Sound processing by local neural populations in the mouse auditory cortex

Thesis submitted for the degree of

“Doctor of Philosophy”

by

Gideon Rothschild

Submitted to the senate of the Hebrew University of Jerusalem

September 2011
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This work was carried out under the supervision of

Prof. Adi Mizrahi and Prof. Israel Nelken
Acknowledgements

Adi, I am grateful to you for introducing me to science, for teaching me how to do it in long days and nights, for taking me through its ecstatic highs and its frustrating lows; it’s been a hell of a ride. Much of what I have learnt in these years, I learnt from you, including the handicraft of neurophysiology and imaging, the aesthetics of science, the efficiency of order and the art of scientific writing. More importantly, you have taught me to fantasize about ideal dream results, and then to stubbornly and resolutely work to try to materialize them.

Eli, scientific research is for you, and has been for me under your guidance, a flow of intellectual challenges, which can only be taken on with a rich toolbox of knowledge and analytic capabilities. Like a Judo wrestler, you have taught me that to land your data exposed on its back, you first have to get intimately close to it. Yet, you have also taught me to keep a wide view to related and unrelated fields. Someone once wrote that "if I have seen a little further it is by standing on the shoulders of Giants". Thanks for the ride.

Yoav, Yoav, Hagit, Lior, Yael, Tal, Ido, Ami, Maya, Hadas and Tohar, I have greatly enjoyed these years with you. I feel the sensitive and friendly atmosphere in the lab, coexisting with the fierce everyday scientific struggle we have all been involved in, is truly unique. I thank you all for your help, invaluable comments and for making this time enjoyable.

Nevo Taaseh, Anat Yaron-Jakoubovitch, Dina Moshitch and Yoav Rubin, thank you very much for your generous assistance in early and shaky stages of my research.

In my graduate years, I have been fortunate to be a part of the ICNC family. The ICNC has always given me the extra push and extra support, and I am very grateful for it. I would especially like to thank Professors Eilon Vaadia, Idan Segev, Udi Zohary, Yossi Yarom and Haim Sompolinsky for this encouraging environment, but also for concrete help with manuscripts, presentations, and invaluable advice. And, of course, I would like to thank Ruthi Suchi whose mere presence (but also lots of administrative assistance) has been a constant comfort.

I would like to thank Judith Baroth, whose generosity with our kids and ourselves knows no limits.

My deepest thanks are to my wife and love Ada, for always being there for me, for inspiring, supporting and enriching me every single day.

I have been fortunate enough to have best friends that are also my brothers. I would like to thank my brothers Yoav and Eyal and my brother-in-law Elad for your support, deep friendship and for being who you are.

Finally, I would like to thank my mother Elisabeth and my father Stefan for everything you have given me throughout the years. Although you are not from the field of science, I have tried to learn from you the most important skills to pursue it: curiosity, ambition, the urge to examine the true origin of matters through ever-present distractions and that hard work and creativity do not oppose but that they are actually symbiotic.
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Abstract

Mammals rely on their auditory system for diverse biological functions such as communication, hunting, danger avoidance, courting, and navigation. Sound processing starts when a sound wave reaches the inner ear, generating a vibration in the cochlea which is transduced into an electrical signal. This signal propagates along various stations of the auditory pathway, through the thalamus, eventually reaching the auditory cortex (AC). The AC is the primary cortical region devoted to processing of sounds, and as such, it is believed to be involved in higher-order processing of sound and having a large influence on auditory perception. However, despite intense research over six decades, the basic questions of "what does AC do, and how?" remain largely unanswered.

To try and answer this question, numerous studies have monitored the physiology of the AC while presenting different auditory stimuli. These studies have mostly concentrated on two distinct levels of spatial resolution: single cell activity monitored using electrophysiological recordings, and large-scale activity monitored using electrophysiological or imaging techniques which sample and/or average the signal over hundreds of micrometers or more. Studies at both of these levels of resolution have revealed important findings about the AC. For example, single-cell studies have found that when presented with pure tones, the receptive fields of neurons in AC are very similar to those at much lower stations of the auditory pathway, in contrast to the hierarchical organization of the visual pathway. However, the receptive fields of single neurons can undergo dramatic changes under different circumstances, such as when the animal is performing a task which involves auditory discrimination. Large-scale physiological studies have revealed, for example, that the AC is organized into several sub-fields, some of which show a smooth tonotopic organization. Moreover, the large-scale organization of the AC may also undergo changes in response to the acoustic environment.

Why is the function of the AC still considered enigmatic, despite a vast amount of information regarding the single cell and large-scale physiology? Partly, this is because the cortex in general, and the AC specifically are extremely complex systems. However, this is partly also because it has so far been impossible to monitor the activity of the AC at the
intermediate spatial resolution, in which much of information processing in the cortex is believed to occur: the level of local neuronal networks.

Information processing in the cortex is believed to be facilitated by the interaction of large numbers of synaptically connected neurons. The combination of the computation individual neurons perform on their input with the pattern of flow of activation between the neurons create population activation patterns which underlie perception and are largely unknown. However, studies have shown that connectivity in the cortex is mostly local: more than half of the synaptic contacts a neuron receives are believed to be from neurons within 100-200 µm away. Thus, monitoring the organization, dynamics and functional plasticity of local neural population is likely to shed new light on information processing in the AC. However, until recently, this has been technically impossible to do in vivo, because electrophysiological recording techniques cannot densely probe neurons at this spatial resolution, while optical techniques to monitor neural physiology have been limited to use at the surface of the tissue.

Recently, this has changed with the development of two photon microscopy and efficient calcium-sensitive dyes. Using two photon calcium imaging (2PCI), it is possible to monitor changes in fluorescence from multiple well-isolated neurons simultaneously at depths of up to 500 µm below the brain surface. Since loaded neurons are very close and their position determined at micrometer resolution, this method has recently been used to probe the organization of local neural populations in a number of sensory cortical regions.

In my research, I have implemented this new method to study local neural populations in the auditory cortex for the first time. Since 2PCI is a relatively new technique, and never previously used in the AC, I carried out a set of experiments to rigorously evaluate, test, and optimize monitoring population activity using this technique. Along with its potential advantages, the main challenges posed by 2PCI for reliable physiological recording stem from the use of an indirect measure of electrical activity, which is optically monitored. In Chapter 1, I examine issues such as the practical temporal and spatial resolutions of 2PCI, its reliability, noise levels, depth limitation, and recording stability. My results have delimited the limitations of 2PCI, thereby allowing to optimize experimental and analytical aspects of this technique to study cortical population activity.
Abstract

Using 2PCI, in Chapter 2 I provided a first look into how local networks of neurons in the AC are organized and activated in response to auditory stimuli. Our data showed that while previously reported large-scale organizational principles do exist, local populations of neurons in the auditory cortex are surprisingly heterogeneous and sparse. Neighboring neurons could respond to very similar or completely divergent tone frequencies and intensities. As a basic assessment of the network dynamics in auditory cortical networks, I calculated pairwise noise correlations. I found that noise correlations were high on average, highly variable between pairs, and that on average noise correlation decreased with the distance between neurons. These results support the existence of heterogeneous local cortical processing modules. This study was published in *Nature Neuroscience* in 2010 (Rothschild et al., 2010).

While the functional organization of local neural populations probably subserves information processing in AC, it may actually be that behaviorally triggered reorganization of these networks is a key feature of AC. In Chapter 3 I studied how the functional connectivity, measured as pairwise and higher-order noise correlations, change in mothers following parturition. To this end, I compared dynamics of local populations in AC in virgins and in mothers 4-5 days following parturition. By this time, mothers had undergone hormonal and physiological changes, as well as experienced maternal care for 4-5 days, in which auditory communication is a central aspect. I found that in contrast to studies in which a single auditory stimulus is instructed with the use of electric shocks, there was no change in the mean response profile of the population. However, pairwise correlations in mothers were twice as high as those of virgins, and higher-order correlations were much more dominant. These results reveal a novel, purely network-level functional plasticity mechanism in AC, associated with a dramatic yet natural change in the state and experience of the animal.

Overall, my research is the first to study local populations of neurons in the AC, allowing to investigate a previously inaccessible level of resolution between single-neuron and large-scale topographic studies. I have described for the first time how cortical networks of neurons in the AC are organized, what their dynamical properties are, and how these dynamics change with the behavioral state of the animal. My findings reveal several novel aspects of auditory cortical organization and processing, advancing our understanding of the unique function of the mammalian AC.
Introduction

The enigmatic (auditory) cortex

Mammals rely on their auditory system for diverse biological functions such as communication, hunting, danger avoidance, courting, and navigation. Sound processing starts when a sound wave reaches the inner ear, generating a vibration in the cochlea which is transduced into an electrical signal. This signal propagates along various stations of the auditory pathway, through the thalamus, eventually reaching the auditory cortex (AC). The AC is the primary cortical region devoted to processing of sounds, and as such, it is believed to be involved in higher-order processing of sound and having a large influence on auditory perception. However, despite intense research over six decades, the basic questions of "what does AC do, and how?" remain largely unanswered (Nelken, 2008; King and Nelken, 2009).

To a large extent, this question remains open for most if not all sensory cortical regions. An expression of the gap between what we would like to know about the cortex and where we actually stand was elegantly expressed in two parts of the title of a recent international conference: "Computations in neocortical circuits: What Does the Cortex Do?" (Janelia Farm Conferences, March 2009). Although much is known about different components of the cortex, such as it anatomy, the response properties of single neurons, and their large-scale functional organization, we currently lack a basic understanding of how information about a sensory stimulus is processed in the cortex.

Neural processing in more peripheral brain regions is generally much better understood. For example, convincing data and theoretical models have been proposed to describe the computations performed in the retina in response to visual stimuli (Gollisch and Meister, 2010) and the processing of sounds between the cochlea and the inferior colliculus (Trahiotis and Hartung, 2002; Nelken, 2008). Why, then, is cortical processing much less understood? Firstly, information about a stimulus reaches the cortex after being processed by multiple brain structures. In the auditory pathway,
information travels through at least 4 synapses before reaching the cortex. Complex and partially understood transformations within and between subcortical nuclei make the input to the cortex only partially understood. Secondly, the cortex itself is an extremely complex system. Each neuron is synaptically connected to thousands of other neurons, and since the precise connectivity between them is generally not available, the transfer of information is practically impossible to monitor in detail. Furthermore, neurons are divided into numerous sub-types, and are organized into layers and columns with varying degrees of inter-and intra-region communication. The cortical circuit is so complex, that an ongoing herculean attempt to model a single cortical column (Markram, 2006) comprising of only 10,000 neurons requires a state-of-the-art supercomputer, capable of performing 500 TFLOPS (5*10^{14} floating operations per second). It is early to say, however, whether this attempt will succeed in revealing new features of cortical processing.

Although we are not yet capable of completing the puzzle of cortical processing, we do have a good description of some of its most important pieces. The identity of the pieces we do understand is largely determined by available technology. Monitoring the information flow across thousands of neurons simultaneously while the animal hears a single sound is technically difficult, but monitoring the activity of a single neuron over thousands of sound repetitions is relatively easy, as is monitoring the average response of many neurons within a confined region. We thus know a lot about single-cell response properties and large-scale functional organization, but very little about neural network physiology. I will next introduce some key findings regarding the physiology and functional organization of the cortex, and specifically the auditory cortex. These findings serve as the starting point for my research into the physiology of cortical networks in the AC.

**Large-scale computation in the AC**

Many cortical sensory areas are composed of multiple sub-regions, segregated based on anatomical and physiological characteristics. The AC is divided into varying numbers of sub-regions in different
mammalian species. For example, six auditory regions have been described in gerbil (Thomas and Lopez, 2003), thirteen in cat (Lee and Winer, 2005), and twelve in monkey (Hackett et al., 1998). The AC of the mouse, the animal model used in my research, has been described as having 5 sub-regions (Stiebler et al., 1997). The primary auditory field (A1) is the largest field, and is tonotopically organized such that low frequencies are represented in its caudal part and high frequencies in its rostral part. The anterior auditory field (AAF) is the second largest field and includes a tonotopic map in a reverse order. The dorsoposterior field (DP), the second auditory field (A2), and the ultrasonic field (UF) are smaller fields which do not show clear tonotopic organization. What can we learn about the function of AC from this structure? In the study which first described this large-scale organization, Stiebler and colleagues did not suggest a function of the division into these subfields and the unique function of each of them. Generally, division of a cortical sensory region into a number of fields with varying response properties is well suited to support parallel processing, where each field is specialized to extract different information from incoming stimuli. However, although differences in response properties between, for example, neurons in A1 and AAF have been described (Linden et al., 2003), they are too small to reveal distinct processing schemes. Thus, it is highly plausible that division of AC into subfields supports auditory processing, but it is unclear how.

Probably the most robust organizational principle of cortex is its columnar organization, first described by Mountcastle in 1957 (Mountcastle, 1957). In his Nobel Prize acceptance speech, David Hubel said that "discovery of columns in the somatosensory cortex was surely the single most important contribution to the understanding of cerebral cortex since Cajal". This organizational principle has been so influential because it suggested that the cortex consists of many small modules which carry out local processing and interact with other modules. Indeed, columnar organization in the visual and somatosensory cortex have been suggested to support different functions in stimulus coding (Erwin et al., 1995; Panzeri et al., 2003). In the auditory cortex, however, columnar processing is still poorly understood (Linden and Schreiner, 2003). Early physiological reports of
columnar organization in the AC of different animals date back to the 1960’s, when electrophysiological recordings revealed neurons with similar response properties along a vertical penetration into the cortex (Suga, 1965; Abeles and Goldstein, 1970; Merzenich et al., 1975; Shen et al., 1999; Wang et al., 2010). In contrast, however, two recent studies in awake rats and anesthetized cats found no evidence for columnar organization in AC (Chechik et al., 2006; Hromadka et al., 2008). The failure to produce a clear model for columnar computation in AC, however, is not for lack of evidence of response similarity along vertical penetrations, but rather close to the opposite— for lack of a systematic change of receptive fields between cortical layers.

Division into layers is a hallmark of cortical organization, and crosstalk between layers has been proposed to facilitate information processing (Grossberg, 2007). The laminar structure of the AC resembles that of other sensory cortical regions, but exhibits some distinct features (Linden and Schreiner, 2003). The primary source of input to the AC from sub-cortical regions arrives from the medial geniculate body (MGB) in the thalamus, and innervates cortical layers 3 and 4. Layers 3 and 4 transmit information to upper layers 2/3, which form dense cortico-cortico projections with multiple layers. The major output layers are 5 and 6, which both project back to MGB, while projections to sub-thalamic regions originate from layer 5 (Oviedo et al., 2010).

Combining this anatomical data with differences in physiological properties in the different layers could reveal what transformations occur between layers. Indeed, in visual and somatosensory cortex, laminar differences in receptive fields have inspired concrete hypotheses about intracolumnar transformations (Brumberg et al., 1999; Martinez et al., 2005). In the auditory cortex, however, studies of laminar differences have failed to produce a consensus on how auditory receptive fields might differ across cortical layers (Linden and Schreiner, 2003). For example, a recent study in guinea pig auditory cortex found differences between response properties of neurons in different layers, but differences were small compared to the variance (Wallace and Palmer, 2008). This lack of a systematic relation between layers and response properties has made it difficult to identify between-layer computations in the AC.
While the strictness of columnar organization in the AC is arguable, organization across the AC shows marked organizational principles. The best described feature of AC organization is tonotopy: a systematic gradient of best frequencies across A1 and other auditory fields. Large-scale tonotopic organization in A1 has been described in numerous studies in different animals (for example, see A1 tonotopy in mice (Stiebler et al., 1997), rats (Polley et al., 2007), ferrets (Sally and Kelly, 1988; Nelken et al., 2004; Bizley et al., 2005), cats (Reale and Imig, 1980), and monkeys (Philibert et al., 2005)). This organization has been linked to normal development of the AC (Zhou and Merzenich, 2007), and has been suggested to minimize connectivity, reduce redundancy, and enhance computational power by eliminating conflicting demands and coordinating multiple algorithmic transformations (Kaas, 1997; Chklovskii and Koulakov, 2004). However, despite the elegance in tonotopic organization, its precision in AC has been a controversial issue for a few decades and remains unresolved to date (Evans et al., 1965; Goldstein et al., 1970; Merzenich et al., 1975; Schreiner and Sutter, 1992; Hromadka et al., 2008). It has therefore been difficult to identify what role tonotopic organization has in AC processing.

Similarly to the overlapping maps of orientation selectivity and ocular dominance in visual cortex, additional maps have been reported in the auditory cortex besides the tonotopic map. It has been suggested that along the isofrequency axis of AC, there is an orderly gradient of other response parameters, resulting in a matrix where each combination of parameters is uniquely represented (Schreiner and Winer, 2007). One response parameter according to which AC has been suggested to be organized is binaural interaction, that is, the neural combination of information from both ears. Organization according to binaural interaction is not as regular as that of best frequency, but neurons forming homogeneous clusters with respect to binaural interaction have been found in many studied species (Imig and Brugge, 1978; Middlebrooks et al., 1980). An additional important response parameter according to which organization has been found is tuning bandwidth. For example, in AI of cats (Schreiner and Mendelson, 1990) and several species of New World monkeys (Recanzone et al., 1999; Cheung et al., 2001; Philibert et al., 2005), clusters of neurons sharply and
broadly tuned to frequency are segregated within the isofrequency domain. In a recent review, Schreiner and Winer wrote that "...physiologic maps [in AC] are fuzzy and variable, with area-, task-, and experience-dependent configurations", and yet, that "Such a stable topographic framework could provide a reference state suitable for perception, learning, and memory". How topographic organization supports these processes, however, is largely unknown.

**Single-neuron computation in the AC**

Understanding the basic, large-scale, organizational principles of a cortical region can promote our understanding of what neural functions this domain may support. However, it provides limited information regarding how this function is executed. To obtain architectural descriptions, the fine resolution of cortical computation via the activity of thousands of synaptically connected spiking neurons is averaged over both time and space. It may resemble the attempt to understand the beauty of a Beethoven string quartet by only identifying that the piece is played by two violin players, a violist and a cellist. To continue with the analogy, one step towards understanding the musical piece might be to study in detail what music the violin, viola and cello may produce individually. Surely, even listening to individual instruments tells you a lot about this form of music (for example, that it is probably not rock music). In neuroscience, this may be compared to recording the activity of single cells using electrophysiological techniques. Electrophysiological recordings have been used to describe the activity and responses of cortical cells *in vivo* for decades. For example, a classic groundbreaking set of electrophysiological studies starting in 1959, describing orientation selectivity of single neurons in the primary visual cortex of cats earned Hubel and Wiesel the Nobel Prize in 1981.

To truly study the computation a single neuron performs, its output must be recorded simultaneously with its input. A small number of such experiments have been performed in vitro (Cruikshank et al., 2002), and in vivo (Miller et al., 2001), starting to reveal important information on
single cell computation in the cortex. However, these are technically difficult experiments, and so far, the more common experimental paradigm has been to record only from AC, while presenting stimulation paradigms which allow testing the unique properties of AC neurons, sometimes by separately recording from neurons in the MGB (Ulanovsky et al., 2003; Las et al., 2005). Thus, instead of the unique computation performed by single neurons in AC, studies more commonly address the related but different question of how sounds are represented in the AC. This may well be changing, as recent technological advances allow monitoring the local activity of single dendritic spines throughout a cell body (Chen et al., 2011).

Neurons in the cochlear nucleus, the first brain structure after the cochlea, often respond to pure tones in a v-shaped response area in frequency-intensity space: at low intensities, a neuron responds to a narrow frequency range around some characteristic frequency, and as the intensity of the stimuli increases, the neuron becomes less selective and responds to a wider frequency range (Rhode and Greenberg, 1992). This response profile is inherited from the hair cells along the cochlea, whose characteristic frequencies are determined by their position along the cochlear membrane. It is quite surprising, however, that neurons in the AC, four or five stations up the auditory pathway, often display a similar tonal response profile (Moshitch et al., 2006). The elegant transformation of receptive fields in the visual pathway, from a center-surround pattern in the retina, to orientation-selective neuron in the cortex, seems to be absent in the auditory pathway, at least when probed with pure tones (Nelken, 2008). Moreover, evidence is lacking for any emergent properties of AC neurons, i.e., stimuli which would trigger a response in AC neurons but not in lower stations (King and Nelken, 2009). Indeed, this has put to question whether the auditory pathway is hierarchical in the same way that the visual pathway is (Nelken, 2008). However, it should be remembered that pure tones are a tiny selection of all possible stimuli, and the responses of neurons to complex stimuli is often only weakly predictable from their pure-tone responses (Bar-Yosef et al., 2002; Wang et al., 2005; Chechik et al., 2006; Bar-Yosef and Nelken, 2007; Ahrens et al., 2008).
Introduction

The immensely complex path for information to transfer from the cochlear nucleus to the AC, makes it highly unlikely that sounds are represented in a similar manner in both regions. What, then, is added along the way? One reasonable possibility would be that neurons in the AC are specialized for processing complex and natural sounds. In support of this possibility, Chechik et al. (2006) found that when presenting natural stimuli, information redundancy decreases in the ascending auditory pathway, possibly reflecting a process of extraction of meaningful structures in the stimulus. A different study found that tailoring stimuli to specific neurons could reveal complex preferred stimuli, although none of these were natural (Wang et al., 2005). However, direct evidence that the AC is specialized for processing natural sounds, such as auditory "grandmother cells" responding exclusively to communication calls, is lacking. Conversely, Bar Yosef and colleagues found that neurons in AC do not preferentially respond to a natural bird chirp, compared to a synthetically manipulated version (Bar-Yosef et al., 2002). These and other findings have earned auditory cortical neurons the title 'promiscuous' (E. Nelken, personal communication).

The lack of data to support AC as specialized for processing complex sounds, has encouraged researchers to look for an alternative unique role of AC neurons. Low stations of sensory pathways, including the auditory pathway, are believed to be designed and optimized to accurately and reliably represent incoming stimuli. For example, the pattern of hair-cell activation along the basilar membrane of the cochlea accurately reflect the frequency composition and intensity of incoming sounds. However, our perception and responses to sounds are not exclusively shaped by their physical properties. For example, a person perceives a loud and sudden shout very differently if he is standing in a crowd and sees his friend is emitting the shout, compared to how he would perceive the exact same shout when he is at home in pitch dark and silence, looking for the source of suspicious sounds. An identical sound is thus perceived very differently under different contexts. In recent years, mounting evidence suggests a central characteristic of auditory cortex function is its functional plasticity, or, context-dependence responsiveness. One example is a decrease in responses to a repeating stimulus, while responses to an even slightly different stimulus remains
strong, a phenomenon termed stimulus specific adaptation (SSA) (Ulanovsky et al., 2003). SSA presumably supports auditory perception by attenuating responses to constant background sounds, allowing improved sensitivity to foreground sounds. However, the unique role of cortical SSA compared to SSA at lower auditory stations (Anderson et al., 2009; Malmierca et al., 2009; Antunes et al., 2010; Bauerle et al., 2011) awaits further examination. While SSA causes reduction in the responses to behaviorally irrelevant sounds, Fritz and colleagues showed that neurons in the AC of awake ferrets change their receptive fields during behavior according to task requirements (Fritz et al., 2003), directly revealing that response properties of single cells are context-dependent. A more recent study showed that the large-scale organization of response properties in AC can undergo large changes during learning, and that these changes enhance perceptual learning (Reed et al., 2011). These studies indicate that a prominent characteristic of single neurons and global organization of AC is its context-dependence, or functional plasticity, at multiple temporal and spatial resolutions. However, plasticity at the level of cortical neural networks, the level most often modeled as the basis of information processing, has been left unstudied.

Going back to the string quartet analogy, electrophysiology has allowed detailed descriptions of what music the violin, viola and cello can produce. However, a complete string quartet piece cannot be deduced from knowing which instruments are playing and what their individual capabilities are. Music of the different instruments may sound beautiful individually, but the interaction between them is what gives the composition much of its meaning. Similarly, since neural activity is composed of multiple interacting neurons, it is reasonable to assume that some properties of the neural code will only be revealed when simultaneously monitoring the activity of multiple neurons.

**Neural-population computation in the AC**

Cortical neurons receive 3000–10,000 synaptic contacts, and more than half of these are thought to arise from neurons within a 100–200 µm radius of the target cell (Shadlen and Newsome, 1998).
Shadlen and Newsome estimated that activation of a few dozen synapses (of the >3000 available) within 10-20 ms, is enough to drive the target neuron to a high discharge rate. They also noted that since neighboring cortical neurons often share similar physiological properties, the conditions that excite one neuron are likely to excite a considerable fraction of its afferent input as well (Mountcastle, 1978; Peters and Sethares, 1991), creating a scenario in which saturation of the neuron’s firing rate could easily occur. In their influential paper, Shadlen and Newsome propose a model for how local populations of neurons compute under these conditions, which they term "high input regime". However, intracellular recording studies do not find supporting evidence for this high input regime (Las et al., 2005; DeWeese and Zador, 2006). Many other computational models have been proposed to elucidate how local cortical computation occurs (Abbott and Dayan, 1999; Salinas and Sejnowski, 2000; Sompolinsky et al., 2001; Averbeck et al., 2006; Shamir and Sompolinsky, 2006). However, it has been impossible to test these models because monitoring the simultaneous activity of multiple individual neighboring neurons has not been technically possible. Thus, central questions regarding cortical processing in the AC have been left unanswered: How are neurons organized to allow efficient computations in local cortical populations? What are the dynamical properties of local neural networks? Are the network dynamics fixed or do the interactions between neurons change under different circumstances? In my research, I have implemented a new technique for monitoring the activity of local populations, for the first time in the AC, and have addressed these and other questions.

**Two photon calcium imaging to monitor population activity**

Monitoring the activity of multiple neurons has, until recently, only been possible to do using extracellular electrophysiological recordings. Electrophysiology offers unmatched reliability for monitoring neuronal activity and is still the gold standard for neurophysiology. In the last decade, however, an alternative approach has emerged with the development and implementation of two
photon laser scanning microscopy (Denk et al., 1990). Traditional optical microscopy techniques are limited to use near the tissue surface (<100 µm), because at greater depths light scattering blurs the image. 2PLSM is based on the idea of excitation of a fluorophore by the simultaneous absorption of two low-energy photons. Since only molecules which absorb two photons simultaneously are excited, this allows exciting only a tiny volume of tissue at the focal spot, eliminating photons from out-of-focus objects, and resulting in clear images down to depths of hundreds of micrometers into the brain. Since the laser beam is very narrow at its focal plane (<1 micron), to image structure or function of neurons the laser beam continuously scans the tissue.

2PLSM has been complemented by an additional technological advance to yield a powerful new technique for monitoring neural population activity in vivo; namely, bulk-loading of calcium indicators into large neural populations (Stosiek et al., 2003). Following a simple extracellular injection, hundreds to thousands of neurons around the injection site are loaded with calcium-sensitive dyes such as OGB-1 AM or Fluo-4 AM. Since an action potential involves a large increase in intracellular calcium concentration, dye-loaded spiking neurons exhibit sharp increases in fluorescence with characteristic dynamics. Using 2PLSM, these fluorescence transients can be monitored from tens to hundreds of neurons simultaneously deep in the living brain, and up to thousands of neurons from each animal (Gobel et al., 2007). Monitoring population activity using 2PLSM offers important advantages along with some limitations which are inherent to the recording technique.

Since tens to hundreds of thousands of neurons are believed to be involved in processing of a given sensory stimulus in the mammalian brain, recording large numbers of neurons simultaneously are essential for extrapolating to the complete population level. For example, recording from pairs of neurons would not allow identifying higher-order correlations between neurons. Using electrophysiological methods, it is currently possible to record the activity of up to ~100 neurons simultaneously with multi-shank silicon probes (Buzsaki, 2004). With 2PLSM, the number of imaged neurons is limited by the number of stained neurons, the size of the field of view of the objective,
and the quality of the fluorescence signal which determines how many pixels have to be sampled from each neuron to yield a high-quality trace. Practically, most in vivo calcium imaging studies have imaged 10-20 neurons simultaneously from each focal plane (Kerr et al., 2005; Ohki et al., 2005; Kerr et al., 2007; Sato et al., 2007; Greenberg et al., 2008). However, recent technological advances such as imaging neurons in different depths simultaneously, have made it possible to image hundreds of neurons simultaneously and thousands of neurons from a single animal (Gobel et al., 2007; Cheng et al., 2011). Although these numbers are still a relatively small fraction from the number of neurons in the population, they are reaching a level where extrapolation becomes realistic.

One drawback of electrophysiological recordings is that the exact locations of the neurons are unknown, thus relating function to structure has been limited to the resolution of hundreds of micrometers. Using 2PLSM for imaging population activity, the precise location of each neuron is known relative to each other as well to the brain surface at a resolution of ~1 µm. This has made it possible to characterize the functional micro-architecture of populations of neurons at distances of 0-200 µm (Kerr et al., 2005; Ohki et al., 2005; Kerr et al., 2007; Mrsic-Flogel et al., 2007; Sato et al., 2007; Greenberg et al., 2008).

Besides the precise positions of the cells, 2PLSM allows relating the imaged activity to the type of the cells. By injecting a marker such as Sulforhodamine 101 mixed with the calcium indicator, astrocytes are distinguishable from neurons by red staining. Using either transgenic animals or targeting of genetic markers using viral vectors, different subpopulations of neurons can be driven to express specific fluorescent proteins. Combining such tagging with population calcium imaging allows dissecting the population to its molecular subparts. Moreover, recent developments in the design of genetically encoded calcium indicators (GECIs) has allowed to monitor the activity of the same population of neurons over periods of weeks and even months (Tian et al., 2009).

However, two photon calcium imaging also comes with substantial drawbacks compared to electrophysiology. Key challenges with two photon calcium imaging stem from the use of an indirect
and slow fluorescence signal as a measure for electrical activity. The calcium, the dye, and the optics might each introduce biases when attempting to extract electrical activity. In contrast to electrophysiology, where solution for inherent challenges such as that of spike sorting have been addressed, developed and optimized for decades (Millar, 1983), the challenges of interpreting the signal obtained with two photon calcium imaging are only starting to be addressed.

In Chapter 1 of the thesis I present experiments and analyses I have performed to study the nature of the fluorescence signal obtained with two photon calcium imaging, the abilities and limitations of this technique, and present my findings regarding good use of this technique.

In Chapter 2 I present a study describing for the first time the functional micro-organization and population dynamics in the primary auditory cortex of the mouse. I found that local populations of neurons in the mouse AC were surprisingly heterogeneous in their mean response properties, yet their tendency to co-fluctuate was strong. Similarity in response properties and tendency to co-fluctuate both decreased within the range of 0-200 µm, suggesting that unique local computation occurs at this resolution. This study was published in Nature Neuroscience in 2010.

In Chapter 3 I examined how response properties of neural populations in the primary auditory cortex of mice change following parturition. To this end, I imaged population activity in mother mice, 4-5 days following parturition, and compared it to that of age-matched female virgins. By the time of the experiments, mothers had undergone physiological and hormonal changes during pregnancy and parturition, and allowed to experience 4-5 days of maternal behavior with the pups. I found that these processes did not change the mean response properties of single neurons in the AC of mothers compared to virgins. Surprisingly, however, the level of correlation between pairs of neurons was doubled in mothers, and higher-order correlations were much more dominant. These results reveal a novel purely network-level functional plasticity mechanism in AC.
Chapter 1: Methodological Issues in Two Photon Imaging

Introduction

Two photon calcium imaging (2PCI) allows monitoring the simultaneous activity of tens to hundreds of precisely localized and genetically identified cells in vivo, and thus allows studying new aspects of neural network organization and dynamics. However, compared to electrophysiology, 2PCI is a new technique, and the possibilities it offers along with its limitations require further testing. In this Chapter I describe experiments and analyses I have performed in order to test this technique and its limitations.

A major concern when using 2PCI for measuring physiology is that the fluorescence signal reports electrical activity only indirectly. The relations between the fluorescence signal and spikes depends on various experimental parameters and thus inferring the electrical activity from the fluorescence signal is a non-trivial task. Many studies do not require detecting individual spike-triggered events, for example when comparing the mean responses of a cell to different stimuli (Ohki et al., 2005). In these cases, averaging the raw fluorescence signal may be a sufficient proxy for neural activity. However, if detecting single events is required, it is necessary to establish the link between spikes and the fluorescence signal. In Chapter 2 I describe simultaneous electrophysiological and calcium imaging experiments which I performed to this end. I found that in our experimental system, fluorescence transients report spikes with high fidelity.

Following a spike, the fluorescence of the neuron typically rises linearly for ~100 ms, and then decays exponentially for ~1 second. Theoretically, if the increase in fluorescence a neuron exhibits was completely fixed, it would be possible to perfectly deconvolve the fluorescence signal to spikes. However, in mammals in vivo the fluorescence trace includes a substantial amount of noise, limiting
deconvolution accuracy and temporal resolution. Moreover, the fluorescence level saturates quickly, such that the fluorescence transients of multiple spikes add up sub-linearly, making the timing of each more difficult to detect. Characterizing the typical time resolution an imaging setup provides would allow asking scientific questions which are plausible to pursue with this technique.

An additional constraint with 2PCI is the sampling rate. The sampling rate is mainly determined by the time it takes for the laser beam to scan the tissue. To date, most two-photon systems use galvanometric mirrors to steer the laser beam, which allow sampling a 512*512 pixel image at a rate of ~1-5 Hz. Since this rate is too slow to measure spiking activity, a common alternative is to use line-scan rather than full-frame acquisition. Using this method, the experimenter draws a line that defines the path of the laser beam, so that only fluorescence from pixels along the line is acquired. By drawing the line through all neurons in the field of view, acquisition rates of 100-200Hz are achieved. However, using this method, only a small area of each neuron is sampled, raising the concern that the fluorescence of the cell is not reliably represented.

Recently, other methods for increasing image acquisition rates have been developed, one of which is using acousto-optical deflectors (AODs) to steer the laser beam, instead of galvanometric mirrors. In an AOD, the deflection is caused by a crystal whose optical properties are set by acoustic energy. The angle of deflection is determined by the sound frequency. This method allows faster acquisition rates, typically around 10-40 full frames per second. However, AOD causes temporal dispersion and broadening of radiation pulses. In addition, the angle of deflection by AODs is wavelength dependent, introducing chromatic aberration (Lechleiter et al., 2002). In this chapter I compared the signal from galvanometric line scans and AOD full frames to describe the advantages and limitations of each method.

In electrophysiological extracellular recordings, multiple neurons are often recorded with varying signal qualities due to varying distances of the cells from the electrode and different procedures are applied to identify signals with sufficient quality. In 2PCI, different technical reasons necessitate
similar data filtering. Putative neurons in the fluorescence images are usually identified manually or automatically based on shape and high fluorescence relative to background. However, a fluorescent neuron could yield poor or no fluorescence transients. One reason for this is that although the resolution of a two-photon imaging system at the x-y (focal) plane is typically sub-micrometer, in the x-z plane (vertical) the resolution is inferior. This means that the signal coming from an optical section is actually an average of some volume, elongated along the vertical axis. If this volume includes, for example, neuropil which has not been loaded with calcium indicator, the resulting signal is expected to be degraded. A converse problem could arise if the neuropil carries a strong fluorescence signal. In such a case, the signal from neurons in the field of view could be contaminated by the neuropil signal. Thus, the resolution of the imaging system, the signal carried by the neuropil, and the degree of optical degradation/contamination should be assessed per experimental system. Furthermore, an evaluation of fluorescence traces should be performed to identify and remove traces with poor signal quality.

Lastly, although two-photon technology allows imaging considerably deeper than with one-photon microscopy, it is still limited. While electrophysiological recordings can be made from any location in the brain, two photon imaging is roughly constrained to depths of up to 500 µm from the brain surface. However, the practical maximal depth depends on many factors such as the dye, and the optical clarity of the volume between the objective and the neurons. Moreover, a given depth corresponds to different cortical layers in different animals, ages and sizes. Since cortical processing is layer-dependent (Brumberg et al., 1999; Martinez et al., 2005; Grossberg, 2007), it is desirable to (1) maximize the range of imaging depth by optimizing optical conditions, and (2) identify the cortical layers of the imaged neurons.
Overall, 2PCI offers studying novel aspects of neural network activity. However, inferring reliable physiological activity from the fluorescence signal requires careful experimentation and analyses. In this chapter, I will discuss in turn:

1. Loading cortical neural populations with calcium indicators
2. The fluorescence signal of neurons, non-neuronal cells and neuropil
3. Assessing the quality of fluorescence signals
4. Comparing the signal from different scanning modes
5. The resolution of our imaging system
6. Effect of the position of the focal plane relative to the soma on imaging quality
7. Signal deterioration over time
Results

Loading cortical neural populations with calcium indicators

2PCI relies on successful loading of neurons with a calcium indicator, followed by monitoring of their fluorescence over time with the two-photon microscope. Using the bolus loading technique (Stosiek et al., 2003), I injected the calcium indicator Fluo-4 AM mixed with Sulforhodamine 101 (SR101) extracellularly at depths of 300-400 µm below the brain surface. Fluo-4 stained neurons, astrocytes and neuropil in a spherical volume with a diameter of ~150 µm (Fig. 1a, green). SR101 selectively stained astrocytes and diffused more readily throughout A1 (Fig. 1a, red). Loading was optimal ~40 min post injection, at which time hundreds of neurons could be detected around the injection site. I calculated the distribution of depths of imaged neurons across a large dataset of experiments (n=895 neurons from N=11 mice) and found that the majority of imaged neurons are 300-450 µm below the brain surface (Fig. 1b). I next performed a set of experiments to identify the cortical layers from which the neurons I image are sampled. I targeted the same region in which I routinely

![Figure 1 Laminar distribution of the neurons in our dataset is largely confined to L2/3. (a) Side-projection of a reconstructed z-stack performed during a calcium imaging experiment. Red cells are SR101-stained astrocytes, green cells are Fluo-4 loaded neurons. Dotted lines illustrate the injection pipette penetration path. Scale bar: 20 µm (b) Distribution of depths below the cortical surface for all neurons in the dataset. Distance in the graph is aligned to the scale in 'a' and 'c'. (c) Photomicrograph of the cortical layers in A1 (green - SMI32 antibody), co-labeled with electroporated neurons (red rhodamine dextran). Electroporation was targeted into the same location of the Fluo-4 injections in the calcium imaging experiments. Scale bar: 100 µm]
performed calcium imaging, and performed electroporation of rhodamine dextran, followed by immunolabeling using the primary antibody SMI32 and the secondary antibody CY5-conjugated goat anti-mouse (N=4 mice). Images of the cortical slices were then acquired using a fluorescent microscope (Fig. 1c). Staining using the SMI32 antibody revealed the laminar structure of the cortex in the location of the injection site (Fig. 1c, green labeling). Somata of electroporated cells expressed strong red labeling. The depth below the brain surface of these cells is consistent with the typical depth of neurons in the calcium imaging experiments (compare a,b and c). With regard to the cortical layers evident by the SMI32 staining, electroporated cells are mostly confined to cortical layers 2/3. I thus infer that the vast majority of our calcium imaging data is from cortical layers 2/3.

**The fluorescence signal of neurons, non-neuronal cells and neuropil**

Following successful loading of calcium indicator and constructing a cranial window, I monitored activity of local neuronal networks by imaging the fluorescence of groups of individual neurons. Neurons in each optical plane were imaged simultaneously, and multiple focal planes were imaged consecutively. To maximize data acquisition speed, I used line scans through neuronal somata (Fig. 2a, left panel). I compared the optical signal coming from neurons, astrocytes and neuropil. Neurons exhibited typical calcium transients with a fast rise time (~100 ms) and a slow exponential decay (500–1,000 ms) (Fig. 2b-d). Astrocytes showed slow fluctuations of fluorescence on the order of seconds and the neuropil showed no obvious signal. To assess the temporal resolution of the fluorescence transients, I aligned all transients of a representative neuron according to their peak (Fig. 2c,d). Although transients were aligned at their peak, they differed by up to ~50 milliseconds in their transient onset (Fig. 2d, green bar).
Assessing the quality of fluorescence signals

As others and I have shown, the fluorescence transients observed in neurons result from spiking activity (see Chapter 2). During an action potential there is a large increase in the intracellular calcium concentration, which binds to the calcium-sensitive dye, resulting in an increase in fluorescence.

Thus, the sensitivity of a fluorescence trace for reporting spiking activity can be generally defined as the ratio of the amplitude of typical transients divided by the amplitude of the ongoing fluorescence fluctuations. The higher this signal-to-noise ratio is, the more reliably it reports spikes.

However, the quality of the signal coming from each neuron depends on many factors, and thus differs between neurons (compare, for example neuron n1 and n4 from Figure 2). It would thus be desirable to identify the neurons with inferior signal and remove them from the data set. In Chapter

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**Figure 2** The fluorescence signal of neurons, non-neuronal cells and neuropil (a) Micrograph of a group of neurons and astrocytes within the imaging volume, divided into red and green channels (right and middle panels), and merged (left panel). In left panel, line marks the path of the laser, circles mark areas whose fluorescence was monitored in b. Red channel shows SR101 staining (astrocytes), and green channel shows Fluo-4 staining (neurons and astrocytes). Scale bar: 10 μm. (b) A part of an imaging session showing ΔF/F traces from the corresponding neurons, astrocytes and neuropil areas marked in left panel in 'a'. Scale bar: 2 seconds, 40% ΔF/F. 'n' - neurons, 'a' - astrocytes, 'p' - neuropil. (c) All fluorescence transients (90 transients) of neuron n1 from 'a' and 'b' (notice 'b' shows only a part of the traces). Transients are overlaid and aligned according to the transient peak. Red trace shows the mean. (d) Same data as in 'c', but zoomed in in time. Two example traces are highlighted in red. Notice time 0±0 here denotes transient peak. Although the two example traces peak at the same time, their transient onset differs by ~50 ms (green bar).
2 I introduce one measure of quality based on simultaneous imaging-electrophysiology experiments. Here I present a more general measure which is less affected by imaging parameters.

For traces which do not show transients (for example, from out-of-focus neurons, see below), the distribution of fluorescence values is roughly Gaussian. Traces which show spike-evoked transients in addition to the Gaussian background noise would have a skewed distribution with a right tail. Thus, a natural measure for signal quality is skewness.

For univariate data $y_1 ... y_N$,

$$\text{skewness} = \frac{\sum_{i=1}^{N} (y_i - \bar{y})^3}{S^3}$$

Where $\bar{y}$ is the mean and $S$ is the standard deviation.

Figure 3a shows three examples of traces, their value distributions and skewness values. The traces are Gaussian noise (top), and two fluorescence traces from imaged neurons (middle and bottom). Of the three traces, only the fluorescence trace with the transients has a distribution with a right tail, and its skewness value is indeed much higher than that of the two other traces. These examples suggest that indeed, skewness quantifies the quality of fluorescence traces.

However, skewness should be used with care. Firstly, slow drifts in the trace (which are not rare) have a strong impact on skewness. Traces should thus be high-pass filtered to remove such drifts. Secondly, skewness is not tailored to the shape of spike-evoked transients, so if any non-physiological source of peaks exists in the trace (such as movement artifacts), these would contribute to skewness. Lastly, skewness is affected by firing rate in a non-monotonic way. The non-monotonic dependence of skewness on firing rate is illustrated using synthetic data in Figure 3b. Introducing an increasing number of large events to synthetic normally-sampled data results in increasing levels of skewness until some point (top three panels). From that point on, introducing additional events reduces skewness (bottom 4 panels). Thus, normalization of the skewness value
according to firing rate should be considered when analyzing neurons with highly differing firing rates.

![Figure 3 Assessing the quality of fluorescence signals using skewness](image)

**Comparing the signal from different scanning modes**

To obtain fluorescence signals from cells of interest with sufficient sampling rates, I used line-scan imaging rather than full-frame acquisition (line-scanning was generously first made available to us by Yoav Rubin, lab of Dr. Jackie Schiller, Technion, Israel). By drawing the line through all neurons in the field of view, I imaged their activity at rates of 100-200 Hz, which are sufficient for acquiring the fast transients. Alternatively, scanning using acousto-optical deflectors (AODs), allows imaging full frames at up to ~50 Hz. However, AODs introduce larger amount of noise into the fluorescence signal. Thus, full frame imaging using AODs allows acquisition of fluorescence from all pixels of all cells in a focal plane, but optical noise is expected to be high. Conversely, using line-scanning with galvanometric mirrors samples only a minority of pixels from each neuron, but noise is expected to be lower. I next compared the fluorescence signals obtained using each method.
To obtain the fluorescence level of a neuron in a single time point, I average the fluorescence of all pixels of the neuron. Using our typical imaging parameters (magnification and image size), I sample 30-50 pixels from each neuron using linescans and ~1000 pixels with full-frame AOD scanning. The increased number of pixels gathered from each neuron with AODs is expected to have a positive impact on the signal quality. However, this depends to a large extent on the amount of noise in the system. If each pixel reports the calcium concentration in the pixel area with little noise, then the average of only few pixels would reliably report the fluorescence of the neuron. Pixels in this case are expected to be highly correlated because the calcium signals in different areas of the cell are highly correlated. In contrast, if the signal from each pixel includes a large amount of noise, pixels would be less correlated and averaging many pixels would improve signal quality, provided (as expected) that the noise is independent between pixels. Thus, in our case correlation between pixels is expected to be inversely related to the amount of noise.

![Figure 4](image_url) Comparing the signal from different scanning modes (a) Example fluorescence trace from one neuron, imaged using line-scanning (top), and the fluorescence of the individual pixels which were averaged to obtain it (bottom). (b) Cross-correlation matrix of the pixels in bottom panel in 'a'. (c) Distribution of correlation values (same data as in lower left part of correlation matrix in 'b'). (d) Correlation as a function of distance between pixels. (e-g) Same analyses as 'a'-d', but for a neuron imaged using AOD scanning. (h) The correlation matrix in 'i', enlarged along one part of the diagonal.
Figure 4a shows a fluorescence signal from one neuron acquired using galvanometric line-scanning (upper panel), and the fluorescence of the 30 individual pixels which were averaged to obtain it (bottom panel). The obvious correlation between pixels appears in the cross-correlation matrix in Figure 4b. The mean of the cross-correlation values was 0.11 (Fig. 4c) and there was a decrease in correlation with increasing distance between pixels (Fig. 4d). Figure 4 e-i shows the same analyses for the fluorescence of a neuron imaged using AOD scanning. Here 1200 pixels were averaged to obtain the fluorescence trace. The cross-correlation matrix and distribution of correlation values seem to show values tightly distributed around 0 (mean=0.0067, Fig. 4f-g). However, the mean value is significantly larger than 0 (t-test, df=748475, p=0), and a closer look around the diagonal of the cross-correlation matrix shows significant correlations between closely-located pixels. Directly adjacent pixels are relatively highly positively correlated, pixels a bit further away are negatively correlated, and at distances larger than ~7 pixels, the correlation is essentially 0 (Fig. 4h-i). While positive correlations between nearby pixels are expected, negative correlations are less so and possibly result from refractoriness of the photon multiplier tube. It thus seems that a good S/N ratio with AOD scanning is obtained by averaging a large number of pixels (~1200) with low levels of mean correlation (or high levels of noise), while a similar signal from linescan imaging results from averaging only a few dozen pixels, which are ~16 times more correlated.
To compare the signals obtained with the two techniques directly, I consecutively imaged the same neurons with both techniques. Figure 5 (left panels) shows examples from two experiments of fluorescence traces obtained from the same neurons using the two methods. The fluorescence trace of each neuron obtained with galvanometric line-scanning appears in blue, and above it the fluorescence obtained with AOD scanning in black. For line scans, 30-55 pixels were averaged for each neuron, and for AOD scans 1000-1300 pixels per neuron. The right panels show the skewness values of the fluorescence traces. These data show that signals obtained from the same neurons using the different techniques do not systematically differ in quality.

A simple statistical rule is that as the noise level increases, the more samples you need to average to get rid of it. Is this indeed the essential difference between linescans and AOD data? To test this, I modeled two populations of pixels, which differ only in their mean correlation levels and compared their resulting signal. First, an arbitrary reference signal was constructed by smoothing a short segment of data (Fig. 6a). Then, 50 pixels were modeled by adding to this segment independent Gaussian noise to a level where the mean correlation between them was 0.11 (Fig. 6b). An additional 1200 pixels were
modeled by adding larger amounts of Gaussian noise, so that the mean correlation between them was ~0.006 (Fig. 6c). These two populations thus represent simple statistical models of linescan and AOD data, respectively. Figure 6d shows the resulting signals after averaging the pixels for each model. The 50 pixels with high correlation yielded a very similar signal to the 1200 pixels with low correlation. To quantify this, I calculated for each model, the mean squared distance of the mean trace from the reference signal, as a function of the number of pixels. In Figure 6e, I plotted the \((\text{mean squared distance})^{-1}\) of the resulting trace from the reference signal, for each of the models. The graph indeed shows that the trace quality when using 50 pixels from the high-correlation model is very close to that obtained when using 1200 low-correlation pixels. Moreover, both lines are roughly linear, meaning that for any signal quality, the ratio of the number of pixels needed is about 24 low-correlated pixels to 1 high-correlated pixel.

This simulation shows that a very simplistic model in which the only difference between two populations of pixels are their sizes and their mean correlation values, which were assigned to be the observed values of AOD/Linescan data, results in signals which are very similar in quality. Since this is also what was observed experimentally, it suggests that indeed the essential tradeoff with AOD/Linescan data is using many noisy pixels (low correlation), compared to using few pixels with less noise (higher correlation), respectively. Practically, for an experimentalist, these results don’t offer an obvious advantage for either alternative because typically the signal is averaged across pixels. However, one implication is that if the excessive optical noise introduced in the AOD optical path is diminished, it would make it a preferred method. Efforts in this direction are in progress (Lechleiter et al., 2002). Since AOD data takes up roughly 20 times more disk space than linescan data, and since the sampling rate with linescan is higher, I chose to use galvanometric line-scans for all experiments. It is important to note, however, that the comparison here was limited to a small range of imaging parameters, and that changing some parameters which were not tested here might further improve imaging quality of either technique.
Chapter 1 - Results

The resolution of our imaging system

Drawing a line for line-scanning is done on an image of a single optical section, and line width is 1 pixel. However, a single pixel in the image will report the fluorescence from a volume around it, whose size is determined by the resolution of the imaging system. To assess the spatial resolution of our imaging system, I calculated its point spread function (PSF). To this end, I imaged tiny latex spheres with a mean diameter of 0.175 µm. I immobilized the beads on a slide, and took z-stacks from below a random bead to just above it. To calculate the resolution at the x-y plane I chose a focal plane crossing a bead at its center (Fig. 7a, top bead). I calculated the fluorescence profile along a line crossing the center of the bead (Fig. 7b), and found that the full-width at half-maximum (FWHM) is 0.9±0.08 µm. To calculate the resolution along the x-z (vertical) axis, I calculated the
profile created by a single bead along the vertical axis (Fig. 7c‐d). I found that the FWHM is 6.1±0.7 µm.

Thus, the true resolution at the x‐y plane is sufficient for imaging structures as small as dendritic spines (~1 µm). However, the z resolution is much poorer, and is such that imaging a cell body (~12 µm in diameter) at its periphery is expected to include fluorescence from outside the cell as well. It is important to note that the resolution level depends on numerous parameters, such as the numerical aperture of the objective and the medium between the objective and the imaged tissue.

Thus, calibration as performed here should be performed per imaging system.

**Effect of the position of the focal plane relative to the soma on imaging quality**

Given the z resolution I found, it is expected that the signal from a neuron which is not crossed by the focal plane at the center of its soma would be contaminated by the neuropil signal. Since the neuropil typically does not show any signal in our experiments (Fig. 2), this would result in a decrease in S/N of the transients. *In vivo*, a single optical section is expected to cross some neurons at the center of their somata and others through their periphery. I thus tested the effect of gradually changing the focal plane relative to the soma of a neuron. Indeed, I found that as the focal plane moves away from the center of the soma, the fluorescence signal is degraded (Fig. 8). This result implies that out of all imaged neurons in a single focal plane, many would not yield a good signal. Indeed, in the studies in Chapters 2 and 3 I identify and discard ~50% of the data, partly due to this reason.
Signal deterioration over time

For the reasons described above (and others), neurons yielding high-quality fluorescence signals often constitute a minority of the putative neurons in the imaging field. I next asked how stable is the fluorescence signal from these neurons? The fluorescence signal could deteriorate over time due to photo-bleaching (i.e., dilution of effective fluorophore due to the laser excitation), overheating of the cells by the laser causing deterioration in their viability, and other reasons. I thus tested how long the effective continuous imaging session is from the same cells.

Figure 9a shows an example of fluorescence traces of simultaneously imaged neurons from one experiment. The cells initially exhibit typical spike-evoked fluorescence transients with good S/N. However, after 100 seconds of imaging, the rate and amplitude of these transients seem to decrease. The signal continues to deteriorate, and by the end of the 5-minute imaging session no transients are visible in any of the cells. To quantify this, I calculated the skewness of the fluorescence traces over 1-minute long windows. Fig 9b shows that the skewness of the fluorescence signal decreases over time. Fig. 9c-d shows an example from a different experiment where deterioration seems to be slower, and Fig. 9e-f shows fluorescence signals from a third experiment where almost no deterioration is evident over more than 8 minutes of imaging. Thus, signal deterioration rate differs between experimental conditions. Although I did
not systematically examine the different factors affecting deterioration rate, I noticed that deterioration occurred most rapidly when using high intensity laser power, long dwell-time per pixel durations, high optical zoom, at shallow imaging depths. To quantify deterioration across the population, I calculated skewness over time as described above for a large imaging dataset (n=85 neurons from 5 experiments). Notably, this dataset only included imaging sessions of up to ~7 minutes. Over this period of time, I found that on average there is a small but significant deterioration of signal (ANOVA, df=662, p=0.02). Performing the analysis for the first 5 minutes of data yielded no significant deterioration (ANOVA, df=514, p>0.08). I conclude that in our imaging system, experimental protocols should be designed to last no more than 5-8 minutes, and in any case a post-hoc analysis should be performed to identify deteriorating signals. Notably, all the data

![Graph](image_url)

Figure 9 Deterioration of the fluorescence signal (a) Fluorescence traces of 5 imaged neurons, showing quick and strong deterioration (b) Skewness of 1-min bins of the traces in ‘a’. (c-d) and (e-f) Same analyses as ‘a’-‘b’, from different experiments, showing slower and weaker deterioration. (g) Skewness as a function of time over 1-min bins, across the dataset. Skewness shows a significant decrease over time (ANOVA, P=0.02).
analyzed here was obtained from the two-photon microscope used for experiments in Chapter 3.
The experiments described in Chapter 2 were performed on a different microscope with a weaker laser, where deterioration was less evident.
Discussion

In this chapter I described a set of experiments and analyses I performed to study the nature of the fluorescence signal obtained using in vivo two-photon calcium imaging in our experimental system. When designed, performed and analyzed meticulously, this technique offers substantial advantages for studying the organization and physiology of neural networks in comparison with other available techniques. However, overlooking the details and limitations of the technique may result in large biases in the data, or the inability to answer the scientific question. In this section I will discuss the critical factors for obtaining successful calcium imaging experiments.

Recent advances, such as the use of genetically encoded calcium indicators, marking of genetically-identified neurons, and imaging of behaving animals open a new era in the field of 2PCI. However, here I limit the discussion to the kind of experiments I have performed, namely imaging of anesthetized animals using synthetic dyes while distinguishing only between neurons and astrocytes.

Experiment design

2PCI is currently probably the best technique for studying the organization of neural populations, often termed micro-architecture, in vivo. In my experiments, I imaged up to 200 precisely localized and well-isolated neurons from each animal, which is impossible to do with any other technique. Organization of sensory maps has been studied extensively for all modalities at a resolution level of hundreds of micrometers (Chklovskii and Koulakov, 2004). However, mounting evidence suggests that many features of cortical computation occur much more locally (Song et al., 2005; Yoshimura et al., 2005; Ohiorhenuan et al., 2010). Thus, organization of local populations is likely to yield new insights about cortical processing.

2PCI also allows studying questions of population physiology, such as how correlated are the responses of multiple neurons. For measuring physiology, however, a major consideration regarding
2PCI is that it yields a noisy signal. Some noise can be substantially reduced using proper experimental and analytical procedures (see below), but some of the noise is inherent to the technique. Although efforts are made to extract the most precise and reliable information from the fluorescence signal, the data from all studies in mammals so far include substantial levels of noise. At a first level, extracting spikes from the fluorescence signal is limited by the S/N of the transients. Currently, some studies report single-spike detection, but even then it comes with some level of error. In Chapter 2 I describe a set of experiments to test the correlation between spikes and fluorescence transients. Moreover, when a spike-evoked transient is correctly identified, the exact number of spikes can usually not be determined. This means that when using 2PCI, some spikes are lost while some events are erroneously detected as spikes. At a second level, I showed that due to different sources of noise, the timing of correctly identified spike-evoked transients is only correct at a resolution of tens of milliseconds. Thus, 2PCI should not be used when single-spike detection and precise timing are central to the scientific question. Rather, this technique is well suited for studies where a statistical measure of spikes is sufficient. This is important to note, because there is an unresolved controversy regarding the nature of the neural code, and specifically what effect do single spikes and their timing have on cortical computation (Kumar et al., 2010).

The strength of 2PCI is thus in studying the population level, while the signal from individual cells is noisy. Thus, it is arguable whether the technique should be used simply for high-throughput data acquisition of single-neuron activity when interactions between neurons are absent or outside the scope of the experiment. Juxtacellular recordings, for example, are technically simpler to perform, and yield a far more reliable trace of the spikes. Since repeated penetrations of juxtacellular recordings can yield dozens of neurons per animal, while calcium imaging experiments can yield one or two orders of magnitude more neurons, the choice between the two becomes a question of quantity versus quality.

In electrophysiological recordings, it is possible to continuously record for periods of hours and even days (Karlsson and Frank, 2009). This allows presenting the animal with long protocols, allowing to
densely probe the response properties of the neurons. In our experimental setup, continuous imaging is limited to <8 minutes. This should be a major consideration when choosing the recording technique. For example, presenting auditory stimuli which densely probe the receptive field of the neurons (50 frequencies, 5 attenuation levels, 30 repeats, 100ms duration and 900ms ISI) would exceed the time available for imaging. Again, this limitation implies that comparisons between the responses of neurons (to fewer stimuli) are better suited to study with 2PCI than detailed probing of single-cell activity.

Animal preparation

Figure 2 shows signals from neurons and astrocytes at ~350 μm below the brain surface, while Figure 1b shows the typical depths of stained neurons. However, in many experiments no neurons are visible at all. From my experience, the two most critical factors for obtaining high-quality signal with calcium imaging is the optical clarity of the imaged brain region, and the dye injection. With two photon imaging, only a small region at the focal spot is excited, and thus only few photons are emitted, but they are all 'informative'. Thus, high signal quality in two photon imaging relies on detecting a large portion of emitted photons. Small optical obstacles, such as the dura mater or minor bleeding, which have no effect on electrophysiological recordings, result in a deteriorated fluorescence signal. Thus, when performing the craniotomy, the last stage of bone removal should be done with blunt forceps when the skull surrounding the to-be removed area is thinned but still intact, avoiding any contact between a sharp edge and the brain. Dura removal should be done with fine but straight-shaped tip forceps (for example, Dumont #5 forceps, cat# 11295-10, Fine Science Tools). Removal should be done under maximal magnification of the binocular and using much light. The brain should be just moist to avoid clinging of the dura to the pia, but still not covered with fluid such that visual inspection is hampered. Injection of the dye should be done under visual guidance of the two-photon microscope to monitor clogging of the injection tip by the dye, which is not
uncommon. Injection pulses should be long (30-60S) and at minimal pressure which still ejects the dye from the pipette. Short, high-pressure injections often result in hyper-correlated fluorescence signal across the image, presumably from either heavy optical contamination due to dye over-loading and/or from epileptic-like synchronous activity due to injured neural tissue. In this case, plotting the fluorescence of all neurons as in Figure 2 would result in near-identical traces.

**Imaging**

A given well-stained brain region can yield highly different signal qualities by using different imaging parameters. Common commercial software for two-photon microscopes allows controlling numerous imaging parameters which influence signal quality, but I will mention only a few. In this chapter I showed that using AOD scanning of full-frame images yields data of similar quality to galvanometric line-scans. I thus performed my experiments using the latter alternative, and will only discuss parameters relevant for this scanning method.

Studying physiology requires sufficiently high acquisition rates. Acquisition rate of line-scanning is determined by the dwell-time per pixel and the length of the line. This seemingly poses a tradeoff: the more pixels are acquired and the longer the dwell time, the less noisy the data is expected to be, but at a cost of slower acquisition rates. However, an examination of the relevant parameters and knowing the timescales of the calcium signal, point to a clear preferred alternative. If I choose images of 512*512 pixels, a typical line crossing all neurons in the image will be 2000-4000 pixels long. Dwell times which do not cause immediate photobleaching are 2-4 micro-seconds per pixel. Thus, even if I take a line of 5000 pixels and select the maximal dwell time, I reach an acquisition rate of 50 Hz. Since calcium transients are on the order of 500-1000 ms, this rate is sufficiently high, and many studies use frame rates of 10-20 Hz. Thus, line length is practically not a limiting factor, and all cells in the image should be sampled by the line. Moreover, sampling the cells more densely by
crossing the somata with wriggling lines might improve the signal while maintaining a sufficient acquisition rate.

Another important parameter is what optical zoom to use. Increasing the zoom yields more pixels per cell, and thus improves resolution, but decreases the field of view. Again, theoretically this tradeoff seems balanced but practically the preferred alternative is quite clear. Using the 40X objective with no optical zoom results in a field of view of ~250*250 µm. This field of view often includes stained neurons surrounded by an unstained region, as the diameter of the area containing loaded neurons is ~150 µm. Thus, zooming in until only stained neurons are visible is recommended. Zooming in further improves the signal from individual cells only slightly, while stained cells are left un-imaged, which undermines the key aspect of these recordings. The proposed rule of thumb is thus to zoom in until the stained neurons take up the entire field of view.

**Analysis**

Raw linescan data comes as tiff images, where each row is one scan along the line. Thus, when the line crosses a bright cell soma, a bright column appears in the image. The first stage of analysis is to mark regions along the line (or image) which correspond to cells. This stage may have a large impact on the results, because cells can be missed or conversely, non-informative cells can be marked (such as out-of focus cells as in Figure 8) and yield low-quality data. This stage may be done manually or automatically, but assuming neither alternative would yield perfect performance, the key question in this stage is which kind of errors the experimenter should prefer: missing cells, or marking low-quality cells. My policy has been the latter. The main reason for this is that both bright and dim columns can yield high or low quality data, and therefore a data filtering stage must be performed based on the quality of the signal. My strategy has thus been to be liberal in the marking stage, i.e., mark all areas whose fluorescence is higher than the background and have a reasonable size for a cell, and identify and exclude low-quality data later based on further analysis.
This policy relies on an effective heuristic/algorithim to assess fluorescence signal quality, that would allow low-quality traces to be discarded. As a preliminary stage, slow DC shifts (usually slow decreases in fluorescence) throughout the imaging session should be corrected using high-pass filtering. In this chapter I suggested skewness as a measure for signal quality, and in Chapter 2 I present an algorithm based on simultaneous imaging-electrophysiology experiments, which is partly used in Chapter 3 as well. This stage is similar to analysis of extracellular electrophysiological recordings, where spike-separation quality is assessed and insufficiently-separated neurons are discarded from further analysis.

Although efforts should be made to minimize photobleaching, signal deterioration throughout a continuous imaging session cannot be avoided completely. Thus, an important stage is to identify deteriorating signals and exclude them from the dataset. In my data, simultaneously imaged neurons in a single focal plane usually deteriorated at a similar rate, presumably because deterioration is a result of excessive laser power (which is similar across the cells). Deterioration can be identified by applying the signal quality assessment described above to consecutive segments of ~1 min from the data.

Lastly, since the fluorescence signal reports spikes indirectly, different studies have analyzed it in two different ways. In some studies where calcium transients have a sufficient S/N to identify events evoked by one or few spikes, events were first identified in the fluorescence traces, and further analyses were performed on the events data similarly to analysis of spike data. Alternatively, other studies do not quantize the data into spikes, but analyze the raw fluorescence signal, based on the correlation between the number of spikes and the amplitude and integral of the fluorescence signal. In this case, the size of the responses is quantified by the integral or amplitude of the trace. Although both alternatives should in principle report the same neural activity, they could yield different results, and are suitable for different kinds of data and questions. First, in many calcium imaging experiments, fluorescence transients are not 500-1000ms long and reporting one or few spikes, but rather are many seconds long, presumably corresponding to spike trains of that duration.
In these cases reconstructing spikes from the fluorescence trace is impossible, and thus the fluorescence signal is analyzed as a measure for neural activity. However, other studies (Kerr et al., 2005; Kerr et al., 2007; Sato et al., 2007; Greenberg et al., 2008; Rothschild et al., 2010) report single-spike detection in the fluorescence trace. In these cases, which analysis method should be preferred?

Converting the fluorescence trace to events allows to directly determine if a neuron fired in a specific trial, thus response probabilities can be calculated, while this is not possible with the analog signal. Furthermore, identifying spike-evoked events is usually done based on the amplitude and shape of characteristic transients. This is an important feature when fluorescence traces show non-spike related increases in fluorescence. For example, optical contamination can cause small but consistent increases in fluorescence from the soma of one neuron whenever a neighboring neuron undergoes a real spike-evoked transient. In this case, the event-identification algorithm would assist in filtering out non-spike related increases. The major drawback in converting the fluorescence trace to events is the errors involved in this procedure. Typical reported error rates are around 5% false positive (wrongly identified events) and 10% false negative (missed spikes). Moreover, as mentioned, identifying individual events is only possible when firing rates are low. The error rate estimates are usually based on recordings of spontaneous activity, while stimuli usually increase firing rate. Since individual events in a train are harder to detect, responses may be underestimated. These errors are not expected to strongly affect results such as single-cell response profiles, but would be more considerable for analyses such as pairwise correlations.

Thus, the preferred alternative depends on the specific question and data characteristics. In Chapter 2, simultaneous electrophysiological recordings were performed and an algorithm was written to identify spike-evoked transients. The majority of this study was devoted to the micro-architecture and thus to mean response properties of individual neurons. Thus, in this study, fluorescence transients were converted to discrete events. The next study, presented in Chapter 3, was performed on a different microscope, using different imaging parameters, limiting the possibility to
apply the same transient-identification algorithm. Moreover, experimental procedures were
optimized such that no optical contamination was evident. Lastly, that study concentrated on
pairwise correlations and did not require determining if a neuron fired in a specific trial. For all these
reasons, in Chapter 3 I chose to analyze the original raw fluorescence trace.
Chapter 2: Functional Organization and Population

Dynamics in the Mouse Primary Auditory Cortex
Functional organization and population dynamics in the mouse primary auditory cortex

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Cortical processing of auditory stimuli involves large populations of neurons with distinct individual response profiles. However, the functional organization and dynamics of local populations in the auditory cortex have remained largely unknown. Using in vivo two-photon calcium imaging, we examined the response profiles and network dynamics of layer 2/3 neurons in the primary auditory cortex (A1) of mice in response to pure tones. We found that local populations in A1 were highly heterogeneous in the large-scale tonotopic organization. Despite the spatial heterogeneity, the tendency of neurons to respond together (measured as noise correlation) was high on average. This functional organization and high levels of noise correlations are consistent with the existence of partially overlapping cortical subnetworks. Our findings may account for apparent discrepancies between ordered large-scale organization and local heterogeneity.

RESULTS

In vivo two-photon calcium imaging in A1

To characterize the functional architecture and dynamics of local networks in A1, we performed in vivo two-photon calcium imaging in anesthetized, freely breathing mice (Fig. 1). We loaded cells in the auditory cortex with a mixture of Fluo-4 a.m. and SR101 using the multicell bolus loading technique14. Fluo-4 stained neurons, astrocytes and neuropil in a spherical volume with a diameter of ~250 μm. SR101 selectively stained astrocytes and diffused more readily throughout A1. Loading was optimal ~40 min post injection, at which time hundreds of neurons could be detected at depths of up to 450 μm, corresponding to cortical layers 2/3 (Fig. 1a, Supplementary Movie 1 and Supplementary Fig. 1). To maximize the data acquisition speed, we used line scans through neuronal somata (Fig. 1a,b). We imaged sequentially from up to ten different depths in each mouse, which enabled us to image the activity of hundreds of cells in a single injection site. To verify that the imaging was performed in A1, we labeled neurons in the same stereotactic coordinates using electroporation of dextran-rhodamine. Consistent with A1-thalamic connectivity15, rhodamine-labeled terminals and retrogradely stained cell bodies were always visible in the ventral division of the medial geniculate body (n = 6 mice; Fig. 1e).

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Received 7 October 2009; accepted 4 December 2009; published online 31 January 2010; doi:10.1038/nn.2484
Because in vivo two-photon calcium imaging has not been used previously in the auditory cortex, we performed several control experiments to evaluate our optical measurements and relate them to known cellular and electrophysiological properties of neurons in A1. We first compared the optical signal coming from neurons, astrocytes and neuropil. Neurons exhibited typical calcium transients with a fast rise time (50–90 ms) and a slow exponential decay (500–1,000 ms) (Fig. 1c,d). Astrocytes showed slow fluctuations of fluorescence on the order of seconds and the neuropil showed no obvious signal (Supplementary Fig. 2). To further analyze the origin of the optical signal, we calculated the pixel-by-pixel correlation along the scanned line. Signals from neighboring pixels inside neuronal somata were substantially correlated, whereas there was little or no correlation between the signals from somata and their neighboring neuropil (Supplementary Fig. 3), confirming that our signal comes from the neurons and not from the neuropil.

We next studied the relationship between the calcium transients and spiking activity. We performed simultaneous calcium imaging and electrophysiological loose-patch recordings from labeled neurons in vivo using two-photon targeted patch16 (n = 10 neurons from 7 mice; Fig. 2a). Calcium transients reliably followed spikes with high consistency (Fig. 2b). Single spikes induced robust calcium transients (14 ± 4.8% ΔF/F). Although the exact number of spikes could not be accurately predicted from the calcium transients, double and triple spike bursts induced significantly higher ΔF/F values as compared with single spikes (23 ± 8% and 29 ± 11.5%, respectively, one-way ANOVA, F(2,76) = 26.6, P = 1.8 × 10−5; Fig. 2b,c). Moreover, slow, nontransient increases in fluorescence were not associated with spikes. These data prompted us to develop an algorithm that identifies spike-triggered calcium

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**Figure 1** In vivo two-photon calcium imaging from dozens of neurons simultaneously in A1. (a) In vivo two-photon micrograph of a single optical plane in A1 after bolus loading of Fluo-4 a.m. (green) and SR101 (red). This optical plane is 381 µm below the pia. Scale bar represents 10 µm. (b) A drawing of the path of the line scan that was chosen to image 27 cells from a. (c) Relative changes in fluorescence (ΔF/F) of the 27 neurons shown in a and b during presentation of a stimuli series (not shown). Scale bars represent 1 s and 50% ΔF/F. (d) A single calcium transient enlarged from c (red dotted box). Scale bars represents 250 ms and 10% ΔF/F. (e) Left, a fluorescent micrograph of a coronal slice following electroporation of dextran-rhodamine into A1 (green arrow). Middle, micrograph of a coronal slice of the medial geniculate body (MGB) from the same mouse. Labeled axons project to the ventral MGB (yellow arrow). Right, high-resolution micrograph of axonal projections in the ventral MGB shown in the middle panel. Scale bar represents 1,200 µm (left), 600 µm (middle) and 20 µm (right).

**Figure 2** Identification of spike-induced calcium transients. (a) In vivo two-photon micrograph of a patch pipette loaded with Alexa 594 attached to a Fluo-4–loaded neuron. Scale bar represents 10 µm. (b) Simultaneous imaging and loose-patch traces of spontaneous activity in vivo (upper and middle traces, respectively). Bottom ticks indicate the events identified by the algorithm as being spike evoked. Scale bars represent 1 s and 20% ΔF/F. (c) A plot of transient amplitude as a function of the number of evoking spikes (n = 120). Means are marked in red (paired t test, ** P < 0.01, ns indicates not significant, P > 0.05). (d) A single calcium transient labeled with the parameters used by the algorithm: transient amplitude (vertical line) and transient area (dashed area). (e) Top, distributions of transient amplitude and transient area. Scale bars represent 20% ΔF/F and 10 arbitrary units. Bottom, examples of raw calcium traces taken from the distribution above. Scale bars represent 30% ΔF/F and 2 s. Distributions and traces are shown for an astrocyte, neuropil, nonactive neuron and an active neuron. Red circles denote the events identified as being spike triggered by the algorithm. Black dots in the distribution correspond to black dots in the traces. (f) A representative example of a calcium signal (top) with its corresponding weighted events (bottom). Scale bars represent 20% ΔF/F and 600 ms.
transients from the optical signal (Fig. 2d,e). These transients were then converted to `weighted events’, whose amplitudes are equal to the calcium transients’ amplitudes. Thus, the time of an event expresses the beginning of a spiking event and the weight of an event is approximately proportional to the number of spikes that triggered it (Fig. 2f).

For each local peak in a trace, we computed two values: the peak amplitude and the ΔF/Φ area of the transient (Fig. 2d). Plotting these values for traces with no typical spike-related transients resulted in a roughly two-dimensional normal distribution (Fig. 2e). Neuronal traces containing calcium transients had a noticeable ‘tail’ in addition to the two-dimensional cloud. This tail corresponded with high precision to the high peaks of the calcium transients (Fig. 2e). The algorithm used the shape and density of the two-dimensional cloud to identify the transients in the tail, and these transients were classified as being triggered by spikes. We tested this analysis on optical traces in which we simultaneously recorded the spiking activity of the neurons. For optically well-separated neurons, 95% of the identified events were spike-triggered (that is, 5% of the events were false positives) and 91% of the spikes were successfully identified. This performance is comparable to that of recent imaging reports in other cortical areas.12,13

In vivo, it is expected that a single optical section will cross some neurons at the center of their somata and through the periphery of others. Because the optical signal coming from the periphery of the soma was degraded compared with the signal from the center of the soma, some neurons were expected to yield weak or no transients (Fig. 1 and Supplementary Fig. 4). To evaluate the quality of the signal coming from each neuron, we computed a separation score of the transients in each trace (Online Methods). Traces that had robust and clear ΔF/Φ transients resulted in high separation scores and vice versa. The higher the separation score, the better the performance of the algorithm in detecting spike-evoked transients (data not shown). We defined a separation threshold and all traces that did not pass it were excluded from the database. In total, we imaged 1,627 neurons in 11 animals, up to 197 putative neurons per mouse, and up to 32 neurons simultaneously. Of these neurons, 895 of 1,627 (55%) passed the separation threshold and were analyzed in detail using weighted, spike-triggered events. For clarity, we classified all neurons according to the quality of their traces and their response properties (Supplementary Fig. 5).
Local organization in A1 is heterogeneous and sparse

To study tonal response profiles, we presented 760 pure tones at each focal plane (19 frequencies from 2–45 kHz, 5 attenuation levels, 8 repeats). We derived average responses of each neuron to the different stimuli and displayed them as frequency response areas (FRAs; Fig. 3a–c). Calcium responses to different stimuli were generally heterogeneous between different neurons and between different trials, although many neurons showed a consistent selectivity to some stimuli (Fig. 3c,d).

A recent study in marmosets proposed that heterogeneity in FRA shapes might be an important component of sound processing in awake animals. Thus, we asked whether our dataset, from anesthetized mice, also reveals heterogeneity in FRA shapes. We classified all responsive neurons with nonpatchy FRAs (comprising 267 of 895 of all imaged neurons) as either V (decreasing frequency selectivity with increasing intensity), I (narrow, level-tolerant tuning), O (nonmonotonic) or sound intensity–tuned FRAs (Supplementary Fig. 6). Of 267 neurons, 54% (145 of 267) had V-shaped, 11% (30 of 267) had I-shaped, 27% (71 of 267) had O-shaped and 8% (21 of 267) had intensity-tuned FRAs. Although these numbers differ from those of awake marmosets, especially in the proportion of O-shaped FRAs (27% versus 64%), this distribution is highly heterogeneous compared to previous extracellular electrophysiological studies in rodents (but see ref. 6), suggesting that our method was not biased toward neurons with specific response patterns.

We next studied the spatial organization of neurons with respect to their FRAs. We plotted anatomical maps in which we positioned each FRA at the location of the neuron from which it was derived (Fig. 4a). These FRA maps revealed a variable array of different FRA shapes even in a single imaging window (~250 × 250 μm). Neighboring neurons could have very similar (Fig. 4b) or very different FRAs (Fig. 4c).

We next examined the organization of tone-responsive and unresponsive neurons. Many neurons did not show any tone-driven responses (25%, 226 of 895) or had significant tone-driven responses (P < 0.05), but did not show significant stimulus selectivity (P > 0.05, 33%, 293 of 895) (Fig. 4a,d). In 10 of 11 experiments, the spatial distribution of unresponsive neurons was not significantly different (P > 0.05) from a random distribution (see Online Methods), suggesting that unresponsive neurons do not cluster together, but are instead scattered in the local circuitry. Moreover, the precise location of a neuron in the circuit does not seem to impose strong constraints on its tone-driven response profile.

To what extent is tonotopy expressed at the level of local circuits? To answer this question, we analyzed neurons with a clear best frequency (n = 241 neurons). As seen previously, the best frequency distribution across the population was not uniform, peaking between 16–32 kHz (Fig. 5a). By plotting the relative position of all these neurons in each mouse, we found that there was no clear local tonotopy in 10 of 11 mice, as demonstrated by the lack of correlation between the position of a neuron along the rostro-caudal axis and its best frequency (Fig. 5b). In one experiment, we imaged neurons from two injection sites, thus covering a larger area. In this case, we observed a clear and significant tonotopic axis (r = 0.76, P < 10⁻⁴; Fig. 5c). It therefore seems that tonotopy exists on large scales (>~250 μm), but breaks down at finer scales.

To quantify local heterogeneity in best frequencies across the data, we plotted the difference in best frequency as a function of the distance between neurons, data grouped from two different experiments. Left and center panels show the positions of the neurons, color coded according to their best frequency in a side view and a top view, respectively. Right, plots of the neurons best frequencies and their relative distances along the rostro-caudal axis (CR). LM, lateral-medial. (c) Data are presented as in b, but for a mouse with two injection sites, covering a larger cortical surface. (d) Difference in best frequency as a function of distance between pairs of neurons, data grouped from all experiments (n = 3,783 pairs). Distance is measured in the two-dimensional rostro-caudal, lateral-medial plane.
As not all frequencies are equally represented in A1 (ref. 8; Fig. 5a), even smooth tonotopy would predict uneven shifts in best frequencies along the cortical surface. The largest shifts in tonotopy that have been reported in large-scale mapping experiments are ~0.5–1 octaves for a distance of 100 µm. Our data, showing substantially larger variability in best frequencies over such distances (up to four octaves per 100 µm and even in 50 µm), argues against smooth tonotopy and in favor of functional heterogeneity in local circuits in A1.

The best frequency of a neuron, although being an important determinant of its sensory responses, does not reflect other response properties such as intensity selectivity. To analyze functional organization on the basis of a more complete representation of the receptive fields, we calculated the signal correlation between pairs of FRAs. Signal correlation measures the degree of similarity between the average responses of pairs of neurons to all stimuli. A maximal signal correlation value of +1 implies perfectly correlated response profiles, whereas a minimum of −1 implies complementary response profiles. We calculated the signal correlation by employing a modified version of signal correlation, which removes the contribution of noise correlations (see Online Methods). As expected from the weak tonotopy, the mean signal correlation between all pairs of simultaneously measured neurons was low (0.082 ± 0.15, 3,926 pairs). Nevertheless, the rather low signal correlation values were significantly higher than those computed between shuffled FRAs (paired t test, P < 10−10; Fig. 6a). The mean signal correlation values between neurons recorded nonsimultaneously from different optical planes were similarly low (0.068 ± 0.15, n = 30,218 pairs).

**Figure 6** Signal correlations between neurons in local networks are low on average and are variable and decrease with distance. (a) Distribution histogram of the signal correlation values between all pairs in the dataset of simultaneously imaged neurons (gray bars) and for shuffled FRAs (black line) (n = 3,926 pairs). (b) Schematic presentation of signal correlation values between a representative group of neurons from one mouse in one optical plane. Each FRA is drawn at the location of the neuron from which it was derived. Color of lines between each pair of FRAs codes the signal correlation value. Scale bar represents 20 µm. SC, signal correlation. (c) Signal correlation values as a function of distance between neurons from a single experiment (blue dots and line, n = 835 pairs). Red line is the best linear fit to the data from all the pooled data (n = 3,926 pairs, n = 11 mice). (d) Data are presented as in c but after randomly shuffling each FRA.

Although the heterogeneous FRA maps suggest a lack of a strict relationship between FRA similarity and distance (Figs. 4 and 6b), there was a significant (although small) decrease in signal correlation with distance (r = −0.14, P < 10−10; Fig. 6c,d). Specifically, high signal correlation values were rare at larger distances, whereas both high and low signal correlation values were found at short distances. These data imply that although tonotopy breaks down at finer scales, there is nevertheless a tendency for neurons with highly similar FRAs to reside in proximity.

**Local dynamics in A1**

Although signal correlation quantifies the similarity between average response profiles of the neurons, the trial-by-trial tendency of neurons to fire together (or not) is not captured by this measure. We therefore calculated the noise correlation between all pairs of neurons that we imaged simultaneously. Noise correlation measures the tendency of neurons to respond together above and below their average response to the specific stimulus on individual trials. Some pairs responded independently across trials, resulting in noise correlation around 0 (Fig. 7a), whereas others had a strong tendency to respond...
in the same trials, resulting in high noise correlation (Fig. 7a). Over the entire population \( (n = 3,926 \text{ pairs}) \), the pair-wise noise correlation values had a mean \( \pm \text{s.d. of 0.183} \pm 0.111 \) (Fig. 7b). Notably, there was a small tail of higher values, indicating that a small number of pairs \( (106 \text{ of } 3,926, 2.7\%) \) had unusually high noise correlations \( (>0.42) \).

We next tested whether the neurons that we imaged simultaneously tended to be active together when not driven by stimuli. We performed the same noise correlation analysis, but in time windows immediately preceding each stimulus presentation. Noise correlations during on-going activity were lower and more variable than noise correlations during tone stimulation (mean \( \pm \text{s.d. of 0.133} \pm 0.136 \)). We then examined whether pairs of neurons that tended to respond together in response to tonal stimuli also tended to fire together spontaneously. We found a strong and significant correlation between the pair-wise noise correlation during tone-evoked and ongoing activity \( (r = 0.46, P < 10^{-10}; \text{Fig. 7c}) \).

Is the amount of noise correlation (during stimulation) between a pair of neurons related to the distance between the pair? To answer this, we plotted noise correlation values as a function of distance between neurons (Fig. 7d). Noise correlation decreased significantly with distance \( (r = -0.17, P < 10^{-10}) \), implying that nearby neurons have stronger tendencies to respond together than distant ones. However, at any distance, different pairs could have a wide range of noise correlation (Fig. 7d).

Finally, we tested whether neurons with similar FRAs also tend to respond in the same trials. This comparison revealed a significant positive correlation between the signal correlation and the noise correlation of pairs of neurons \( (r = 0.275, P < 10^{-10}; \text{Fig. 7e}) \). Despite this trend, there were still pairs with a high signal correlation that were not noise correlated and vice versa.

**DISCUSSION**

*In vivo* two-photon calcium imaging enabled us to characterize the functional architecture of neuronal populations in layer 2/3 of the mouse primary auditory cortex with high precision. Because neighboring cortical neurons \( (<100 \mu\text{m} \text{ apart}) \) have a high probability of sharing common input\(^{21,22} \) and being synaptically connected\(^{21-23} \), one might expect that local populations of neurons would be homogeneous in their response properties. However, our data revealed a highly heterogeneous local population in which neighboring neurons could have very similar or very different response properties.

Despite the local disorder, large-scale organizing principles do exist. Tonotopy and gradual decrease of signal correlation with distance were found when examining larger distances. It thus seems that local heterogeneity is embedded in larger-scale order in A1. Furthermore, imaging dozens of neurons simultaneously allowed us to unravel temporal interactions between thousands of neuronal pairs as measured by noise correlations. Despite the heterogeneous organization with regard to signal correlation, neurons tended to have similar noise correlation during on-going and tone-driven activities, suggesting that noise correlations reflect structure in the local network (see below).

**Calcium imaging of cortical circuits in A1**

Sensory responses in A1 have mostly been studied with electrophysiology\(^{9,19,24} \). Unlike electrophysiology, imaging allows monitoring of the activity of dozens of precisely localized neurons simultaneously\(^{10-13,25,26} \). Furthermore, imaging is an unbiased sampling technique, relatively non-invasive, and enables coverage of hundreds of neurons in a small cortical volume. The resulting large datasets not only increase efficient data collection for experimentation, but also enable thorough sampling of local neuronal populations.

Imaging, however, measures light rather than voltage, thus reporting voltage fluctuations only indirectly. It was therefore important to calibrate our optical signal with simultaneously recorded spikes (Fig. 2). Only 55% of our original neurons reliably reported spikes, at least partly as a result of variability in the position of the neurons relative to the focal plane (Supplementary Fig. 4).

We used line scans, in contrast with the full-frame acquisition used in some previous studies\(^{11-13} \). Although line scans sample fewer pixels per neuron, they provide substantially higher sampling rates as compared with full frames \( (250-300 \text{ Hz versus } 1-30 \text{ Hz, respectively}) \); such rates are essential for capturing the fast rising phase of the calcium transients, whose duration is fairly short \( (50-90 \text{ ms}) \) and cannot be adequately captured at sampling rates below 100 Hz. In addition, we used the indicator Fluo-4 instead of the more widely used OGB, as Fluo-4 gave rise to substantially less optical signals from the neuron\(^{13} \) (Supplementary Figs. 2 and 3).

We conducted our experiments under ketamine-medetomidine anesthesia. Ketamine was selected primarily because this anesthetic was used in a number of studies serving as important references for large-scale tonotopic organization in mouse A1 (refs. 8,27). In addition, ketamine-medetomidine anesthesia allows for future time-lapse experiments, which is one advantage of *in vivo* imaging\(^{28} \). Despite these advantages, anesthesia clearly has its drawbacks. Specifically, ketamine-medetomidine has been shown to increase tonal selectivity in the cat and increase response variability in rats, suggesting that tonotopy would actually appear to be more strict and regular under anesthesia\(^{29,30} \). Thus, the heterogeneity that we found might be even greater in the awake state. However, the precise effects of anesthesia on A1 neurons of mice are still not known and await evaluation, preferably on the same neurons before and during anesthesia\(^{29} \).

**Order and disorder in A1**

Overall, our data indicate that the organization of neurons responding to pure tones in A1 is sparse and heterogeneous, as manifested by a number of measures. First, tone-responsive and selective neurons comprised only 42% of all imaged neurons \( (376 \text{ of } 895) \), whereas V-shaped FRAs, which have been reported to dominate A1 (ref. 31), comprised only 16% of all imaged neurons \( (145 \text{ of } 895) \). These results indicate that tone-responsive neurons are sparsely scattered in the local population in A1. Unresponsive neurons \( (25\%) \) of all imaged neurons were intermingled with responsive neurons rather than being restricted to specific domains. Recently, electrophysiological experiments have shown that responsive and unresponsive neurons are located in nearby penetration sites \( (~50-100 \mu\text{m} \text{ apart}) \) and even along a single penetration site from different cortical layers\(^9 \). Our results support and augment these findings by showing that nonresponsive neurons are an integral part of L2/3 networks in A1 at single-cell resolution. These neurons might be involved in something other than simple pure-tone coding, such as processing of complex sound features\(^{22} \).

Second, heterogeneity was evident by the wide distribution of FRA shapes. FRA diversity, and specifically O-shaped FRAs, was recently proposed to be important for auditory object recognition in awake animals\(^{17} \). Although the proportions of the different FRA types that we found were different (which may be a result of anesthesia, species specificity or cortical layer specificity), the heterogeneity of FRA shapes that we found in small volumes supports the idea that neurons with different FRA shapes may interact to process complex sounds\(^{17} \). This study focused only on responses to pure tones, whereas the local organization with regard to complex sounds awaits future investigation.

Third, heterogeneity was manifested as a lack of local organization according to best frequency (Figs. 4 and 5). At first, these data seem...
difficult to reconcile with numerous studies showing smooth, large-scale tonotopic organization in A1 (for example, see A1 tonotopy in ferrets\textsuperscript{19}, mice\textsuperscript{8}, rats\textsuperscript{18} and monkeys\textsuperscript{39}). In fact, the precision of tonotopic organization in A1 has been a controversial issue for a few decades and remains unresolved to date\textsuperscript{34–37}.

Previous reports of smooth tonotopic maps may have resulted from techniques that average the responses over multiple neurons. Electrophysiological studies that reported precise tonotopy used electrode penetrations that were spaced by more than 200 μm and used multi-unit activity to determine frequency tuning\textsuperscript{8,18,36,38}. Because multi-unit recordings sample spikes from neurons located at distances of up to 100 μm\textsuperscript{19}, such studies may have observed the larger-scale tonotopy while missing the local diversity of single-neuron responses. Another possible explanation for the apparent discrepancy is a sampling bias of extracellular recordings toward highly active neurons\textsuperscript{40}, raising the concern that calcium dye loading might be biased as well, penetrating preferably into subtypes of neurons with unique response profiles. Although this concern has not been thoroughly ruled out, it seems unlikely because, in at least one study, GABAergic interneurons were loaded just as efficiently as neighboring pyramidal neurons using similar methodology\textsuperscript{41}. Other studies revealing tonotopy using imaging techniques such as intrinsic imaging\textsuperscript{27,42} or voltage-sensitive dyes\textsuperscript{43} averaged responses over many neurons and are prone to overlook the local heterogeneity that we found here.

Heterogeneity was also apparent when comparing complete FRAs of all responsive neurons. The average signal correlation between all pairs of neurons was low and variable, inconsistent with the view of a highly homogeneous primary sensory cortex. In addition, local populations included neurons with highly variable FRA shapes (Supplementary Fig. 6).

Despite the large heterogeneity, there seems to be some order in the disorder. Nearby neurons were, on average, more similar than distant neurons. Highly correlated neurons were scarce at distances >100 μm and tonotopy was observed at distances >250 μm. Thus, the apparently different views of A1 (that is, heterogeneity versus tonotopy) may be reconciled; although rough tonotopy exists at large scales, it breaks down at fine scales (below ~250 μm), as local heterogeneity is embedded in the large-scale order. This feature may in fact be ubiquitous throughout primary sensory cortices of rodents. Studies in the visual\textsuperscript{44} and somatosensory\textsuperscript{12,13} cortices have shown that although global architectural features exist, neighboring neurons could have very different response properties.

It is not known whether this functional organization holds true for other species. On the one hand, there may be differences in developmental constraints between small animals such as mice, in which the whole tonotopic axis is represented in ~1 mm of cortex, and larger animals in which the tonotopic axis occupies a substantially larger area. In support of this possibility, the local organization of visual cortex may be different in rats and cats\textsuperscript{10}. On the other hand, our data are consistent with the results of electrophysiological experiments that found high heterogeneity in neuronal response properties in A1 of both cats and rats\textsuperscript{6,52} and low signal correlation between nearby neurons\textsuperscript{45}. Consistently, there is evidence that tonotopy, at least in the cat, is an average characteristic\textsuperscript{24,35}.

We focused our experiments on the tonotopic organization and used pure tones. However, A1 presumably processes additional sound properties using mechanisms that are not engaged by pure tones. For example, neurons in A1 can respond in a sustained manner by their preferred stimuli, which are often more complex than pure tones\textsuperscript{46}. Therefore, other functional organization principles that are based on other sound features (for example, organization by periodicity\textsuperscript{47}) may well exist in A1. In this respect, imaging could serve as a powerful method for measuring response properties that go beyond pure tone coding.

Neural networks may have very different behaviors depending on the trial-by-trial correlation between neurons and may require different read-out mechanisms for the information encoded in the neural responses\textsuperscript{1}. We found that neuronal pairs had a mean noise correlation of 0.18. Notably, this value is similar to that reported in V5 of monkeys\textsuperscript{2,20}. Although a noise correlation of 0.18 for a given pair of neurons could be seen as rather weak, the effect on information storage of the network at these values could be marked\textsuperscript{20,48,49}. Generally, this level of correlations would substantially limit the advantage of pooling over large populations of neurons to increase the signal-to-noise ratio of the sensory responses. This would in turn support a model in which small subgroups of neurons respond to each stimulus presentation.

Local organization in A1

Our main finding consists of highly heterogeneous local populations with relatively large correlations between a minority of neighboring neurons. What type of local connectivity might give rise to these results?

The simplest connectivity model to consider would be that of a tonotopic input combined with locally random (noisy) connectivity. This model could partially explain our results, including the heterogeneous micro-architecture and the decrease of signal and noise correlations with distance. Our data, however, put additional constraints on this connectivity model. Specifically, at short distances, a minority of the neurons are coupled rather strongly, whereas such coupling is absent at longer distances. These findings are consistent with the random connectivity model provided that there is at least one component of the overall connectivity that is strong, sparse and decreases fast with distance. Such a model would result in the formation of small subnetworks of highly correlated neurons, partially overlapping in space.

A number of our findings support such a subnetworks model. Notably, a subnetworks model would account for the details of the dependence of signal correlation and noise correlation on distance. Specifically, at short distances we observed both very large and very small correlations, while at longer distances we observed only smaller correlations. In consequence, the scatter of correlations and distances had a wedge shape for both signal and noise correlations. In contrast, a smooth gradient model would presumably result in a constant variance with distance. In the subnetworks model, there are pairs belonging to both the same and to different subnetworks at short distances (resulting in high and low correlations, respectively, and overall in high variability), whereas only pairs from different subnetworks would be found at long distances. In support of this argument, we performed a set of computer simulations to compare our signal correlation versus distance data to different organizational layouts and found that it was most consistent with a subnetworks model (see Supplementary Discussion and Supplementary Fig. 7). In addition, the strong correlation between the signal correlation of pairs of neurons (generally attributed to common input), and the noise correlation between them (generally attributed to direct synaptic connections) supports a model of strongly coupled subnetworks that share common input. Such a model, with partially overlapping, strongly connected subnetworks that share common input, has already been suggested for L2/3 neurons in the visual cortex\textsuperscript{50}.

Finally, our data describing strong correlation between noise correlation during on-going activity and during auditory stimuli support the idea that distributed groups of interconnected neurons are coactivated during tone stimulation.

Although our data seems to best fit the overlapping subnetworks model, it is only one interpretation of our results. Different experimental procedures, including direct mappings of synaptic
connectivity in local cortical circuits, would be required to reveal the underlying organizational principles of the auditory cortex.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureneuroscience/.

Note: Supplementary information is available on the Nature Neuroscience website.

ACKNOWLEDGMENTS

We thank H. Sompolinsky and E. Zohary for critically commenting on early versions of this manuscript. We thank N. Taaseh and A. Yaron-Jakoubvitch for their kind technical assistance during the early stages of this project. We thank Y. Rubin and J. Schiller for the software module of the line scan. We thank J. Linden for her help on cortical recordings in mice. We thank all the members of the auditory cortex. Cereb. Cortex 15, 1637–1655 (2005).


ONLINE METHODS

Animal preparation. We used male and female NMRI mice (total of n = 28 mice, 8–12-weeks-old), anesthetized using ketamine (100 mg per kg of body weight) and medetomidine (0.83 mg per kg). Depth of anesthesia was assessed by monitoring the pinch withdrawal reflex. Dextrose-saline was injected subcutaneously to prevent dehydration. Body temperature was maintained at 36–38°C. The skull was exposed, cleaned and dried. A metal pin was glued to the skull and attached to a custom-made head holder allowing precise orientation of the head relative to the objective. The muscle overlaying the left auditory cortex was removed and a craniotomy (~3 × 3 mm) was performed. The dura was gently removed and the cortical surface was kept continuously moist. Following each experiment, animals were killed by overdose with sodium pentobarbital. All experiments were approved by the Hebrew University Animal Care and Use Committee.

Dye loading and two-photon imaging. The auditory cortex was loaded with Fluo-4–a.m. (FL4201, Invitrogen) usingmulticell bolus loading. Fluo-4–a.m. was dissolved in 20% Pluronic F-127 in DMSO (vol/vol, P-6687, Invitrogen) to a concentration of 10 mM and further diluted tenfold in external buffer containing 125 mM NaCl, 5 mM KCl, 10 mM glucose, 10 mM HEPES, 2 mM CaCl2, 2 mM MgSO4, and 0.1 mM sulforhodamine 101.

Injections were performed under visual guidance using two-photon excitation. The solution was slowly injected into the cortex (duration 1–3 min) using a quartz pipette into a diameter of ~250 µm per injection. In one experiment, two injections were made in a distance of a few hundred microns. The craniotomy was sealed with a thin layer of 2.5% agarose (wt/vol, type IIIa, Sigma-Aldrich), glass coverslipped and secured with dental cement. The mouse was placed under the microscope and the cranial window was oriented perpendicular to the microscope and the cranial window was oriented perpendicular to the objective lens. Imaging was carried out on an Ultima microscope (Prairie Technologies) with a 40× (0.8 NA) IR–Achromat water-immersion objective (Olympus). A femtosecond laser (Mai-Tai Spectra Physics) was used to excite Fluo-4 at 488 nm with a 40× (0.8 NA) IR–Achroplan water-immersion objective (Olympus).

Imaging was performed using two-photon excitation. The solution was slowly injected into the cortex (duration 1–3 min) using a quartz pipette into a diameter of ~250 µm per injection. In one experiment, two injections were made in a distance of a few hundred microns. The craniotomy was sealed with a thin layer of 2.5% agarose (wt/vol, type IIIa, Sigma-Aldrich), glass coverslipped and secured with dental cement. The mouse was placed under the microscope and the cranial window was oriented perpendicular to the objective lens.

Background noise (coming especially from the laser chiller and galvo scanners) was attenuated by filtering the signal using a programmable attenuator (ED1, PA5, Tucker Davis Technologies). The muscle overlying the left auditory cortex was removed and the dura was gently removed and the cortical surface was kept continuously moist. Following each experiment, animals were killed by overdose with sodium pentobarbital. All experiments were approved by the Hebrew University Animal Care and Use Committee.

Data analysis. Statistical tests are considered to be significant at the 0.05 level. All data is presented as mean ± s.d. Low-pass filtering of all ΔF/ F traces was performed using a finite impulse response filter designed by the Matlab function fir1. The duration of the impulse response was 50 ms and the cutoff frequency was the sampling frequency divided by 10. To keep the temporal features of the calcium signal in their correct locations, we used the function filtfilt to achieve zero-phase filtering, effectively corresponding to two passes of the low-pass filter.

To study the relationship between the calcium transients and spikes, we used the simultaneous imaging and electrophysiological experiments from which we developed an algorithm to automatically identify spike-evoked calcium transients as follows. For each local peak in a trace, we computed the transient peak amplitude, calculated as ΔF/F peak – ΔF/F peak – 100 ms, and the transient area, calculated as ΔF/F peak – 100 ms, and the transient area, calculated as

Loose-patch recordings from Fluo-4–loaded neurons. Fluo-4–loaded neurons were recorded in a loose-patch configuration (n = 10 neurons from 7 mice). Quartz pipettes (tip resistance 7–9 MΩ) contained 140 mM potassium glutonate, 4 mM NaCl, 0.5 mM CaCl2, 5 mM Mg-ATP, 5 mM EGTA, 10 mM HEPES and 0.05 mM Alexa 594 (pH = 7.2). Fluo-4–loaded neurons were targeted using two-photon targeted patch. Unlike the imaging-only experiments, the craniotomy was not sealed with glass, but only covered with agarose. Spikes were recorded using an intracellular amplifier in current clamp mode (Multiclamp 700B, Molecular Devices) and acquired at 10 kHz (Digidata 1440A, Molecular Devices). Calcium imaging was performed simultaneously as described above.

Auditory stimulation. Auditory stimuli were generated online using custom-written software (Matlab) through an electroacoustic loudspeaker driver and a programmable attenuator (ED1, PA5, Tucker Davis Technologies). The loudspeaker (ES1) was placed ~10 cm from the right ear of the mouse. Acoustic stimuli consisted of a series of randomly presented pure tones at 19 frequencies ranging from 2–45 kHz and at five to six attenuation levels ranging from 35–75 dB SPL. Each combination of frequency attenuation was presented eight times. Tone duration was 50 ms, including 5-ms ON and OFF linear ramps, with a 400-ms inter-stimulus interval.

Histology. We labeled cortico-thalamic projections (n = 6 mice) by electroporation of dextran-rhodamine into the same location as the imaging experiments, as described previously. To verify the cortical layer of imaging, we combined targeted electroporation with immunolabeling (n = 4 mice) using the primary antibody SMI-32 (ab28029, Abcam, 1:1,000) and CY5-conjugated goat antibody to mouse as a secondary antibody (1:400, 115-175-003, Jackson ImmunoResearch Laboratories). Images were acquired on a fluorescent microscope (Olympus IX70), processed off-line using ImageJ or Matlab and adjusted for brightness and contrast.

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the sum of all events in a 10–200-ms time window following stimulus onset. The best frequencies of the FRAs were determined manually, but were chosen blind to the specific experiment from which the FRA originated. If a best frequency could not be clearly determined, the neuron was classified as not having a clear best frequency. All FRAs in each imaging session were color-scaled to the maximal and minimal pixel values for all FRAs in the session. FRA shapes were classified manually according to the following criteria. FRAs showing decreasing frequency selectivity with increasing intensity were classified as being V shaped, constant frequency selectivity with increasing intensity was classified as being I shaped, responsiveness to a small range of intensities and frequencies was classified as being O shaped if the peak response was not to the highest intensity, and high selectivity to 1–2 intensity levels with no clear selectivity for frequency was classified as being intensity tuned.

To estimate whether a neuron was responsive to pure tones, we compared its responses during the 200 ms just preceding and just following stimulus onset using a paired t test (P < 0.05). This is a conservative test given that many stimuli did not elicit a response, but were nevertheless included in the test. Response selectivity of a neuron was tested using a one-way ANOVA on the 95 stimuli, with the single-trial responses as repetitions.

To test whether unresponsive neurons were spatially clustered, we calculated the sum of the distances between all unresponsive pairs. We then calculated distances between the same number of random neurons in the imaging volume and plotted this distribution for 1,000 runs. In 10 of 11 experiments, the distance between the unresponsive neurons was not in the lowest 5% of the distances provided that r ≠ r′. If r = r′, the expected value of the product would include a contribution resulting from noise correlations. In our case, the noise correlation was typically higher than the signal correlation, and it may contribute substantially to the estimates of the signal correlations. To avoid errors in the estimates of signal correlation by noise correlation contamination, we then removed these terms from the sum. Thus, we estimated the contribution of stimulus s to the covariance:

\[ \frac{1}{\# \text{s}} \sum_{s} (c_{i}(s) - <c_{i}>) \times (c_{j}(s) - <c_{j}>) \]

If we denote the last expression as corrected_product, then

\[ \text{cov}_{\text{corrected}}(c_{i}, c_{j}) = \frac{1}{\# \text{s}} \sum_{s} \text{corrected_product} \]

and the correlation coefficient becomes

\[ R_{\text{corrected}}(c_{i}, c_{j}) = \frac{\text{cov}_{\text{corrected}}(c_{i}, c_{j})}{\sqrt{\text{cov}_{\text{corrected}}(c_{i}, c_{i}) \times \text{cov}_{\text{corrected}}(c_{j}, c_{j})}} \]

The correlations between the fluctuations of the single-trial responses of pairs of neurons around their mean response were used as the pair-wise noise correlations. From each single-trial response of a neuron, we subtracted the mean response of the neuron to that stimulus. For each neuron, this resulted in a vector of fluctuations around the mean responses to the different stimuli. The noise correlation was the correlation coefficient between these vectors.
Chapter 3: Network Dynamics in Auditory Cortex of Mothers Following Parturition

Introduction

The transformation between incoming stimuli and their neural representations lies at the heart of sensory processing research. Classical studies in this field describe the neuronal responses to systematically varying stimuli, thereby characterizing neuronal receptive fields. For example, tonal receptive fields of single neurons in the auditory cortex have been often described as v-shaped in frequency-intensity space, i.e., a neuron is tuned to some best frequency (BF), but responds to an increasingly wide range of frequencies around the BF at increasing intensity levels. As receptive fields characterize how sensory information is processed, encoded, and mapped to guide perception and behavior, it may be expected that they would remain stable over time to form a reliable and consistent representation of the sensory environment. However, compelling evidence suggests that sensory cortical neurons can undergo large changes in their receptive fields. For example, one study showed that neurons in the primary auditory cortex of trained ferrets change their receptive fields during the performance of a tone-discrimination task (Fritz et al., 2003). Changes occurred within minutes, and sometimes lasted for many hours after the task was over.

If we extrapolate from these single-cell findings to the population, it is likely that cortical neural networks process sensory information in a context-dependent manner. In the study by Fritz and colleagues, more than 70% of A1 neurons changed their receptive field during the tone discrimination task, usually involving an increase in responsiveness to the target tone. Thus, the portion of the population responding to a specific tone increased during the task.
Population responsiveness plasticity and its relation to behavior were recently studied using nucleus basalis stimulation (Reed et al., 2011). In these experiments, stimulation induced rapid cortical map plasticity, and this plasticity was shown to enhance perceptual learning.

These studies indicate that the mean response properties of numerous individual neurons undergo large changes to facilitate changing behavioral demands. However, in these experiments a single artificial auditory stimulus was given critical importance using electrical stimulation, casting doubt on the ethological relevance of these results. It is possible that natural, unguided experience would be expressed differently in the brain. In addition, the net change a population of neurons undergoes and its relation to behavior cannot be inferred solely from single-cell responses. Since a given stimulus is coded in the brain by a complex activation pattern of large populations of interacting neurons, characterizing changes in network dynamics are likely to shed new light on sensory processing plasticity. Our main goal in this study was to examine how dynamics of neural networks change under naturally varying conditions of behavioral relevance and experience.

To this end, I examined neural activity before and after a dramatic change in the life of female mammals: giving birth and becoming a mother. Becoming a mother, especially for the first time, involves physiological, hormonal, behavioral, social and psychological changes. These changes, together with the experience with the pups during the first days after birth, define a unique "behavioral state" in mothers compared to virgin mice. Following birth the mother switches from taking care only of herself to taking care of her pups with a wide range of maternal behaviors (Smotherman et al., 1974). These behaviors rely on specific calls emitted by the pups, such as ultrasonic vocalizations (USVs) and low-frequency wriggling calls, each inducing a specific maternal behavior almost exclusively in mothers. Thus, the behavioral state of mothers after birth, and specifically the acuity of their auditory sensitivity, differs greatly from that of virgin mice.

What in the brain of mothers may underlie these differences? In one study (Liu et al., 2006), multiunit recordings were performed in the auditory cortex of mothers and virgin mice while
presenting pup call bouts. The authors found that neuronal responses were entrained to repeated stimuli extending up to the natural pup call repetition rate (5 Hz) in mothers, but not in virgins. In another study (Liu and Schreiner, 2007) applying a similar experimental setup, the authors found that the information neural responses convey about pup communication calls reaches a larger and earlier peak in mothers compared to virgins. However, what changes occur at the population level remains unknown.

To study changes in the activity of populations of neurons under different behavioral states, I performed in vivo two-photon calcium imaging in the primary auditory cortex of mother mice 4-5 days following birth, and compared it to that of age-matched virgin females. This method allowed us to examine changes ranging from the level of single neurons to the population. I found that while single-neuron response properties were very similar between mothers and virgins, population dynamics, measured as pairwise and higher-order correlations, varied considerably between the groups. Our results indicate that populations of neurons in the auditory cortex of adult mice undergo large changes in their network dynamics under different behavioral states. I describe the nature of these changes and discuss how they may affect network coding and behavior.
Chapter 3 - Methods

Methods

Animal preparation

We used female NMRI mice (n=12, 8-10 weeks old), anesthetized using Ketamine (100 mg/kg) and Medetomidine (0.83 mg/kg). Virgin mice are females that were never housed with males or pups after they had been weaned at PD21. Mothers are females 4-5 days following parturition, nursing a litter of at least five pups.

Depth of anesthesia was assessed by monitoring the pinch withdrawal reflex. Dextrose-Saline was injected s.c. to prevent dehydration. Body temperature was maintained at 36–37°C. The skull was exposed, cleaned and dried. A metal pin was glued to the skull and attached to a custom made head holder allowing precise orientation of the head relative to the objective. The muscle overlying the left AC was removed and a craniotomy (3 mm in diameter) was performed. The dura was gently removed and the cortical surface was kept continuously moist. Following each experiment, animals were sacrificed with sodium pentobarbital overdose. All experiments were approved by the Hebrew University Animal Care and Use Committee.

Dye loading and two-photon imaging

Dye loading was performed as previously described, targeting L2/3 neurons of primary auditory cortex (A1) (see Chapter 2). A1 was loaded with Fluo-4 AM (F14201; Invitrogen, Carlsbad, CA) using multicell bolus loading (Stosiek et al., 2003). Fluo-4 AM was dissolved in 20% Pluronic F-127 in DMSO (P-6867; Invitrogen) to a concentration of 10 mM and further diluted 10-fold in external buffer containing (in mM): 125 NaCl, 5 KCl, 10 Glucose, 10 Heps, 2 CaCl2, 2 MgSO4, and 0.1 Sulforhodamine 101. Injections were performed under visual guidance using two-photon excitation. The solution was slowly injected into the cortex (duration 30-180 seconds) using a quartz pipette. In some experiments, several injections were made within a distance of a few hundred micrometers.
The craniotomy was glass cover-slipped and secured with dental cement. The mouse was placed under the microscope, and the cranial window was oriented perpendicular to the objective lens.

Imaging was carried out on an Ultima microscope (Prairie Technologies, Middleton, WI) with a 40X (0.8 NA) or a 16X (0.8 NA) water immersion objective (Olympus). A femtosecond laser (Mai-Tai Spectra Physics, Mountain View, CA) was used to excite Fluo-4 at 820 nm. Line-scan images were acquired at 100–300 Hz, depending on the length of the line. Neurons were imaged at depths of 250–450 µm under the pia, corresponding to layers 2/3 of the cortex. Imaging was stable with no movements caused by heartbeat or respiration. Imaging started 30 minutes post injection and lasted for ~3 hours. Scan timings, stimuli delivery and electrophysiological recordings were acquired using a standard data acquisition board (Digidata 1440A, Molecular Devices, Sunnyvale, CA) on a separate PC.

**Auditory stimulation**

USVs were recorded with a 1/4 inch microphone, connected to a preamplifier and an amplifier (Bruel & Kjaer) from P4-P5 pups isolated from their mother, and placed in a custom built sound-shielded box. Vocalizations were sampled at 500 kHz using a Digidata 1322A (Molecular Devices, Sunnyvale, CA). USVs were identified offline, and three were chosen based on high SNR. Auditory stimuli were generated online using custom written software (Matlab), through an electrostatic loudspeaker driver and a programmable attenuator (ED1, PAS, Tucker Davis Technologies). The loudspeaker (ES1, TDT) was placed ~10 cm from the right ear of the mouse. Acoustic stimuli consisted of pure tones at 10, 20 and 60 KHz, a synthetic wriggling call composed of pure tones at 3.7, 7.4 and 11.8 KHz, broad band noise, and three recorded ultrasonic vocalizations, for a total of 8 stimuli. Each stimulus was presented 20 times per series, pseudo-randomly shuffled, at a single intensity level (70 dB SPL). Synthetic stimuli duration was 100 ms including 5 ms ON and OFF linear ramps, while USV duration was ~70 ms. Inter-stimulus interval was 700 ms.
**Data analysis**

Statistical tests are considered significant at the 0.05 level. All data is presented as mean ± standard deviation unless specified otherwise. All ΔF/F traces were smoothed using a finite impulse response low-pass filter. To keep the temporal features of the calcium signal in their correct locations, the function filtfilt was used to achieve zero-phase filtering.

Response strength was quantified as the integral of the fluorescence signal within a window of 250 ms following stimulus onset. Others and I have shown (see Chapter 2) that transient integral correlated with the number of evoking spikes. Thus, the integral statistically reports the number of spikes. In this study, I chose to refrain from converting the fluorescence signal to discrete events for a number of reasons. Firstly, errors inherent to event-identification procedures may strongly bias calculations of correlations, which are at the center of this study. Secondly, optical contamination between neurons and between neurons and neuropil, which is a central reason to identify only typically-shaped transients, is virtually non-existent in our data due to use of minimal amounts of Fluo-4 dye and slow and gradual injections. Lastly, in this study I was not interested in questions requiring binary indication of response, such as would be needed for computing response probabilities.

To determine if a neuron was responsive to a specific stimulus, I tested whether its fluorescence following stimulus presentations was significantly larger than the fluorescence preceding stimulus presentations. To this end, the integral of the fluorescence in windows of 250 ms following and preceding stimuli was used. A t-test (at a significance level of 0.05) was performed on the signal in the 20 post-stimulus windows against the signal in the corresponding 20 pre-stimulus windows. This allowed us to determine to which stimuli each neuron is responsive. A neuron was assigned as responsive if it was responsive to at least one of the 8 stimuli. While no correction was applied for multiple comparisons, the rate of false positives was expected to be on average 0.34 stimuli per
neuron; the observed average number of stimuli evoking a response was about twice as large as that (see Results), suggesting that false positives did not dominate the data.

The correlations between the fluctuations of the single-trial responses of pairs of neurons around their mean response were used as the measure of pairwise noise correlations. From each single-trial response of a neuron, I subtracted the mean response of the neuron to that stimulus. For each neuron, this resulted in a vector of fluctuations around the mean responses to the different stimuli. The noise correlation was the correlation coefficient between these vectors. To calculate stimulus-specific noise correlation, only trials of the specific stimulus were included. To calculate ongoing noise correlation, responses were defined as the integral of the fluorescence signal over a time window of 250 ms preceding stimulus presentation.
Results

To characterize the responses of single neurons and neuronal populations in A1, I performed *in vivo* two-photon calcium imaging in anesthetized, freely breathing mice while presenting both synthetic and natural sounds. I imaged two groups of mice: lactating mothers 4-5 days after parturition (n = 6 mice) and age-matched virgin females, serving as controls (n=6 mice). I loaded cells in the auditory cortex with the calcium indicator Fluo-4 AM using the multicell bolus loading technique (Stosiek et al., 2003). Fluo-4 stained cells within a sphere of ~150 μm in diameter around the injection site. I imaged neurons at depths of up to 400 μm below the surface of the brain, corresponding to cortical layers 2/3 (see Chapters 1 and 2). I imaged sequentially from multiple depths in each mouse, with an average of 9±4 neurons imaged simultaneously in each focal plane (Fig. 1). I measured relative changes in fluorescence of dozens of neurons in each mouse using line-scans, reaching sampling rates of 100-150 Hz. Under these conditions, neurons exhibited typical calcium transients with a fast rise time (~100 ms) and a slow exponential decay (500–1,000 ms) (Fig. 1c,f). I found no systematic
differences in the amount, quality, or timescales of optical signals from single neurons in lactating mothers as compared with those of naïve virgins. In this study, I analyzed the data based on the continuous fluorescence signal (see Chapter 1 and Methods). Responses were defined as the integral of the $\Delta F/F$ signal from 0-250 ms following stimulus onset.

**Single neuron response profiles following parturition**

To test how synthetic as well as natural sounds are coded in mice after parturition, I presented 8 different auditory stimuli. Specifically, I presented three different pure tones (10, 20 and 60 KHz), broad-band noise (BBN), a synthetic wriggling call (WC, composed of pure tones at 3.7, 7.4 and 11.8 KHz, (Stiebler et al., 1997)), and three ultrasonic vocalizations (USVs, recorded from 4-5 day old pups). For each focal plane, I presented each stimulus 20 times for a total of 160 stimuli presentations, in a pseudo-random sequence. Overall, in both mothers and virgins, some neurons showed clear stimulus selectivity (Fig. 1f, cell7/10KHz), while others were less selective (Fig. 1c, cell1). Responses to different stimuli ranged in consistency from 0-100% of the trials (see e.g. Fig. 1c, Cell5, 60K, WC and BBN). As I described in Chapter 2, nearby neurons could show similar or highly divergent response selectivity (Fig. 1c, cells 1&3 vs. 1&7; stars indicate significant responses, see Methods).

We defined a responsive neuron as one that responded significantly to at least one stimulus. Of all neurons in lactating mothers (n=418 from 6 mice) and virgin mice (n=283 from 5 mice), a mean±s.e.m of 37.7%±7.4% and 41.9%±9.6% of the neurons were responsive. There was no significant difference in the fraction of responsive neurons between the two groups (t-test, df=5, p=0.65, Fig. 2a). However, the average number of stimuli evoking a significant response was different between the groups. Neurons in mothers responded to significantly less stimuli than neurons in virgins (Number of stimuli; mothers=0.59±0.8, naïve=0.85±1.05, t-test, df=282, p<0.001). This was also true when considering responsive neurons only (Number of stimuli;
mothers=1.39±0.62, naïve=1.69±0.87, t-test, df=142, p=0.005). Although I used only 8 different stimuli, this result suggests that L2/3 neurons in mothers are more selective than neurons in virgins (Fig. 2b).

Do neurons in lactating mothers respond to a different set of stimuli as compared to neurons in A1 of virgins? To answer this question, I averaged responsiveness per stimulus for the different experimental groups. The mean response profile of mothers and virgin mice did not differ significantly (3-way ANOVA, No significant effect of state: F(1,111)=1.44, p=0.23; significant effect of stimulus: F(7,111)=6.93, p<0.0001, no interaction between state and stimuli: p=0.96; Fig. 2c). In both groups, neurons were mostly responsive to pure tones of 10 and 20 KHz, and to synthetic wriggling calls, while few neurons were responsive to USVs, BBN and the 60 KHz tones. The lack of responses to USVs and 60 KHz tones and high rate of responses to 10 and 20 KHz tones and wriggling calls is probably due to consistent imaging from central A1 rather than from the ultrasonic field (UF, Stiebler et al., 1997). The low rate of responses to BBN are probably a unique feature of layer 3 neurons (Oviedo et al., 2010), which is our target layer.

Pairwise noise correlation following parturition

The above analyses of single-neuron response properties reveal a high degree of similarity between mothers and virgin animals. I next tested if this similarity translates to the level of the neural network as well. One basic measure for
network dynamics is pairwise noise correlation (NC). NC measures the tendency of two neurons to respond above and below their means, or 'fluctuate', together. To calculate NC, I subtracted from the response of each single-trial the mean response of that neuron to the specific stimulus. This resulted in a 'fluctuation vector' for each neuron, describing how the response in each trial deviated from the mean response (160 values in total). To calculate NC between a pair of neurons, I cross-correlated the fluctuation vectors of the pair. Given that NC measures correlation in time, only pairs that were imaged simultaneously were analyzed. To calculate stimulus-specific NC, I followed the same procedure but included responses to a specific stimulus only (i.e. vectors carried 20 values rather than 160).

Figure 3a shows an example of such vectors for a pair of neurons imaged simultaneously, and showing a strong tendency to co-fluctuate. This tendency is depicted in Figure 3b, showing a scatter plot of the fluctuations of one cell from Figure 3a against the fluctuations of the other. The strong correlation is obvious, and NC in this case is high (NC=0.77). In contrast, Figures 3c-d shows the same analyses for a pair with low NC (NC=0.008).
Across our dataset, the average NC in virgin mice was 0.18±0.14 (mean±standard deviation, n=1103 pairs), consistent with our previous results (see Chapter 2). Surprisingly, NC in lactating mothers was almost twice as high on average (0.34±0.17, n=2148 pairs), and this difference was highly significant (two sample t-test, df=3249, p=0). These unusually high NC values in mothers were consistent across individual animals (Fig. 3e).

To calculate NC distributions, I included NC values from all pairs of simultaneously imaged neurons. An alternative way to look at the distribution of NC values in a population is to test the significance of each individual pairwise NC value. Vertical dashed lines in Figure 3e mark the significance cutoff (at 0.05) for the NC values in our dataset. All values that are outside the area delimited by these lines are statistically significant. Using this classification, I found that 55% of the neuronal pairs in virgins had NC values that significantly differed from 0, while in lactating mothers the rate was 85%. Furthermore, significant NC values were almost exclusively positive, highlighting the increased level of NC in lactating mothers.

The finding that NC values in mothers are much higher than in virgins prompts the question whether this difference originates from responses to a specific subset of stimuli. In the context of elucidating the role of the primary auditory cortex, an attractive possibility would be that complex or natural calls would be processed in a different dynamic mode than behaviorally meaningless pure tones. I therefore calculated stimulus-specific NC in mothers and virgins. For both mothers and virgins, NC varied significantly with stimulus identity (Fig. 4a, ANOVA, mothers: F(7,17176)=14.32, p=0, virgins: F(7,8816)=4.85, p<0.0001). However, the magnitude of variation was small: std/mean NC across stimuli was 0.063 for mothers, and 0.097 for virgins. Furthermore, natural calls did not induce different levels of NC compared to pure tones. NC was larger in mothers than virgins for all stimuli, and the differences between NC in lactating mothers and virgins were much larger than the variation.
in NC between stimuli within each group. These results indicate that the increased NC in mothers are not stimulus-specific.

The relatively small effect of stimulus identity on NC in both mothers and virgins raises the question of how much of the NC is due to stimulus-evoked activity to start with. To test this, I calculated NC for periods when no stimuli were presented (‘ongoing NC’, measured in the time windows preceding each stimulus). Ongoing NC were significantly larger than 0, and were higher in mothers than in virgins (Fig. 4a, dashed lines). However, ongoing NCs were lower than stimulus-evoked NC by ~50%, indicating that stimuli robustly enhanced the tendency of neurons to co-fluctuate. I next asked whether neurons that tended to co-fluctuate during ongoing activity also tended to do so during sound presentation, or are these tendencies independent? Consistent with our findings in Chapter 2, I found a strong correlation between the ongoing and stimulus-evoked NC (Fig. 4b-c). This correlation was significant in both virgins and mothers (corrcoef, mothers: r=0.68, p=0, virgins: r=0.46, p=0). Taken together, these data show that both ongoing NC and stimulus-evoked NC are increased in lactating mothers.

![Diagram](image)

**Figure 4** Noise correlation is stimulus driven but only weakly stimulus specific (a) Stimulus-specific NC in mothers (red) and virgins (blue). Horizontal dotted lines indicate mean NC values during ongoing activity (b) Scatter plot of stimulus-evoked NC as a function of ongoing NC per neuronal pair in virgins (c) Same as ‘b’ for mothers
High order correlations following parturition

The size of pairwise NC is an important descriptor of the activity of a network. Positive mean pairwise correlations, as I found in mothers and virgins, imply that pairs of neurons tend to co-fluctuate more than expected by independence. This naturally leads to the question of co-fluctuations of larger groups of neurons. I next examined population fluctuations in mothers and virgin mice.

To analyze high order noise correlations I grouped fluctuation vectors of all simultaneously imaged neurons into 'fluctuation matrices'. In each matrix, each row describes the fluctuations of one neuron to all 160 individual trials, ordered chronologically. Representative matrices from 3 virgin mice and 3 lactating mothers are shown in Figure 5a,b (top panels). Note that, as previously described, the mean response of the neuron to a specific sound is subtracted from all single-trial...
responses to this sound, eliminating any effect of sound preference. "Population fluctuations" are evident in the data from lactating mothers as brighter or darker vertical lines in the fluctuation matrices, indicating that in a given trial most or all of the neurons fluctuated up/down together. Qualitatively, population fluctuations are less pronounced in the matrices from virgin mice. Plotting the mean fluctuation of all neurons per trial (Fig. 5a,b middle panels) results in seemingly Gaussian fluctuations of the population in the virgin data, while the data from mothers shows large-amplitude transients and a right-skewed distribution of the average fluctuations (Fig. 5a,b bottom panels).

To compare the probability of observed network pattern activations to a model with independently responding neurons, I first quantized fluctuations to positive or negative (±1). For each imaging session of N neurons (usually N= 8-14), I then calculated for each of the $2^N$ possible activation patterns, what is its expected rate based on an independent model and compared it to the actual observed rate (see Methods for details). The top left panel in Figure 5c shows such an analysis for one set of neurons imaged in a virgin mouse (same data as top middle panel in 'a') and the top right panel shows an example from a mother (same data as top middle panel in 'b'). Each point indicates the expected rate of a specific activation pattern based on an independent model, as a function of its observed rate, color-coded according to the number of active neurons in the pattern. For example, the green dots in the upper right corner indicate single-cell activation patterns, and by construction their observed rates are identical to the expected rates. For patterns with two or more active neurons, virtually all activation patterns occur more often than expected by an independent model. These high order noise correlations are eliminated by shuffling responses of the neurons in time (Fig. 5c, bottom panels). While patterns occurring more often than expected by the independent model were evident in both mothers and virgins, the ratio of observed/expected was much higher in mothers. Many patterns of activation occur ~1000 times more often than expected in this example and across the data (Fig. 5d). These data suggest that common fluctuations of large neuronal populations are prevalent in AC of mothers.
Discussion

We used in vivo two photon calcium imaging (2PCI) of L2/3 neurons in the primary auditory cortex of lactating mothers and virgin mice to study how the difference in behavioral state is expressed in the activity of local neural populations. Our main finding is that while single-neuron response properties are similar between virgins and mothers, pairwise and higher-order correlations are considerably higher in mothers.

Possible biases

Although our findings highlight novel network-dynamic changes associated with behavioral state, a number of caveats should be mentioned. The first is that our experiments were performed on anesthetized animals, while the effect of behavioral state on neural activity would ideally be studied in awake animals. Anesthesia induced by Ketamine and Medetomidine has been shown to modify single-cell response properties such as stimulus selectivity (Zurita et al., 1994) and response variability (Kisley and Gerstein, 1999). However, while absolute values of responsiveness and NC are likely to differ to some extent in awake animals, our main findings stem from comparisons between mothers and virgins, and these results are thus less prone to be biased by anesthesia. In fact, behavioral-state-dependent differences are likely to be accentuated in awake animals. I thus hypothesize that the differences in NC between mothers and virgins would be even greater in awake animals.

Secondly, 2PCI measures physiological activity indirectly, introducing the risk of biases due to the nature of the calcium signal. Low temporal resolution and noise in the signal are inherent characteristics of the technique. However, as mentioned above, our main results show large differences between population dynamics in mothers and virgin mice, which were imaged using the same technique. Although an electrophysiological signal from the neurons would allow...
characterizing the differences in higher temporal resolution, the differences I found could not be attributed to the recording technique alone.

Lastly, it is important to note that both single-cell and population activity in the auditory cortex is laminar-dependent (Sakata and Harris, 2009; Oviedo et al., 2010). The neurons I imaged were in cortical layers 2/3. Dynamics in deeper, thalamo-recipient layers may show different characteristics.

**Previous studies of NC changes**

Many previous studies on modulation of NC have been performed on awake and trained monkeys performing a behavioral task, while recording electrophysiologically from different brain regions. These studies were thus unbiased by anesthesia and allowed monitoring changes associated with stimuli, attention, learning and behavior. For example, NC has usually been found to decrease during attention (Cohen and Maunsell, 2009; Mitchell et al., 2009), and decrease or increase during behavior (Ahissar et al., 1992; Vaadia et al., 1995; Cohen and Newsome, 2008). Findings regarding attention and behavior are difficult to relate to our results, but a number of studies examined stimulus-dependence of NC. For both mothers and virgins, I found that mean NC varied only weakly with stimulus, although responsiveness levels to some stimuli were more than 5 times higher than to other stimuli. These findings are consistent with a number of monkey studies showing that correlation between neurons changes little or not at all in response to different stimuli (Bair et al., 2001; Kohn and Smith, 2005; Gutnisky and Dragoi, 2008). These results suggest that functional connectivity within the network is relatively stable on short time scales and depends only weakly on the nature of the stimulus.

While studying anesthetized animals comes with obvious drawbacks, our experimental approach allowed us to study naturally occurring correlation changes on a timescale not studied previously, one that I believe most fits the definition of "behavioral state". Instead of momentary changes in correlation on the scale of seconds, I describe a large jump in mean NC, which is probably stable.
over days or longer, and is associated with a difference in the baseline state of the animal. I found that the state of recent motherhood itself, irrespective of specific stimuli or tasks, is correlated with a global increase in mean NC levels.

"Behavioral state" in mothers and virgins

What differs between mothers and virgins mice? Mothers and virgin mice were all females, age-matched, and raised in the same environment. The 'behavioral state' of mothers and virgins thus differed by two main factors: experience and physiological state. By the time of imaging, mothers had been introduced to a male mouse, mated, undergone pregnancy and birth, and then spent 4-5 days of maternal care with the pups. To test if this experience underlies increased NC levels, continuous recordings from the auditory cortex of mothers throughout the first days following parturition are required. However, learning to take care of the pups shares some attributes with learning to perform a behavioral task, which has been extensively studied. For example, a recent study implemented in vivo 2PCI in the motor cortex of behaving rats while they were learning a discrimination task (Komiyama et al., 2011). The authors found that correlation between pairs of neurons increased throughout learning, and suggested that correlated activity in specific ensembles of functionally related neurons is a signature of learning-related circuit plasticity. Although maternal skills are probably more instinctive for the mother than learning to perform an artificial discrimination task, it is likely that the increased level of correlations I observed is at least partly a result of a similar learning process.

In addition to experience, mothers 4-5 days following parturition differ from virgins in their physiological state. During pregnancy and following parturition, the neuro-endocrinological state of mothers undergoes large changes, which may have profound effects on sensory processing (Brunton and Russell, 2008). Levels of hormones such oxytocin, oestrogen, progesterone and prolactin are upregulated, and have been shown to facilitate maternal behaviors (Brunton and Russell, 2008).
In this study, I did not attempt to differentiate between the contributions of experience and physiological state on the observed differences between mothers and virgins. One way to do so could be to expose naïve virgins to similar experience with the pups as mothers and monitor their neural population activity. A recent electrophysiological study on olfactory-auditory interaction in the auditory cortex of mother mice did just that (Cohen et al., 2011). This study found that many but not all behavioral and neurophysiological phenomena that occur in mothers but not in virgins, also occur in virgins who had gained experience with pups. It is thus probable that a combination of internal physiological processes and experience modulate neural activity in the auditory cortex of mothers.

We next discuss what the nature of this change is and what could be its implications on sensory processing. I start by discussing changes in the most basic level of population activity: single-cell response properties.

**Single-neuron changes**

Our data shows that most properties of single neuron activity do not differ between mothers and virgins females. These findings are consistent with a previous study, which performed single and multi-unit electrophysiological recordings from mothers and virgin mice while presenting USVs (Liu and Schreiner, 2007). The authors found that the mean spike count of single neurons in response to USVs was similar in mothers and virgin mice, and that the auditory intensity threshold also did not differ. Only more subtle response properties, such as timing and information, differed between mothers and virgins. Using calcium imaging, our data is limited in its temporal resolution, and I could not examine these properties. However, I found that the main difference in single-neuron responses was that neurons in mothers were significantly more selective compared to virgins. In light of the large differences I found in pairwise correlations between virgins and mothers, I suggest that changes in the behavioral state of the animal are expressed primarily at the neural population level.
Small changes in single-cell response properties along with large changes in pairwise correlations have been previously reported to underlie changes in attention levels (Cohen and Maunsell, 2009; Mitchell et al., 2009). These studies found that attention-modulated changes in pairwise correlation improve the signal-to-noise ratio of pooled neural signals substantially more than attention-dependent increases in firing rate. In our study, I found that although single neuron responses did not seem to depend strongly on behavioral state, they did depend on the identity of the stimuli, as neurons were highly stimulus selective. Based on these findings, an appealing possibility would be that the hierarchical nature of behavioral meanings of the stimuli I used is matched by a hierarchy of coding strategies in cortical processing: while the physical nature of the stimulus is encoded very similarly by responses of single neurons in mothers and virgins, the behavioral significance of the stimuli is encoded by correlations at the population level. Whether this is a general feature of cortical processing requires further investigation.

Possible mechanisms of NC changes

What is the origin of noise correlations and what is the mechanism underlying their increase in mothers? Responses of cortical neurons to presentation of the same stimulus typically vary between trials. The computation a neuron performs on a given input is believed to be relatively stable, and thus fluctuations in responses are usually attributed to fluctuations in presynaptic input to the neuron. Thus, the tendency of two neurons to fluctuate together, i.e., their noise correlation, is usually attributed to common input or direct connectivity between the pair. Anatomical, physiological and modeling studies have estimated that a pair of nearby (<200 µm away) cortical neurons share between 5%-30% of their input (Hellwig et al., 1994; Shadlen and Newsome, 1998; Ecker et al., 2010), while the probability for a pair of nearby neurons to be directly synaptically connected has been reported to be ~10%-20% (Sjostrom et al., 2001; Song et al., 2005). A recent study has strongly reasserted the relation between NC and synaptic connectivity by calculating NC
between pairs of neurons \textit{in vivo} and then performing multiple whole-cell recordings from the same neurons \textit{in vitro} (Ko et al., 2011). The authors found a significant correlation between the NC of pairs of neurons and the probability of synaptic connectivity between them.

If NC indeed reports anatomical connectivity, what process underlies the increased level of NC I observed in mothers? While cortical circuits were once believed to lose their plastic ability after an initial critical window of development, there is now much evidence for experience-dependent synaptic plasticity in adulthood (For example, reviewed in Holtmaat and Svoboda, 2009; Fu and Zuo, 2011). Increased spine and synapse densities have been reported after rearing in enriched environments (Moser et al., 1994) and also after long-term sensory stimulation (Knott et al., 2002; Livneh et al., 2009) and deprivation (Zuo et al., 2005; Holtmaat et al., 2006; Keck et al., 2008). In the days following parturition, mother mice are exposed to novel sensory stimuli and undergo a learning process which are at least as striking as those used in these studies. Experience-dependent synaptic plasticity is thus very likely to occur in mothers at this stage. Interestingly, whereas NC has been shown to decrease on short timescales during attention and stimuli processing, our results of increase in NC are consistent with the increased synaptogenesis observed following experience on longer timescales.

\textbf{Computational implications}

While NC have been reported between pairs of cortical neurons in a large number of studies (Ahissar et al., 1992; Zohary et al., 1994; Vaadia et al., 1995; Bair et al., 2001; Kohn and Smith, 2005; Yoshimura et al., 2005; Cohen and Newsome, 2008; Cohen and Maunsell, 2009; Mitchell et al., 2009; Cafaro and Rieke, 2010; Ohiorhenuan et al., 2010; Rothschild et al., 2010; Komiyama et al., 2011), their potential influence on network computation is controversial. In an early study (Zohary et al., 1994), the researchers found a similar level of NC in area MT of the monkey as I report for virgin mice. Based on a simple model in which a downstream neuron codes a stimulus by averaging
responses of the pre-synaptic neurons, the authors have argued that these levels of NC have a detrimental effect on efficiency of the population. These results were extended and generalized in a later study (Sompolinsky et al., 2001), describing a model in which mean positive pairwise correlations decrease the Fisher information of a network relative to a model where noise is independent. However, recent studies reveal a more complex picture. One study (Gu et al., 2011) reported that perceptual learning reduces NC between pairs of neurons in macaque visual cortex, which, based on the studies mentioned above, might have been expected to improve coding efficiency. However, the authors found that the reduction in noise correlations leads to little change in population coding efficiency. Additional recent studies have shown that mean positive correlations do not necessarily hinder network computation and that in some cases mean positive NC improve coding (Abbott and Dayan, 1999; Averbeck et al., 2006; Shamir and Sompolinsky, 2006; Cafaro and Rieke, 2010). While the structure and assumptions of the models in all these studies differ in many ways, they point at the same general rule: if mean response properties of individual neurons in the population are homogeneous (as was the case in all early computational studies), correlation will decrease information capacity of the network. However, if mean response properties are heterogeneous, coding may tolerate and even be improved by means of positive correlations. For example, Shamir and Sompolinsky (2006) have demonstrated that introducing even a small level of heterogeneity prevents information saturation due to positive correlations in large neural populations.

In Chapter 2, I characterized the functional micro-architecture of the auditory cortex with the same experimental setup, but using a large array of stimuli, making it possible to derive neuronal receptive fields. I found that even for neighboring neurons, receptive fields were highly heterogeneous, to the extent that they resembled a random organization more than a homogeneous one. The definition and assumptions of the model described by Shamir and Sompolinsky (2006) make a quantitative comparison with our results difficult. However, in their study, even small deviations from homogeneity prevented information saturation while the primary result of our study and others
(Ohki et al., 2005; Kerr et al., 2007; Sato et al., 2007; Greenberg et al., 2008; Hromadka et al., 2008; Komiyama et al., 2011) is that populations of neurons in rodent cortical L2/3 are highly heterogeneous. It is thus possible that information coded by populations of neurons in mothers is not lowered, and might even increase, relative to virgins.

In any case, it is important to note that the quality measure in most studies mentioned above, the Fisher information, is not the exclusive measure with which to assess the coding efficiency of a system. For example, it is possible that the auditory cortex in mothers optimizes transmission efficiency to downstream regions rather than stimulus discrimination. In support of this alternative, it has been shown that pairwise correlations are expected to increase the variability of the population output spike train to downstream regions (Salinas and Sejnowski, 2000). A combined computational and experimental study, in which the theoretical model can be directly tested by population recordings, would allow a more in-depth understanding about the effects of increased NC on computation.
Discussion

Measuring physiology with 2P calcium imaging

The brain, its function and dysfunction, have been a matter of investigation for thousands of years (Edwin Smith Papyrus, ca. 1600 BCE). However, the history of measuring its activity, or in its common term, neurophysiology, is much shorter. For example, EEG has been used in neuroscience research for a century (Pravdich-Neminsky, 1913) electrophysiology has been practiced for over 60 years (Lennox and Lennox, 1947), and fMRI for 20 (Belliveau et al., 1991). From the time these techniques were first introduced, besides being used for studying brain function, researchers have studied the techniques themselves: their reliability for reporting neural activity, their temporal and spatial resolutions, and other aspects regarding the nature of the physiological signal. For all of these techniques, these investigations are still ongoing (Harris et al., 2000; Logothetis et al., 2001; Yitembe et al., 2011).

The first major study using in vivo 2PCI to study population activity was published 6 years ago (Ohki et al., 2005) (roughly at the same time as when I started by graduate studies), drawing a lot of attention and excitement (Hubener and Bonhoeffer, 2005). However, whether or not this technique turns out to be attractive for studying brain function depends on many details which have not yet been sufficiently explored, and the technique is still at a time where it has to justify itself. Do the advantages it has to offer compared to existing techniques outweigh its disadvantages? To answer this question, careful examination, assessment, and comparisons have to be made. In Chapter 1, I conducted a set of experiments to study the nature of the fluorescence signal, the advantages it offers and the inherent limitations to the technique.

The limitations of 2PCI, which I examined in Chapter 1, stem from two main factors. The first is that regardless of how it is monitored, the calcium signal is an indirect and slow measure for electrical activity. The temporal resolution I found is on the order of 50 milliseconds, while even with optimal
optical conditions the reliability for reporting spikes is not perfect. In Chapter 2 I found that the error rate of spike identification is 5% false positives and 9% false negatives.

The second limiting factor is that the signal is monitored optically, which has a number of limiting implications. First, in my experiments, imaging depth was limited to ~450 µm, below which image quality was poor. This meant that I could only monitor activity in layer 2/3. Secondly, optical noise from different sources is mixed with the signal, and its impact must be assessed. Lastly, photo-bleaching limited imaging duration to ~8 minutes. So, does 2PCI justify itself?

Which of the numerous available techniques to record brain activity should 2PCI be compared against? Functional Magnetic Resonance Imaging (fMRI), Electroencephalography (EEG), Positron Emission Tomography (PET), Intrinsic Signal Imaging (ISI) and Voltage Sensitive Dye (VSD) imaging are all powerful methods to measure brain activity, but none of them offers single-cell resolution in vivo. For studying true network activity, currently the only attractive alternative to 2PCI is electrophysiology.

For nearly all practical applications, temporal resolution with electrophysiology is optimal. Neurophysiological cellular activity is on the order of one millisecond or more, while the signal may be acquired at Megahertz rates or higher. Reliability is a more delicate question, and often overlooked in electrophysiology. Intracellular and juxtacellular (loose-patch) recordings allow monitoring spikes with high reliability (Chorev et al., 2009). However, these are single-cell recordings. When monitoring the activity of multiple neurons, using an electrode, tetrode, multielectrode array or a silicon probe, identification of spikes and especially separating spikes coming from different neurons is a non-trivial computational challenge that involves errors. Moreover, the error rates of commonly used methods for spike separation were estimated to be up to 30%, while the estimated optimum is up to 8%, depending on different parameters such as the firing properties of the cells (Harris et al., 2000). This performance is similar or worse than the estimated performance of spike-identification algorithms using calcium imaging (Kerr et al., 2007;
Sato et al., 2007; Rothschild et al., 2010). Thus, spike identification is a common challenge of 2PCI and electrophysiological population recordings.

I would suggest that reliability is similar using the two methods, electrophysiology is far superior in terms of time, but the opposite is true for space. 2PCI is favorable in the spatial dimension in two aspects: 1. the location of each imaged neuron is known at a micrometer resolution, and 2. dozens to hundreds of imaged neurons are concentrated within a diameter of ~200 µm. In Chapter 2 I found that pairwise signal and noise correlations decrease with distance between 0 and 200 µm, suggesting that unique computation occurs at this range (see below). Such findings could not be obtained using electrophysiology or any other available experimental technique. With electrophysiology, the location of units recorded in a single electrode can be anywhere within ~150 µm from the tip (Buzsaki, 2004), and thus inter-unit distances between 0-300 µm are indistinguishable. This is still true when using tetrodes, multi-electrode arrays or silicon probes, only the area and number of recorded cells is larger. Moreover, when using multi-electrode arrays, distances between neurons recorded on different electrodes are usually larger than 100 µm. Thus, 2PCI is highly suited for studying the functional micro-architecture and dynamics of populations of neurons at a spatial resolution of 0-200 µm. Is this level of spatial resolution relevant for sensory processing?

**What is unique about the scale of 200 µm?**

2PCI has allowed us, for the first time, to probe the organization and dynamics within the smallest network structures in the AC. In the discussion of Chapter 2, I describe the heterogeneous micro-organization I found, which was surprising in light of previously described smooth large-scale tonotopic maps in the AC. Here, I would like to discuss our results in a more general context, not restricted to the specific organization of AC.
A number of our findings suggest that networks at a scale of 0-200 µm are unique in their organization and dynamics, forming perhaps the smallest computational modules in the cortex. In Chapter 2, I presented two measures for similarity between the responses of pairs of neurons: signal correlation (SC), which measures the similarity of mean response properties, and noise correlation (NC), which measures the similarity in fluctuations from the mean responses. Although these measures are largely independent and describe complementary aspects of response similarity, I found that both were positive at close distances but significantly decreased with distance between neurons from 0-200 µm (Chapter 2, Fig. 6c and 7d). Average SC decreased from ~0.1 to ~0 over this range, while NC decreased from ~0.2 to ~0.1. Thus, the levels of SC and NC I found are largely restricted to pairs of neurons at distances of 0-200 µm. I thus suggest that networks of neurons in this range in the mouse AC form unique functional modules.

Neurons in the brain are grouped at multiple spatial scales. The size of the mouse AC, for example, is ~2X2 mm (Stiebler et al., 1997), over which neurons can form long-range horizontal connections by sending their axons to distances of a few millimeters (Lubke et al., 2003). Neurons are further organized into columns, which have a diameter of 300-600 µm (Mountcastle, 1997). Lastly, minicolumns of 50-100 µm in diameter have been suggested to be the smallest functional cortical unit (Mountcastle, 1997), grouping neurons with similar response properties (Favorov and Diamond, 1990; Mountcastle, 1997). Minicolumns have been described in numerous species and cortical regions (although not always named as such), including the auditory cortex (Shamma et al., 1993; Sugimoto et al., 1997; Chance et al., 2006). Our findings regarding local modules in AC seems to be consistent with the scale of one or a few minicolumns. As expected from neurons within a minicolumn, I found that pairs of neurons within these distances showed the highest correlations in their mean responses and response fluctuations, while correlations between pairs at larger distances were weaker. Additional supporting evidence for a unique functional module at these scales comes from a recent elegant study in macaque visual cortex, which compared network dynamics at varying scales (Ohiorhenuan et al., 2010). Using multi-tetrode arrays, Ohiorhenuan and colleagues recorded
small groups of neurons which were either at distances of 0-300 µm (recorded on same tetrode), 600 µm (neighboring tetrodes) or 1000-1250 µm (distant tetrodes). They found that although pairwise correlations existed in all scales, higher order correlations that could not be explained by pairwise correlations only existed at the 0-300 micrometer scale. These results support our findings regarding unique cortical functional modules at these distances.

**Heterogeneously organized minicolumns**

Although our results of a unique functional module at a scale of 0-200 µm are consistent with the basic idea and scale of minicolumns, they challenge some accepted aspects of it. A defining feature of cellular minicolumns in sensory cortex is that neurons within the same minicolumn exhibit similar physiological characteristics and receptive fields (Mountcastle, 1957, 1978, 1997). However, our results indicate that in L2/3 of mouse AC, pairs of cells as close as 20 µm may exhibit both similar and divergent response properties (Chapter 2, Fig. 4-6). How can these results be reconciled? One possibility is that homogeneous minicolumns would appear when presenting different stimuli than those I used, such as complex sounds. However, I find this unlikely because tone frequency is the strongest organizational principle described in auditory cortex. A different possibility is that minicolumns are homogeneous in some, perhaps larger species, such as cats or monkeys, while they are heterogeneous in mice. In support of this possibility, Ohki et al. (2005) found precise columnar organization in the cat, but not in the rat visual cortex. However, one study on monkey motor cortex found that both similarly tuned cells and cells with opposite tuning could be found within distances of 50-100 µm (Amirikian and Georgopoulos, 2003). A third possibility is that within-minicolumn heterogeneity is unique to the AC. Opposing this possibility, recent studies reported local heterogeneity in the somatosensory (Sato et al., 2007) and visual (Mrsic-Flogel et al., 2007) cortex.

The most plausible reason for the discrepancy between our findings of local heterogeneity and previous findings of large-scale order is the inherent spatial averaging of previously used recording
techniques. Multiunit recordings, intrinsic signal imaging, voltage sensitive dye imaging and other large-scale imaging techniques which have been used to describe columnar organization lack single cell resolution and instead average the signal over many neurons. In support of this reasoning, recent studies implementing highly unit isolation in the rat AC, found that nearby neurons could have divergent response properties (Luczak et al., 2007; Hromadka et al., 2008; Luczak et al., 2009). Moreover, in the same issue of Nature Neuroscience as the one our study was published in, another study which performed calcium imaging in the AC of mice was published (Bandyopadhyay et al., 2010). Although the authors used a different calcium indicator, auditory stimuli, analyses methods and other experimental parameters, their findings regarding local heterogeneity were remarkably similar to ours. I thus suggest that at least in the rodent brain, minicolumns group interconnected neurons with increased response correlations, but that still allow a heterogeneous micro-organization.

**What does local heterogeneity tell us about the brain?**

Ordered cortical maps have been suggested to offer different advantages for neural development and function. Perhaps the strongest argument comes from the wiring optimization principle originally proposed by Ramon Y Cajal over 100 years ago, and recently reintroduced as a central principle of neural organization (Kaas, 1997; Chklovskii and Koulakov, 2004). According to this principle, wiring in the brain (axons and dendrites) is costly, and there is thus an evolutionary pressure to minimize connectivity by positioning synaptically connected neurons near each other. This principle was used to explain ordered cortical maps by arguing that interacting neurons are expected to share similar response properties. How can our results of local heterogeneity be explained in light of the wiring optimization principle?

One possibility is that wiring optimization drives connected and similarly responsive neurons to be close, but not necessarily adjacent. In other words, the organization I observed may be viewed as a
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perfectly smooth organization where neurons with similar receptive fields are connected and adjacent, which was scrambled by shifting neurons a few tens of micrometers, while maintaining their connectivity. In such a case, wiring optimization is not maintained at local population levels. Heterogeneity in this case refers only to the spatial distribution of the cells, and not to the connectivity diagram. An alternative possibility is that wiring optimization is indeed maintained at our resolution. In such a case, the observed local heterogeneity is an indication that neurons with dissimilar response properties are connected, implying heterogeneity in connectivity and computation as well as in the spatial distribution. One extreme example for such a case could be an inhibitory interneuron involved in lateral inhibition of a neighboring excitatory neuron. The receptive field of the inhibitory neuron might be flanking and non-overlapping that of the excitatory neuron, although they are connected. Connections between two excitatory neurons with differing receptive fields might also be advantageous for different network functions. Computationally, given that neurons exhibit noise correlations, heterogeneous networks of neurons are advantageous in terms of information processing (Abbott and Dayan, 1999; Averbeck et al., 2006; Shamir and Sompolinsky, 2006; Cafaro and Rieke, 2010). In such networks, common noise is 'cancelled out' by neurons with differing receptive fields.

The two extreme alternatives are thus: 1. Subnetworks of neurons with similar response properties are distributed in space but are connected only between themselves (thereby wiring optimization is not maintained), and 2. A neuron is connected to the neurons adjacent to it, some of which have dissimilar receptive fields (thereby wiring optimization is strictly maintained). Our data does not allow to fully determine which alternative is correct. However, our data is most consistent with an intermediate alternative: neurons form few strong connections with more distant neurons with similar receptive fields, and many weak connections with adjacent neurons whose receptive fields are different. Support of this alternative may be found in Fig. 7e of Chapter 2, where I calculated SC (receptive field similarity) as a function of NC (functional connectivity). I found a significant correlation between SC and NC, indicating that indeed, neurons with similar response properties are
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on average more strongly functionally connected. However, the correlation was pretty weak (r=0.275), and many pairs with uncorrelated receptive fields were functionally connected.

Such a model of network connectivity has been previously proposed (Song et al., 2005; Yoshimura et al., 2005). However, while the emphasis in these studies have been on the few strong distant connections which group neurons with similar response properties, I would suggest an equally important role of the many weak local connections with neurons with dissimilar response properties. Indeed, each type of connection may facilitate a different function. One possible advantage of connecting neurons with identical receptive fields, sometimes termed "sister cells" (Dhawale et al., 2010), is increasing response reliability. If, for some reason, the input one neuron received from the thalamus in response to some stimulus was below average, the sister neuron which was driven by the same stimulus might induce the neuron to fire. In contrast, one advantage of connecting neurons with dissimilar response properties, is performing association between stimuli. Suppose that the BFs of neurons X, Y and Z are 3.7, 7.4 and 11.8 KHz, respectively, and suppose they all connect to a neuron K, which is driven to fire only when X,Y and Z fire together (resulting in sensitivity to synthetic wriggling calls). In this toy example, the receptive field of neuron K will greatly differ from those of all the neurons it is connected to. Such a mechanism to associate different stimuli into auditory objects may take place in auditory cortex.

I thus suggest that the organization I found in AC may tell us something about its function. Namely, that output reliability and stimulus associations might both be central functions of AC, facilitated by local population dynamics.

Network-level plasticity in AC

The classic view of primary sensory cortical regions is that of high-order stimuli analyzers. For example, the proposed main function of the visual cortex, the most studied sensory cortex, is applying spatiotemporal (Gabor) filters on incoming visual information, which may carry out
neuronal processing of spatial frequency, orientation, motion, direction, speed, and many other spatiotemporal features. The auditory cortex has been suggested to perform similar processing. For example, one study found that the preferred stimuli of AC neurons consists of complex temporally and spectrally modulated sounds (Wang et al., 2005). However, increasing evidence from studies at different levels of spatial and temporal resolution, emphasize the role of AC in functional plasticity (Fritz et al., 2003; Valentine and Eggermont, 2003; Weinberger, 2004; Norena and Eggermont, 2005; Zhou and Merzenich, 2007; Kim and Bao, 2009; Reed et al., 2011). The main findings of all of these studies can be summarized as follows: in AC, representations of a "distinguished" sound is larger than that of control sounds. Such a sound may be a target sound in a task (Fritz et al., 2003), a sound which is paired with electrical shock (Weinberger, 2004), a sound which is paired with stimulation of nucleus basalis (Reed et al., 2011), or a sound which is repeatedly played to the animal (Kim and Bao, 2009). However, the ethological relevance of all of these manipulations is limited.

In Chapter 3 I constructed an experimental system to examine what changes occur in the AC following a dramatic, yet natural, change in the life of mice (and all female mammals): giving birth and becoming a mother. Compared to virgins, mothers had undergone different physiological and hormonal changes, and gained a few days of experience with the pups. The combined effect of these factors was termed as a change in the 'behavioral state' of the animals. I found that under these conditions, when no specific stimulus is being artificially instructed, responses of single neurons in mothers did not differ much from those in virgins. The overall responsiveness of neurons in mothers and virgins was similar, as was the mean response profile. Concomitantly, however, correlation between pairs of neurons doubled in mothers.

Our results thus demonstrate a novel purely neural network-level change in AC associated with a change in behavioral state in AC. This suggests that changes at the single-cell and network level are distinct, and may engage different plastic processes. It seems that when a single stimulus is given critical importance (such as instruction with an electrical shock), single neurons shift their response
Discussion

properties, but when the set of new experienced stimuli is large and the experience is unguided, the change is to a large extent expressed in functional connectivity between neurons.

Coding at the level of the network connectivity rather than at the level of single cell response profiles may, in fact, be advantageous for a number of reasons. First, neuronal receptive fields across a population form a representation of the world, and should thus be reasonably stable over time. The computation a downstream neuron performs on inputs from AC neurons may change its meaning if receptive fields of its presynaptic neurons are changed. Secondly, in contrast to heterogeneous changes of individual neurons, a general sway of the receptive fields of a population towards the same direction necessarily involves a decrease in representation of other stimuli. In normal conditions, it is desirable to attain a representation of a new sound without losing the representation of another. Lastly, increasing the representation of an important sound seems impractical when multiple new sounds are experienced, for example, when the animal enters a new environment. I thus suggest that our results of a stable mean response profile of the population, coexisting with a large change in functional connectivity is an efficient strategy for representing naturally occurring and complex changes.

In fact, changes in connectivity between cortical neurons are believed to be the network mechanism underlying memory (Frankland and Bontempi, 2005). According to the classic model of memory consolidation, interaction between the hippocampus and cortex modifies network connectivity in the latter. Our demonstration of changes in functional connectivity associated with experience with pups thus shares some attributes of memory formation. However, in the memory formation model, connectivity is changed between different pairs such that the net connectivity is stable, while our results indicate a large global increase in connectivity. This phenomenon does not conform to the standard model of memory formation. Instead, our results seem to indicate a similar mechanism as reported by a recent study which monitored structural changes in mice visual cortical neurons following monocular deprivation (Hofer et al., 2009). Following monocular deprivation, neurons in the deprived region become responsive to stimuli from the open eye. The authors found that
following this dramatic experimental manipulation, spine formation in the visual cortex doubled. These spines most likely carry synapses (Knott et al., 2006; Nagerl et al., 2007), thereby increasing connectivity in visual cortex populations. Although parturition and monocular deprivation are very different experiences, the observed neural changes in both cases may be manifestations of a similar process following a sudden and dramatic change in the input of auditory and visual information, respectively. I suggest that parturition involves an overall increase in connectivity between AC neurons which facilitates reorganization of cortical networks, possibly allowing adaptation of the mother’s auditory system to support maternal behavior.

**Summary**

My research is the first to study local populations of neurons in the AC, allowing to investigate a previously inaccessible level of resolution between single-neuron and large-scale topographic studies. In my research I studied how cortical networks of neurons in the AC are organized, what their dynamical properties are, and how these dynamics change with the behavioral state of the animal. Our findings reveal several novel aspects of auditory cortical organization and processing. First, I found that within the large-scale order in the AC, which was previously described in numerous studies, local populations of neurons are surprisingly heterogeneously organized. For example, I found that tonotopy, a hallmark of auditory cortical organization, breaks down at distances <200 µm. At the same time, I found that similarity in responsiveness between pairs of neurons (both the mean response profiles and the fluctuations from the mean) decay at these distances, supporting the existence of a local processing module, the microcolumn, with a diameter of about 100 µm. I next studied how the network dynamics in the AC change following parturition. Mothers, who had undergone physiological and hormonal changes as well as experienced maternal behavior, exhibited no difference in their single-cell response properties. However, the correlation between pairs of neurons was doubled in mothers, and higher-order correlations were substantially more dominant.
These results introduce a novel, purely network-level, functional plasticity mechanism in auditory cortex, associated with a dramatic, yet natural, change in behavioral state.

Can my results assist to solve the enigma of the function of AC? The unique organization and dynamics I found in local neural populations in the AC challenge the suggested role of AC as a representational map of sound attributes. To maintain a representational map, neurons would be expected to be orderly organized with respect to their receptive fields, and functional connectivity would be expected to remain stable over time. Instead, I showed that local populations are heterogeneously organized and that functional connectivity is plastic with respect to the behavioral state of the animal. My findings thus suggest that cortical networks in AC are better designed to perform sound association and undergo functional plasticity. I believe that 2PCI holds huge potential to shed additional light on cortical processing in general and specifically in the AC, and that pursuing the research on local neural networks in the AC will have a large contribution to solving the enigma of the AC.


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Bibliography


תקציר

יונקים משתמשים במערכת השמיעה למגוון תפקודים ביולוגיים כגון תקשורת, ציד, הימנעות מסכנות, חיזור ונווט. עיבוד שמיעתי מתחיל כאשר גל קול מגיע לאוזן הפנימית ומרטיט את הקוכליאה (cochlea). הרטט זה מותמר לאות חשמלי העולה במעלה מערכת השמיעה דרך התלמוס עד לה eqlאת קליפת המוח השמיעתית (auditory cortex). קליפת המוח השמיעתית הנה האור המרכז בקפלת המוח המנérica בעיבור חלקים."כっとו, מאמינים כי הוא מעורב בעיבוד מסדר גבוה של צלילים ויש לו חלק מרכזי בעיצוב תפישה שמיעתית. סך כל, לענות על שאלה זובכדי, מחקרים רבים עקבו אחר הפיסיולוגיה של קליפת המוח השמיעתית בזמן השמעת גירויים אודיטוריים שונים. מחקרים אלו התרכזו בשתי רמות של רזולוציה מרחבית: פעילות תא בודד הנמדדת ברישום אלקטרופיסיולוגי, טרופיסיולוגיה או שיטות הדמיה ומעקב אחר פעילות של אזורים נרחבים באמצעות אלקטרוסטס郵פע של מאות מיקרומטרים או יותר. מחקרים בשתי רמות רזולוציה זו חשפו מידע חשוב לגבי פעילות קליפת המוח השמיעתית. למשל, מחקרים על הטונים טהורים נוירונים בודדים מצאו שכאשר משמיעים לחי, השדות הרצפטיביים של נוירונים בקליפת המוח השמיעתית דומים מאוד לאלו של נוירונים ברמות הרבה יותר נמוכות במערכת השמיעה. אך מחקרים גם מצאו, שהשדות הרצפטיביים של המוח השמיעתית יכולים לעבור שינויים גדולים בתרחישים שונים, לדוגמה, כאשר החיה מבצעת משימה הכוללת אבחנה שמיעתית. מחקרים על פעילות של אזורים נרחבים בקליפת המוח השמיעתית מצאו, למשל, שה❧ "מקוללת" של קליפת המוח השמיעתית היא מחולקת לתתי-אזורים ושחלק מהם מסודרים באופן טונוטופי. מעבר לכך, הראו כי הסדר התפקודי של קליפת המוח השמיעתית יכול גם כןを通して שינויים גדולים בהתאם לסביבה האקוסטית בה החיה נמצאת. 

מדוע, אם כן, ניתןмыслיל קבלת תפקוד הפעילות של קליפת המוח השמיעתית ככה חידה? ת borr אני טווח במעטע לאחר פעילות, מקימה על מבנה מוחה של אזורים גרובים בקפלת המוח השמיעתית, שמישתה את המחלקה האבסטרקטית. מחקרים על פעילות של אזורים גרובים בקפלת המוח השמיעתית מראים, למשל, שעון מחולקת לחללים. מחקרים אחרים בומרים את המפרשים את פונקציות של קבלת תפקוד המוח השמיעתית, מה של קליפת המוח השמיעתית יולג. הם גם לעבר פעילות של המוח השמיעתית בקפלת המוח השמיעתית, ואתל תפקודים שונים של קליפת המוח השמיעתית.
על כן, מעקב אחר הסידור המרחבי, תפקודית של אוכלוסיות מקומיות של נוירונים(Expected), מ揭露 היבטים לא ידועים של עיבוד מידע בקליפת המוח.

עד לאחרונה היה זה בלתי אפשרי לעשות זאת מסיבות טכניות בגוף החי מכיוון שרישומים אלקטרופיזיולוגיים אינם יכולים לדגום בצפיפות תאים בנפח כה קטןainside, ורתיות מוגבלות לשימוש קרוב לפני שטח המוח בלבד. שיטות הדמייה מס.

לאחרונה השתנה מצב זה, עם הפיתוח של מיקרוסקופיית 2 פוטונים וצבעים הרגישים לריכוז סידן.

בעזרת הדמיית סידן בטכנולוגיית 2 פוטונים, ניתן לעקוב אחר שינויים ברמות ריכוז הסידן של מספר רב של תאים במקביל, בעומקים של עד כ 500 מיקרומטרים מעל השטח המוח.

מכיוון שבשיטה זו ניתן לעקוב אחר מספר גדול של תאים הקרובים جدا זו אלו ואלו מיקומם ידוע ברזולוציה של מיקרון בודד, ומצטמצמים בפרטיות זו האלאחרונה לא פיטיוס אוכלים או פיקודים שלเอוכלקספוס או מוסר של מים.

במחקר שלי, יישמתי טכניקה זו הראשונה לחקור פעילות אוכלוסיות תאים בקליפת המוח השמיעית.

מכיוון שהדמיית סידן בטכנולוגיית 2 פוטונים היא שיטה חדשה יחסית אשר מעולם לא ניסתה בעבר בקליפת המוח השמיעית, ביצעתי סדרה של ניסויים להעריך, בוחן ולייעל מעקב אחר פעילות תאים בשיטה.

לצד היתרונות אשר שיטה זו מציעה, קיימות בה גם מספר מגבלות הנובעות מהשימוש במדד עקיף לפעילות חשמלית, וממעקב אחריו באמצעים אופטיים.

בפרק 1 אני בוחן היבטים שונים של השיטה כגון הרזולוציה הזמנית והמרחבית, מידת המהימנות, רמת הירזום, אורכה זמן מגבלת העומק ויציבות הרישום לא ומקומיות של נוירונים.

הפרטים מקומית של נוירונים הוא שיטה חדשה יחסית, לפי שיטה זו של נוירונים fueron veröffentlicht בעיתון Nature Neuroscience בשנת 2010.

בעד שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מופיע지만, הוא מופיעמצן מתכתי במחלקת החיבוריות של השכ🎄בשל משפחת, כך שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מتلكט ייחודי במחלקת החיבוריות של השכ🎄בשל משפחת, כך שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מפילט ייחודי במחלקת החיבוריות של השכ🎄בשל משפחת, כך שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מפילט ייחודי במחלקת החיבוריות של השכ🎄בשל משפחת, כך שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מפילט ייחודי במחלקת החיבוריות של השכ🎄בשל משפחת, כך שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מפילט ייחודי במחלקת החיבוריות של השכ🎄בשל משפחת, כך שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מפילט ייחודי במחלקת החיבוריות של השכ🎄בשל משפחת, כך שהארגון המרחבי של אוכלוסיות מקומיות של נוירונים כנראה משרת עיבוד מידע בקליפת המוח השמיעית, הוא מפילט ייחודי במחלקת החיבוריות של השכ🎄בשל זו האלאחרונה לא פיטיוס אוכלים או פיקודים של אוכלוסיוזוס מול מים.

למרות זאת, וה yan ימי לאחר המלטה, בין 3-5 ימים לאחר המלטה, יזב ברפנות של מפרוש או מתוק ב-4-5 ימים, וב

הפרטים מקומית של נוירונים הוא שיטה חדשה יחסית, לפי שיטה זו של נוירונים fueron veröffentlicht בעיתון Nature Neuroscience בשנת 2010.
There was no change in the response profile of the population in mothers compared to postpartum. However, correlations between pairs of cells were twice as high in mothers, and higher-order correlations were more dominant in a statistically significant manner.

Similarly, the study conducted in the auditory cortex slice revealed a plastic auditory mechanism that is related to a dramatic and natural change in the state and experience of the animal.

In summary, my research is the first to follow local populations of cells in the auditory cortex slice, which act as a high-resolution that has not been accessible between the isolated cell rate and the activity of full areas.

I described for the first time how cells in this area are ordered spatially, how their dynamics change with the animal's behavior. The results of my study reveal several aspects of organization and activity in the auditory cortex slice, which contributes to our understanding of the unique activity of this area in the human brain.
עבודה זו נעשתה בהדרכתם של פרופ' עדי מזרחי ופרופ' ישראל נלקן

פרופ' צבי מזרחי ופרופ' יسرائيل בלוקן
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