maternally motivated state these animals were in, which would drive a quick return of the isolated pups to the nest. It is widely postulated that pup stimuli, as well as hormones, positively impact maternal motivations systems, which in turn can activate general motivation systems in the mouse brain (Numan, 2007). Furthermore, it is hypothesized that these increased motivational states can speed up responsiveness to pups (Numan, 2007), which is shown by the MTs fast behavioral response to isolated pups in our data.

4.2 COMMUNICATION CALLS DURING PUP RETRIEVAL

PR behavior is mainly triggered through distress calls emitted by isolated pups (Noirot & Pye, 1969; Sewell, 1970; Ehret, 1975). Silent pups are not retrieved as often as vocalizing pups (Schiavo et al., 2020). Hence, pup USVs are an important factor in eliciting pup retrieval behavior. They are thought to act partly via heightening maternal attentional states, by for example attracting MTs to the end of a y-maze arm with pup calls being played from there (Bell, 1974; Smotherman et al., 1974). Surprisingly, I found in my experiments that not only pups emitted USVs, but also adult females vocalized (**Fig. 3.4, 3.5**). Previous studies reported that female mice can emit calls, but this was quite rare and not described in a PR context (Portfors, 2007). These rare occasions in which females vocalized included female-female interactions, as well as so-called "confusion calls", when pups were held above the home cage of MTs (Ehret, 1975, 2005). I detected adult calls when analyzing SA experiments, in which pups were absent (**Fig. 3.4**). After the baseline session, females started emitting USVs during SAs, with MTs peaking in session one and EVs and NVs emitting most calls

in session four. This observation led us to implement a computational method to discern adult from pup calls. I found adult animals also vocalized during PR, irrespective of experimental group, and in higher rates than during SA. Similar to the time course of the amount of adult USVs in SA, adults USVs in PR progressively increased per group (**Fig. 3.6C**).

4.2.1 *Potential biological relevance of female USVs*

What might be the biological meaning or relevance of these adult calls emitted in the context of pup retrieval? There are three main potential explanations for this: (1) It is known that mice vocalize in social contexts of elevated arousal. For instance, male mice emit USVs when in close contact with a female and prior to mating, indicating that the male is sexually motivated (Ehret & Haack, 1984; Holy & Guo, 2005; Portfors, 2007). And females were shown to vocalize when encouraging a male partner to retrieve pups (Liu et al., 2013). Following this idea of USVs being a display of arousal state, adult USVs emitted in the context of pup retrieval could simply reflect the heightened arousal state of a female. Mice in our experiments were habituated in the behavioral arena before the baseline session, but had not encountered pups before the start of the experiments. Therefore, the first encounter with isolated pups, which vocalize themselves, could increase internal arousal levels and thereby elicit USV emission in these adult animals. (2) These adult calls could be the aforementioned "confusion calls", recorded when removing pups from the home cage of female mice (Ehret, 1975). Such confusion calls were emitted in the context of pups, and might be always elicited once offspring is relocated from the home cage. (3) The last explanation for these adult calls lies in the their ethological purpose. With the notion that mouse vocalizations either promote or prevent social interactions among conspecifics (Portfors, 2007), our adult USVs could be more than a mere display of heightened attention. Pups are deaf up until roughly P12 (Sonntag et al., 2009), so it is unlikely that these adult calls are directed to pups in our experimental setting. Instead, it could be that these calls are supposed to recruit other females to help care for and retrieve pups. This concept originates from the fact that in the wild, mice often live in communal nests and share pup caring tasks among females (Weidt et al., 2014). This is also the case for laboratory mice (Southwick, 1955). Moreover, female USVs were essential in triggering a retrieval behavior in male partners and could therefore have a similar effect on other females (Liu et al., 2013). Taken together, likely a combination of these three potential reasons provoked adult vocalizations in a pup retrieval context.

4.2.2 *Potential factors influencing USV emission*

Which factors might influence the emission of USVs in the adult? USVs emitted by adult mice are quite variable depending on the individual, but also on the mouse strain (Holy & Guo, 2005; Kalcounis-Rueppell et al., 2006). Therefore, the adult calls measured in our experiments could be specific to the CBA/CaJ strain. Nevertheless, the CBA/CaJ strain is very social compared to other strains, which might thus facilitate vocalizing in a pup retrieval context (Anisman et al., 1998; Shoji & Kato, 2009; Pedersen et al., 2011). In addition, it was shown that the type of housing can influence the vocal repertoire of animals (Portfors, 2007). As our mice are always co-housed with at least one other individual in environmentally enriched cages, conspecific communication should be promoted under these conditions.

4.3 NEURONAL RESPONSES TO PUP USVS IN THE AUDITORY CORTEX

The exact coding mechanisms and rules by which the rodent AC integrates and represents auditory information are still largely undescribed. It is fairly well understood how sound waves reach the inner ear and are represented tonotopically in the cochlea (Hudspeth, 1989). For example, the intricate circuits and key processing features in brainstem nuclei for spatial localization of a sound source are better understood (Grothe et al., 2010). However, already at the level of the MGB in the thalamus, a plethora of various input types coming from either other sensory systems, neuromodulatory systems or top-down feedback from higher order brain regions converge at this nucleus (Edeline, 2003). Hence, the AC input from MGB is already multimodal. Additionally, the AC itself gets direct input from other sensory modalities, such as the visual and the somatosensory cortex, but also motor areas (Budinger et al., 2006; Schneider et al., 2014), It is thus evident that single neuron activity in the AC is modulated by several different input sources and usually not associable with any given uni- or multi-modal input. Moreover, several studies have shown that different non-sensory features can be represented in sensory cortex aside from relevant sensory stimuli (Poort et al., 2015).

In the experiments, I recorded Ca^{2+} signals as a readout of neural activity in multiple neurons of the AC while mice were actively engaged in a task (**Fig. 3.7**). This meant concomitant neural signals deriving from general locomotive movements and head motion, could influence AC neuron activity (Schneider et al., 2014). Additionally, since I placed pups into the arena and probed both behavioral responses to pups and neural responses to pup calls, uncontrollable task parameters originating from pups included: Pup odors, pup movement and the amount and features of emitted pup calls. Jointly, these natural factors could each influence AC responses and complicate analysis. On the other hand, their combined effect allows measurements of responses to the naturally occurring pup, with all the richness this entails. Since pup calls are the main initial trigger of PR behavior (Noirot & Pye, 1969; Sewell, 1970; Ehret, 1975) and the AC bears an essential role in this behavior (Marlin et al., 2015), we hypothesized we would encounter several single cells clearly responding to those calls. Instead, we found heightened population neural activity around pup call onsets when calculating medians across all data per group (**Fig. 3.12A**). When looking at single neural responses to pup calls in the AC, we found that the majority of neurons did not show clear pup call related responses (**Fig. 3.11A,B**).

4.3.1 *Response features of AC neurons*

What does the scarcity of pup call responsive neurons and the diffuse population averaged onset responses tell about pup call processing in the AC? First, a vast body of literature has described the sparsity and unreliability of AC neurons to sound stimulation e.g. (Hromadka et al., 2008; Tischbirek et al., 2019). In other words, the majority of single neurons in the AC have low firing rates, with a few neurons spiking above 20 Hz in response to an auditory cue, and responses to sound stimuli are quite variable from trial to trial (Hromadka et al., 2008; Tischbirek et al., 2019). We could confirm these findings in our experiments of head-fixed, anesthetized and freely behaving mice that were presented with auditory stimuli (**Fig. 3.8, 3.9**). Tone-evoked responses were detected with a one-photon miniscope, similar to responses measured with a benchtop two-photon microscope. However, tone-evoked responses were scarce, in particular to short tone pulses. These results were similar to miniscope imaging experiments in mice that passively listened to sounds, but were allowed to freely move in the behavioral arena (**Fig. 3.9, 3.10**). A few single cells showed clear tone-evoked responses, while the majority of neurons did not respond. These features render the identification of neural responses challenging, especially in the freely behaving situation of PR, where a consistent trial structure is absent. Among others, this entails that there is no true repetition of an emitted pup call or any other stimulus in the task. This therefore adds even more variability to the system.

4.3.2 *Single cell AC responses in freely behaving mice*

So far, AC responses in the awake freely behaving animal are poorly described and AC responses to live vocalizations emitted from another individual have so far not been studied to my knowledge. Most studies utilized experimental approaches in which animals were either anesthetized or awake but head-fixed (Cohen et al., 2011; Bathellier et al., 2012; Rothschild et al., 2013). In these studies, tone evoked responses were not very abundant, as expected, but it is unknown how these responses would change once the mouse is allowed to move freely. In one study, AC responses were monitored in mice carrying a miniscope (Yao et al., 2019). Here, the miniscope was mounted on top of a prism that deflected excitation and emission light at a 90-degree angle. It is noteworthy that additional optical elements generally decrease the resolution of an optical system. Nevertheless, they could record tone-evoked responses in their preparations, albeit a small percentage, i.e. less than 8%, of all images cells responded clearly to a tone (Yao et al., 2019). A second study investigates neural responses measured by electrophysiology to play-back versions of pup call in the AC, and finds that responses to pup calls increase prior to PR behavior (Carcea et al., 2019). However, it is unclear, how many neurons responded to pup calls and how the population of neurons reacted to the calls.

One key study addresses how much AC responses are modulated by movement, and finds that responses to auditory cues are generally inhibited during motion (Schneider et al., 2014). Interestingly, in the same study, it was shown that activity in the AC was suppressed to the same extent when animals vocalized, (Schneider et al., 2014). These self-vocalization-inhibited AC responses were not observed in our study (**Fig. S1**). In SA experiments, in which pups were absent, the median population activity elicited by self-vocalizations was low and indistinguishable from control traces (**Fig. S1**). One potential reason for why these dynamics were not resolved in our study could be the slow kinetics of the calcium indicator (Grienberger & Konnerth, 2012).

4.3.3 *Broad pup call stimulus space*

As mentioned above, we cannot control the amount and type of calls pups emit during PR. Naturally, I standardized the experimental protocol to guarantee that pups were in a comparable state across trials, and equally prone to call once placed into the arena. Nevertheless, we observed a high degree of variability in pup calls, for example in dominant frequency (**Fig. 3.5B**), as has been described before (Liu et al., 2003). Additionally, pup calls are usually emitted in trains of several pup syllables across seconds at a typical emission rate of 3-8 Hz (Schiavo et al., 2020). The duration of such a train can also be quite variable, and sometimes single pup syllables were recorded outside a typical train (**Fig. 3.11A**). Hence, there was effectively no stimulus repetition for the adults. Since this variability among pup calls is naturally occurring, it must be advantageous for pup-caring females to recognize all kinds of pup calls equally well. We therefore hypothesized that the AC of mothers would be able to generalize across several naturally occurring incarnations of pup calls. This could potentially be implemented by representing pup calls as an overall category. This overall category is sometimes referred to as an auditory object (Griffiths & Warren, 2004; Nelken, 2004). An auditory object is represented in the AC by binding relevant auditory and non-auditory information together, thereby instantiating an auditory percept, such as one for pup calls.

4.3.4 *Time integration of communication calls in the AC*

Generally, brain stem nuclei of the auditory system operate at extremely fast time scales to ensure sound source localization (Joris & Yin, 1992). Instead, processing stations higher up in the auditory system analyze sounds at slower time scales (Asokan et al., 2021). When analyzing speech in humans or vocal communication in birds for example, integration time scales lower than 1 Hz are necessary. Global features, such as the type of arrangement in which simple auditory cues are embedded, can be extracted from mouse AC neuron

activity when integrating over a time window of roughly 2 seconds (Asokan et al., 2021). In the study from Asokan and colleagues, a simple white noise stimulus was embedded in rhythmic or random arrangements. This revealed that the cortex slowly adapts to rhythmic stimuli by reducing the amount of spiking, but maintaining high spike rates during unpredictable random patterns (Asokan et al., 2021). Communication calls, such as pup calls, typically come in trains of single syllables, and under natural conditions several pups calls can be emitted in close temporal succession (**Fig. 3.11A**). Currently, it is unknown whether a certain neural summation process underlies the processing of the natural calls that are not temporally controllable, as is the case with play-back versions. Such a neural summation process would define how many single syllable have to be perceived to elicit a measurable above threshold response in AC neurons. Moreover, considering that 1) the AC detects unpredictable salient stimuli better (Fritz et al., 2007; Yaron et al., 2012), and 2) the AC of pup caring females would generalize across several pup call variants, we cannot exclude that some sort of adaptation to reoccurring pup calls across a single trial might additionally play a role during our PR experiments. Adaptation refers to the concept of diminished neural responses to the same stimulus (Polterovich et al., 2018). The role of adaption in our paradigm remains to be studied in depth, considering the high variability of stimulation.

An additional layer of complexity is the behavioral context in which calls are perceived. For instance, it is unclear whether pup calls are equally represented in the AC when the adult female is about to retrieve a pup, compared to when it has just retrieved a pup and is already on the nest.

4.3.5 *Behavioral states*

Several studies have highlighted that cortical responses in general, and the AC in particular, are highly susceptible to the internal state of an animal (Fritz et al., 2007; Schönwiesner et al., 2007; Polack et al., 2013). By state, I am referring to states of vigilance, such as high alertness or arousal during exploratory behaviors, versus states of low arousal, fear and hiding (Gyorgy & Andreas, 2004). Top down attention signals allow the AC to selectively pay attention to momentary relevant auditory cues, and enhance neural responses to those cues (Fritz et al., 2007). The state of an animal was shown to severely affect cortical coding, and can additionally influence the process of learning (McGinley et al., 2015; Yao et al., 2019; Hennig et al., 2020). The group from Nikos Logothetis argues that the AC of humans and macaque monkeys employs a so called "saliency map" which determines, based on elaborate feature extraction, which sounds to attend to (Kayser et al., 2005). Nevertheless, it remains challenging to measure attention experimentally and adequately (Fritz et al., 2007; McGinley et al., 2015). Moreover, it is

difficult to assess how much attention an animal requires to perform a given task(Boudreau et al., 2006; Fritz et al., 2007). In our study, distinct attentional states most likely influenced the processing of pup calls during pup retrieval. For instance, if a mouse is distracted from the task and is in an exploratory mode or tries to escape the behavioral arena, less attention will be paid to current pup calls. This different attentional state could influence AC activity such that no clear pup call related responses could be measured in this scenario. Contrastingly, if a mouse is in a high attentional state, and for instance is about to leave the nest to search for pups, it is much more likely that cells in the AC will respond to any perceived pup call. Hence, gauging the influence of attention during PR experiments is nontrivial, but might have a substantial impact on measured neural responses.

4.3.6 *Multisensory input*

Similar to other cortical areas, the AC is highly interconnected with other sensory cortices, and additionally receives a vast amount of subcortical multisensory input (Musacchia & Schroeder, 2009). Therefore, both ascending inputs to the AC already carry a certain amount of multimodality, and additional inter-cortical connections provide further modulation by other senses. In the context of motherhood, one study demonstrated that olfactory cues combined with stimulation of pup calls enhanced neural responses in the AC of MTs and EVs, but not in NVs (Cohen et al., 2011). Additionally, the AC gets input from the somatosensory cortex and, tactile stimulation was shown to facilitate responses in the AC (Budinger et al., 2006; Rao et al., 2014). Furthermore, higher visual areas project strongly to the AC (Bizley et al., 2007) and can affect neural activity. How exactly all these senses come together in a naturalistic task such as PR remains obscure, and well controlled experiments are needed to disentangle these effects. Notably, it needs to be elucidated how the AC weights the different sensory inputs at different phases of PR. Mice in our experiments have access to all those sensory modalities, making our PR task more naturalistic and with little artificial constraints imposed. With the auditory object theory from section 4.3.3 in mind, it is possible that the diverse pup-originating sensory stimuli generate a more complete and distinct representation for pup calls in the AC, and potentially in other associated cortical areas as well.

4.3.7 *Methodology*

Aside from the points discussed above our chosen imaging methodology has some drawbacks. First of all, it is possible that the FOV I imaged was too small to capture the neural activity underlying pup call processing, especially given our findings pointing to a sparser code where every cell counts. In the surgeries, I targeted the primary

AC, but some experimental evidence obtained by labeling for the immediate early gene c-fos (Geissler & Ehret, 2004), shows that the DP region and the secondary AC (A2) harbor pup call responsive neurons. Secondly, as mentioned in the introduction oxytocin plays an important role in pup retrieval. In particular, neurons in the PVN that secrete oxytocin project to AC (Marlin et al., 2015), and oxytocin receptor expression increases in the AC of MTs and EVs. Furthermore, these oxytocin receptor positive neurons to responded to pup calls. Thus, limiting our imaged cell population to a specific subset of neurons, such as these oxytocin positive neurons, might have revealed more consistent responses. Additionally, I might have still recorded below the threshold of sufficient cell numbers to explain how communication calls are represented in the awake behaving mouse. Notably, the complexity of AC responses described above might need neuron numbers at orders of magnitude exceeding our current ones (Abbott et al., 1996). Although a few neurons in the AC can be highly informative about stimulus identity, these neurons might spatially not be very localized but distributed across the AC (Ince et al., 2013).

Besides the spatial sampling, the temporal resolution of neural responses might additionally mask biological effects. In general, the use of calcium indicators, such as GCaMPs, as a proxy for neural activity imposes non-linearities on the recording of neural activity. The non-linearities arise for the most part from the non-linear relationship between fluorescence and the number of underlying action potentials (Tian et al., 2009). These characteristics of calcium imaging, together with the sparse and unreliable responses in the AC, might jointly hinder the readout of some neural responses. Electrophysiology does not suffer from these drawbacks. It is from electrophysiological studies that we learned single AC neurons can time lock their activity to the presentation frequency of a repeating stimulus, comparable to syllables in a vocalization (Asokan et al., 2021). Such a result is beyond the reach of calcium imaging given the recording frequencies. Another important aspect is that source extraction pipelines for miniscopes are restricted to active cells only (Lu et al., 2018). Finally, it is important to have the right amount of virus expressed in the target cells, as their health and amount of fluorescence need to be balanced for optimal imaging conditions.

4.4 REPRESENTATIONS OF NON-AUDITORY CUES IN THE AC

The richness of neural activity in the AC is already detectable by eye when inspecting the overall ethograms for single PR experiments (**Fig. 3.11A**). These ethograms prompted us to investigate neural activity in response to more global event types, such as pup retrieval events themselves or nest entries and exits. When analyzing responses to these events by calculating median triggered averages, we found that the AC in fact shows onset responses to pup retrieval events in all experimental groups (**Fig. 3.11A**). This was very interesting and at the same time agreed with recent publications, showing that global events - such as movements - are well represented in the cortex and explained a lot of ongoing neural activity (Musall et al., 2019; Stringer et al., 2019). Also, in our data we could decode the mouse's position, as well as its speed, during a trial well above chance, based in the neural activity alone (**Fig. 3.16A**). The representation of mouse location within an experimental arena such as ours, as well as the speed, is most likely a very relevant aspect to a freely behaving mouse, and therefore might be represented broadly in many cortical areas. This information about position of a mouse for instance might bring other sensory cues into context, and potentially allows the mouse to better classify stimuli as currently relevant or irrelevant. Interestingly, we could also decode the nest position of the mouse and saw in nest entry and exit triggered medians of MTs, a significant increase of neural activity after these event onsets (**Fig. 3.12C, 3.16A**). Already in 2019, Carcea and colleagues found that in mice performing pup retrieval, neural activity in the PVN was enhanced upon nest entry (Carcea et al., 2019). This was most prominent in oxytocin-positive PVN neurons. Therefore, enhanced neural activity in the AC upon nest entry could be caused by specific oxytocin-positive neurons in the PVN projecting to the AC.

4.5 POPULATION CODES IN THE AUDITORY CORTEX

In a population code, information is jointly represented by the activity of multiple neurons. Three broad types of population codes have been described based on their trade-off between fine-tuning and sufficient generalizability (**Fig. 1.4**) (Beyeler et al., 2019). On the two extremes are a distributed versus a local code (Rolls & Treves, 1990; Beyeler et al., 2019). In a distributed code, the information about a stimulus is distributed across many neurons, so that each neuron contributes to the encoding of that stimulus. At the same time, each neuron can be part of the representations for many stimuli, a feature referred to as mixed selectivity. In a local code, only a few neurons participate in encoding a stimulus, and no mixed selectivity is found. Here, the interference among different stimulus representations is minimal. In the middle lies the sparse code. In a sparse code, a few neurons encode a given stimulus and can also show mixed selectivity. A sparse code has a few advantages over the others, such as increased storage capacity, less noise susceptibility and less energy demands (Barlow, 1972; Rolls & Treves, 1990). Increased storage capacity refers to the fact that only a few neurons will be activated by a stimulus, and therefore the whole population can represent many stimuli without interference between them. It was shown that, for associative learning networks, a sparse neural code is superior to a fully distributed code, as a higher number of neuronal activity patterns can be stored and discriminated from each other when considering the same number of neurons (Rolls & Treves, 1990). In sensory cortices, such as the visual cortex, sparse coding is advantageous to represent natural scenes for example (Olshausen & Field, 1996). In a distributed network, in contrast, a stimulus activates cells more or less evenly across the entire network (Rolls & Treves, 1990). Such a highly distributed network would have a lot of redundancy in it and thereby makes sure that a given stimulus is well identified. This might be crucial for stimuli that need to be well detected on a global scale. Stimuli of faces were found to be represented in a distributed code in monkey visual temporal areas (Abbott et al., 1996).

The neural code for pup calls during PR can be better described as a sparse or local code, as we observed a small number of highly informative neurons (**Fig. 3.14**). When comparing classifier weight distributions between experimental groups, it became clear that MT neuronal populations had unequal weight distributions, indicative of a rather sparse or local code. EVs and NVs in contrast had both slightly reduced weights and more homogeneous weight distributions than MTs. Therefore, EVs and NVs use a more distributed code that MTs (**Fig. 3.14**). This segregation of weight distributions among experimental groups could reflect the pup exposure regime, and the way in which pup calls are represented in the AC. The more pronounced the exposure to pups, the more weight distributions shifted to higher values (**Fig. 3.14B**). In particular, MTs are heavily involved in pup caring in their home cage, have an optimized sparse neural

code that represents pup calls. On the other hand, NVs, mice that have contact with pups solely in an experimental setting but not in their home cages, have a neural code less adapted to the rather novel pup calls they experience in the task. In this scenario, EVs are found in the middle, since they did not give birth to pups, but are co-housed with a new MT, and closely experience offspring care in their home cage. When analyzing how much the addition of neurons ranked by their assigned weights influenced decoding accuracies, we found that the last steps of neuron addition increased the accuracy more than previous steps in all groups (**Fig. 3.14C**). If the code was distributed, these curves would have a steady increase with each step, as shown for visual face stimuli presented to monkeys (Abbott et al., 1996). This finding highlights the general unequal assignment of weights to AC neurons in representing pup calls. In this analysis, we could not find a difference among groups, probably since this local or sparse coding might be a general feature in representing pup calls. We also found that neurons with high decoding accuracies for pup calls did not significantly overlap with high weight neurons for other behavioral events (**Fig. 3.16B**). However, some of these event types were quite distinct from each other, and therefore it might not be surprising how little high weight neurons overlapped for these event types. It is possible that, when comparing stimuli that are not as distant from each other, one would obtain a different picture. It would be interesting to examine how other pup related cues, such as pup odors, integrate with these results and reveal potential mixed selectivity or the absence of it. In ferret AC it was shown that single AC neurons showed mixed selectivity to pitch, timbre and azimuthal position of a sound (Walker et al., 2011). Theoretical studies have shown that mixed selectivity reduces decoding errors, and is therefore a general coding scheme in the brain (Rigotti et al., 2013; Jeffrey Johnston et al., 2020).

Beyond the sparsity of the code, we found that the neural code for pup calls is very dynamic. In other words, a decoder trained on matched neurons of one PR trial was often not a good predictor for decoding pup calls in another PR trial (**Fig. 3.17**). Moreover, the assigned weights to matched neurons of pairs of PR were sometimes quite unrelated to each other, just as the decoding accuracies. This dynamic rearrangement of weight assignment and decoding based on the same sets of neurons could potentially be a true feature of the neural code, although it could also be a consequence of our limited sampling of the AC, as described above.

Dynamic population codes have been described for other brain regions, such as the posterior-parietal cortex, known to be involved in sensory motor associations (Driscoll et al., 2017). In this study, it was shown that the same neurons that responded to one choice during a learned navigation behavior were not the same neurons that responded to the same choice a few days later, and the population of highly informative neurons drifted over time (Driscoll et al., 2017). Despite the single cell variability, the population activity pattern measured

by principal component analysis remained stable. The authors argue that such a dynamic representation might facilitate the incorporation of new associations into the existing network, thereby highlighting the computational advantages of a dynamic versus a stable code (Driscoll et al., 2017). In barrel cortex instead, sensory neurons remain relatively stable across days, pointing to higher stability of sensory neurons when compared to those in association areas such as the posterior parietal cortex (Margolis et al., 2012; Driscoll et al., 2017). The AC however could act in a different manner than other sensory cortices, since AC neurons are generally less reliably and sparsely responding (Hromadka et al., 2008; Tischbirek et al., 2019). It remains to be answered how much the high weight neurons that encode pup calls contribute to the correct searching and retrieving of pups in the task. Experimentally, it would be interesting to investigate how well a mouse would retrieve pups when these high weight neurons are silenced. This could be implemented by expressing activity dependent light-gated inhibitory ion pumps, such as halorhodopsin, in exactly those neurons. Well-controlled behavioral deficits when silencing these neurons during the task would be a strong indication of their causal role.

Since a large network of neurons can exhibit complex coding schemes representing relevant stimuli in an intricate manner, a population approach might reveal true coding in a neural network better than the sole focus on single cell activities - especially in the AC, where there exists a high heterogeneity of responses types among neighboring neurons (Rothschild et al., 2010).

4.6 CONCLUSIONS

Altogether, I have developed a new approach to quantify behavior in a pup retrieval task, while at the same time, I imaged the engaged neural population in AC. Behaviorally, we showed that pup exposure regimes affected the ability with which mice retrieved pups. While NVs steadily increased their behavioral performance, a strong initial increase was found in MTs and EVs, which had additional contact with pups outside the task. Notably, EVs showed superior pup retrieving performance, potentially based on their co-housing with a MT and putative pheromone induced activation of existing maternal circuits in the brain. Also, these animals could have undergone observational learning from the co-housed MT. In MTs, an additional hormonal component could have facilitated pup retrieval, as it was already shown in other studies to reliably trigger maternal behaviors and fast responsiveness to offspring. Moreover, I found that not only pups vocalized, but also adult mice. The amount of emitted adult calls increased across sessions for each group. The underlying trigger and the behavioral meaning of these calls remain unclear.

In addition to behavioral measurements, I recorded the activity of several neurons simultaneously and analyzed their responses to pup calls. While single cell responses to in-task pup calls were scarce, a dynamic population contained information about pup call presence throughout repeated sessions. When characterizing this population code, a few highly informative neurons were able to efficiently decode pup call presence in a PR task. We found the strongest single cell contributions to pup call decoding in MTs, followed by EVs and NVs. The latter group had the smallest weights, and therefore a more distributed code than MTs and EVs. Pup exposure regimes, and the associated learning of pup retrieval, possibly impact the pup call representations in the AC. This data suggests that the improved detection of pup calls assists them in retrieving pups better in the task. In contrast, the representation of pup calls in the NV AC remained at similar levels throughout sessions, indicative of a differential "relevance" assigned to pup call detection for these two groups, and despite NVs similar steady increase in behavioral performance.

In summary, we have shown in this study that an essential parental behavior in mice is carried out differently in animal groups of disparate experience regimes. Distinct neural signatures in the AC for pup calls and other pup related cues accompanied these behavioral discrepancies. Employing basic natural behaviors such as maternal behaviors as model paradigms to interrogate brain circuits is a powerful approach to shed light on sensory cortex function. By means of the ubiquitous presence of parental behaviors, the underlying biological relevance is out of question and thus serves as an ideal candidate to study social learning and plasticity. Our data contributes to the current understanding of pup call processing, a key element in maternal behavior of mice, and connects this to behavioral quantifications in

the same animals. Further studies may expand on on these findings by taking the mouse's environment and its current state into account in a more comprehensive fashion, thereby strengthening our current understanding of sensory representations and social learning.

5

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SUPPLEMENTARY FIGURES

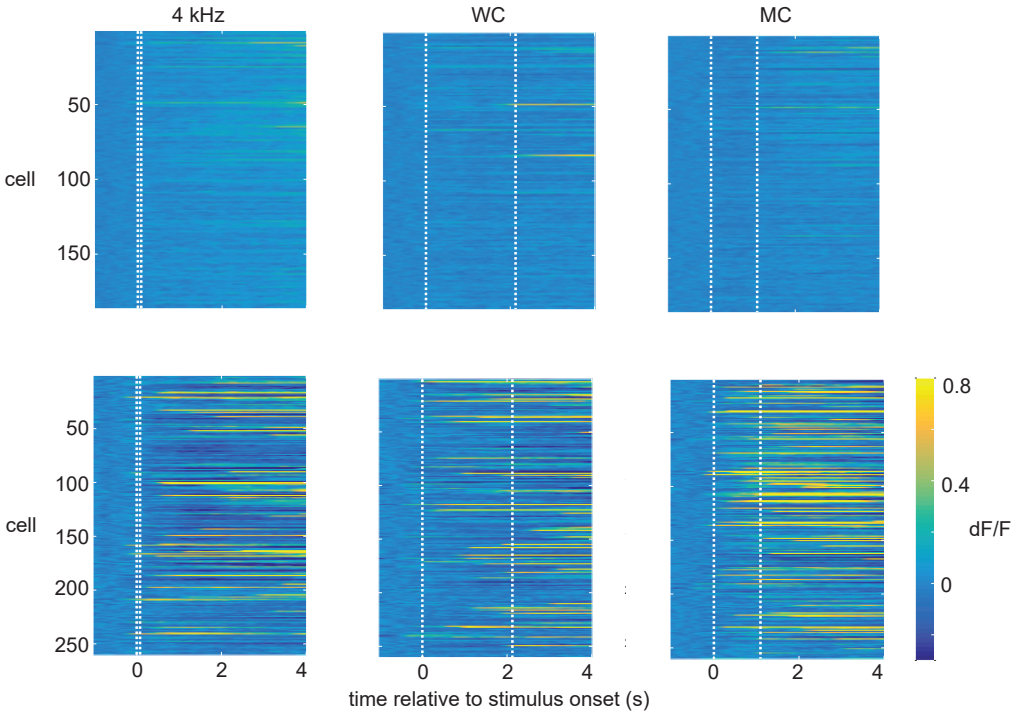

Figure S1 | Sound-evoked activity under different states. *Top*: Exemplary Ca^{2+} traces of manually extracted single AC neurons imaged with the miniscope in an isoflurane-anesthetized mouse, while auditory stimuli were presented. Exemplary auditory stimuli include a 80 ms long 4 kHz pure tone pulse (*left*), a natural wriggling call (WC) (*middle*) and a natural mating call (MC) (*right*. *Bottom*: Same field-of-view imaged 12 days later while mouse was awake and freely behaving in the behavioral arena.

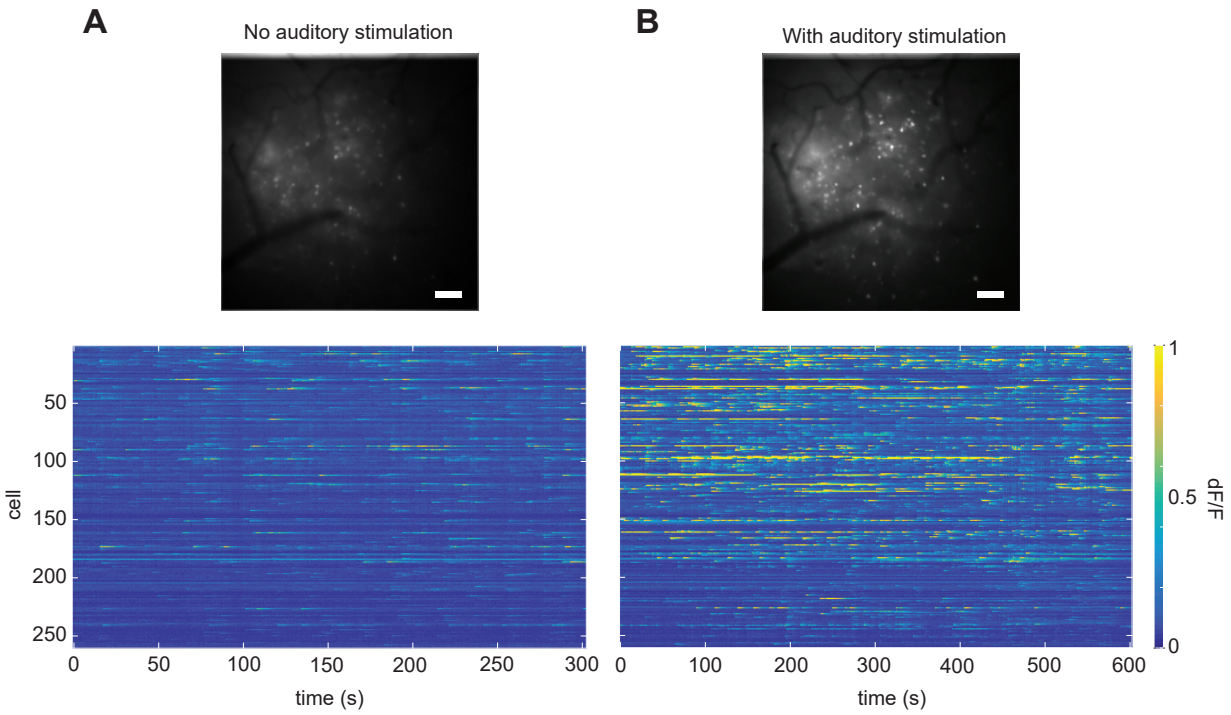

Figure S2 | Awake AC activity with and without auditory stimulation. **A** *Top*: Exemplary standard deviation projection of a miniscope field-of-view containing Cre-GCaMP6s transduced CaMKII^{+} neurons in the auditory cortex of an awake freely behaving mice, scale bar=100 µm. *Bottom*: Ca^{2+} traces of manually extracted single neurons from the same field-of-view as shown above. **B** Same mouse and cells as shown in A, but this time the freely behaving mouse was passively listening to auditory stimuli.

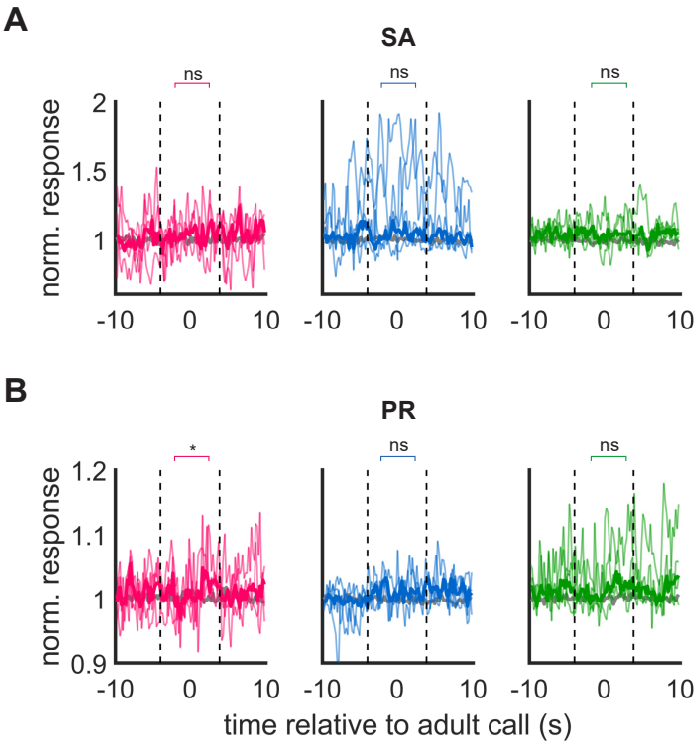

Figure S3 | Neural activity to self-emitted vocalizations. A Adult call triggered medians during SA experiments, dotted lines indicate time window for statistical comparisons between 2 sec before call onset compared to 2 s after call onset per group, Wilcoxon rank sum test with $p<0.05$. **B** Same responses for PR experiments.

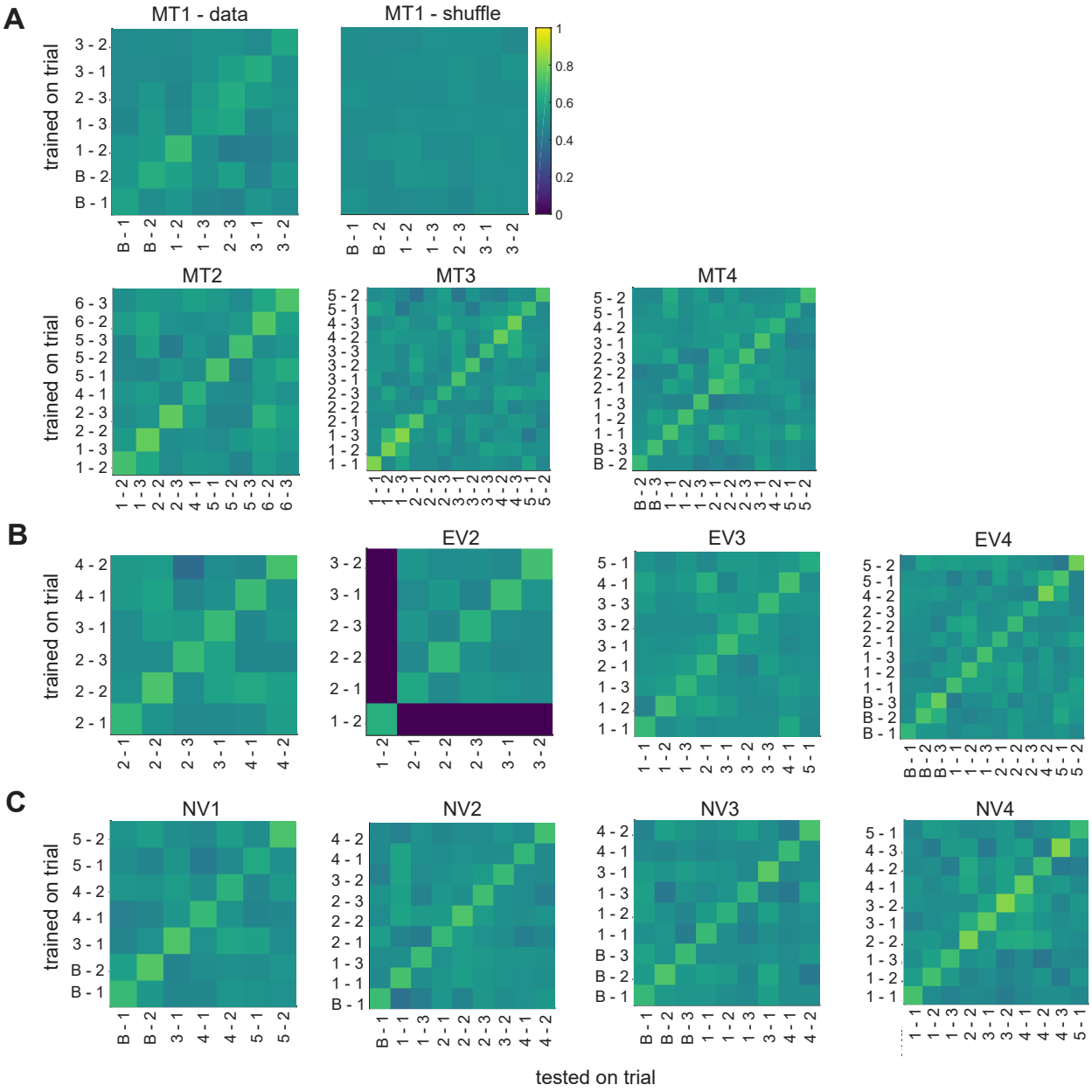

Figure S4 | SVM analysis to decode and generalize pup calls across pup retrieval trials. Pairwise decoding accuracies after training a SVM classifier on one trial (rows) and testing it on another trial (column) among matched cells. **A** *Top left*: Heat map indicates pup call decoding accuracies of MT1 from correctly labeled trials. *Top right*: Heat map represents decoding accuracies for shuffled controls. All shuffled controls looked similar to the exemplary one of MT1 and are therefore not shown. *Bottom*: Heat maps of correctly labeled data for MTs 2-4. **B** Same analysis as in A, but for experienced virgins. **C** Same analysis as in A and B, but for naive virgins.

LIST OF FIGURES

ABBREVIATIONS

A1 - primary auditory cortex
A2 – secondary auditory cortex
AAF – anterior auditory field
AC – auditory cortex
AM – amplitude modulation
BLA – basolateral amygdala
DA – dopamine
DCN – dorsal cochlear nucleus
DNLL – dorsal nucleus of the lateral lemniscus
DP – dorsoposterior field
EE – excitatory-excitatory
EI – excitatory-inhibitory
EV – experienced virgin
FM – frequency modulation
FRA – frequency response area
GABA – gamma-amino-butyric acid
GBC – global bushy cell
GECI – genetically encoded calcium indicator
IC – inferior colliculus
ILD – interaural level difference
ISI – inter-syllable interval
ITD – interaural time difference
LSO – lateral superior olivary complex
MGB – medial geniculate body
MGD – dorsal MGB
MGM – medial MGB
MGV – ventral MGB
Miniscope – Miniaturized fluorescence microscope
MNTB – medial nucleus of trapezoid body
mPOA – medial preoptic area
MSO – medial superior olivary complex
MT – mother
NAC – nucleus accumbens
NV – naïve virgin
OT – oxytocin
PBS – phosphate buffered saline
PC – principal cell
PCTM – pup call triggered median
PFA – paraformaldehyde
PFC – prefrontal cortex
PPD – postpartum depression
PR – pup retrieval
PS – passive stimulation
PV – parvalbumin
PVN – paraventricular nucleus

SBC – spherical bushy cell
SA – spontaneous activity
SC – superior colliculus
SOC – superior olivary complex
SPL – sound pressure level
STRF – spectro-temporal receptive field
TC – thalamocortical cell
TRN – thalamic reticular nucleus
UF – ultrasonic field
USV – ultrasonic vocalization
VCN – ventral cochlear nucleus
VP – ventral pallidum
vnBST – ventral bed nuclei
VNLL – ventral nucleus of the lateral lemniscus
VTA – ventral tegmental area

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AFFIDAVIT

Hiermit versichere ich, Isa-Maria Gross, an Eides statt, dass ich die vorliegende Dissertation *Combined Behavioral and Neural Investigations of Pup Retrieval* selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

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