Integrative Properties of Cortical Pyramidal Neurons

Boudewijns, Z.S.R.M.

2015

document version
Publisher's PDF, also known as Version of record

Chapter 6

General discussion

The involvement of the cerebral cortex in a wide range of cognitive processes has been known for over a century but the actual mechanisms through which individual cortical neurons achieve these processes remain largely unknown. Understanding the connectivity scheme of the cortex together with the physiological properties that enable neurons to integrate different information streams is thought to be key in revealing how cognitive processes emerge from cortical activity. The process of coincidence detection (Larkum, 2012) is a vital concept, as it constitutes an electrophysiological mechanism by which segregated anatomical inputs are integrated at the level of single neurons. The mechanism was first described in brain slices, where backpropagating single action potential combined with depolarization at the apical dendrite, which likely occurs during simultaneous feedback and feedforward activation, cause a burst of action potentials that is dependent on calcium spikes (Larkum et al., 1999a). To fully understand the functional significance of coincidence detection, a more detailed analysis of the anatomical circuit and physiological activity profiles of cortical neurons *in vivo* are required. More precisely, the anatomical connections need to be understood at the subcellular level as they define which segregated streams exist and where they could potentially be integrated. The presence of action potential bursts and the generation of calcium spikes *in vivo* are functional readouts of the presence of coincidence detection. Together, the integration of anatomical and physiological data will reveal how cortical activity underlies cognition.

The studies described in this thesis were aimed at revealing both anatomical and physiological properties that contribute to coincidence detection in different brain areas. In **Chapter 2**, axons of L5A and L5B neurons were fully reconstructed using a newly developed semi-automated method further explained in **Chapter 3**. The technique enables full 3D reconstructions of axons labeled *in vivo* and is therefore a major advancement in the ability to investigate cortical circuits. The reconstructions showed that the two neuronal subtypes in L5 project in a orthogonal fashion, with slender tufted neurons projecting primarily to supragranular layers while the projections of thick tufted neurons are oriented laterally, indicating that the segregated thalamic input that these neurons receive is maintained in their intracortical projections. In addition, the full 3D reconstructions revealed that the spread of axonal projections reach over several millimeters, which further stresses the importance of labeling neurons *in vivo*. In **Chapter 4**, both *in vitro* and *in vivo* techniques were used to study the dynamics of

high frequency bursting of action potentials and the generation of calcium spikes in prefrontal cortex (PFC), an area known for its integrative properties during cognitive performance. *In vivo* recordings showed that the critical frequency at which action potential bursting can generate calcium spikes is lower in L5 neurons compared to L2/3 neurons. In awake animals, supracritical burst spiking at these frequencies was observed more often in L5 compared to L2/3 neurons. The study reported in **Chapter 5**, was designed to investigate whether activity during an attention task was associated with layer-specific activation of neurons in different areas of the prefrontal cortex by analyzing the expression of an activity-dependent gene. Even though we did not find layer-specific correlates of attention, the study did provide valuable insights for the design of future studies.

Full 3D axon reconstructions are essential for understanding the cortical circuit

The availability of increasingly powerful computers has led to the proposition of a number of initiatives aimed at fully modeling cortical columns (Helmstaedter et al., 2007) or even whole brains (Markram, 2006; Kandel et al., 2013). Such initiatives are very promising but their success will depend on the quality of the data that is used to model the cortical circuit. Previously, models of the cortical column were based on reconstructions from neurons labeled in brain slices of at most 400 micrometer thickness. Due to the slicing procedure axons are cut and it is therefore impossible to achieve a realistic model of the cortical circuit which makes the value of models based on this *in vitro* data questionable. The ability to fully quantify axonal length with micrometer precision described in this thesis is therefore a major step forward. For realistic reconstruction of the cortical circuit and modeling of the flow of information precise quantification of dendritic and axonal morphology is an absolute necessity. The semi-automated reconstruction technique discussed in **Chapters 2 and 3** of this thesis is therefore a crucial step in the development of realistic cortical models by allowing full quantification, drastically reducing errors made while tracking axons and reducing the amount of manual labor from over 90 hours to around 10 hours. Therefore, the method paves the way for large-scale reconstructions of full 3D axon morphologies and a meaningful reconstruction of the cortical circuit. The development of the semi-automated axon reconstructing technique is part of a larger effort aimed at a realistic, *in vivo*-based reconstruction of a cortical column. A full cell count has been performed to determine the precise number of neurons

in all layers of a barrel column (Meyer et al., 2010a). Reconstructions of thalamocortical axons combined with dendritic profiles provide the layer-specific profile of cortical afferents and potential thalamocortical connection sites (Oberlaender et al., 2012). The description of a standardized barrel field allows precise localization of individual neurons (Egger et al., 2012). The ability to fully reconstruct cortical axons for all layers then is the final step towards the realistic reconstruction of the anatomy of a cortical column, which then allows detailed computational modeling of signal flow to reveal mechanistic principles underlying sensory guided behavior.

The full 3D axon reconstructions described in **Chapter 2** have revealed a number of important conclusions. First, L5 slender and thick tufted neurons show parallel axonal projection profiles and thereby maintain the segregated information stream that can already be observed in thalamic inputs to the cortex. More specifically, slender tufted neurons project primarily to L2/3 while thick tufted neurons show predominantly lateral projections to infragranular layers. For both types of neurons the axons can be found over a range of more than a millimeter, stressing the importance of 3D reconstructions performed on neurons labeled in intact brains. Finally, the most important result was the realization of a semi-automated reconstruction technique that allows the full quantification of axonal length of these neurons, something that was not possible before. The reconstructions of L5 neurons showed that the total length of the axons of slender tufted neurons is almost threefold that of thick tufted neuron. Furthermore, the ability to fully quantify axonal length also enables a full appreciation of the amount of axons found in each of the cortical layers, which can be used in combination with reconstructions of dendrites to estimate the connectivity scheme of the cortex. Reconstructing axon morphology is crucial to understand the cortical microcircuit and the flow of information in the cortex. Many studies have performed axon reconstructions, usually through single neuron labeling in intact animals or brain slices (Gilbert and Wiesel, 1979; Lund et al., 1979; Zhang and Deschenes, 1997; Schubert et al., 2001; Schubert et al., 2006b; Lubke and Feldmeyer, 2007). However, axon reconstructions based on sliced material suffer from an underestimation of the axonal length as axons are cut during the slicing procedure. Typically, brain slices used for *in vitro* studies are between 300 and 400 micrometer thick. The axon reconstructions described in this thesis showed that axons of both L5A and L5B extend over a millimeter in all directions. This is an important finding, as it shows that neighboring columns are very

importants target for L5 neurons. In fact, for slender tufted neurons the amount of axon that is found outside the principle column is almost four times that found within the principal column. Among thick tufted L5 neurons the total axonal length outside the principal column is three times that found within the principal column. This observation is in line with previous observations that, in spite presence of functional columns (Mountcastle, 1957), considerable lateral spread can be observed among cortical axons (Gilbert and Wiesel, 1983). The wide lateral spread of the axons from both types of L5 neurons described in this thesis suggests that activity in the primary somatosensory cortex potentially spreads very rapidly to neighboring columns. Cortical coding of whisker information is therefore likely a process that involves multiple columns. Interestingly, when mice are trained to use their whiskers to guide them when crossing a gap, the decision to cross is made twice as fast when multiple whiskers are present compared to when only one whisker is available (Celikel and Sakmann, 2007). This observation illustrates the importance of integrating multiple whisker information in behavior.

Even though axon reconstructions are vital for the reconstruction of the cortical circuit, the true value only becomes apparent when combined with functional studies. For example, it is crucial to know whether axons connect to excitatory or inhibitory neurons as the effect of such connections would be opposite. L2/3 neurons, which project primarily laterally within L2/3 and L5 (Lubke and Feldmeyer, 2007; Bruno et al., 2009), have been shown to have an inhibitory effect on neighboring neurons in L2/3 but activate neurons in L5 (Adesnik and Scanziani, 2010). These results illustrate that the functional postsynaptic effect of cortical neurons can be layer specific, presumably through differential recruitment of inhibitory interneurons. Regarding the strong projections of slender tufted L5 neurons to L2/3 discussed in Chapter 2, there are various potential postsynaptic targets. There is a high density of interneurons in L2 (Meyer et al., 2011) as well as apical dendrites of thick tufted L5 neurons (de Kock et al., 2007a; Oberlaender et al., 2011a) and dendrites from L2/3 neurons (Shepherd and Svoboda, 2005; de Kock et al., 2007a; Bruno et al., 2009; Oberlaender et al., 2012). Neurons in L5A have been shown to preferentially activate L2 neurons located in the septa between barrels compared to columnar L2 or L3 neurons (Shepherd and Svoboda, 2005) but otherwise little is known about the postsynaptic targets of slender tufted L5 neurons. These observations show that purely anatomical studies are not sufficient to understand the mechanisms underlying cortical processing.

Nevertheless, anatomical studies do provide an indispensible guide for designing functional studies by providing the target areas of axonal projections.

The ability to study functional connections is greatly increased by the development of optogenetics, a novel technique that uses light stimulation to activate or inhibit neurons (Yizhar et al., 2011). Through the viral expression of a light sensitive excitatory ion channels or an inhibitory chloride pump (channelrhodopsin and halorhodopsin, respectively), the activity of neurons can be modulated using light of the appropriate wavelength. One major advantage of the technique is that it can be expressed in specific neurons, either through the use of genetic techniques (Josh Huang and Zeng, 2013) as has been shown for instance for cholinergic interneurons (Witten et al., 2010) and L6 pyramidal neurons (Olsen et al., 2012) or through *in utero* electroporation, as has been shown for L2/3 neurons (Adesnik and Scanziani, 2010). Before the development of optogenetics, activation of neurons could only be achieved by electrical stimulation and inhibition was usually realized through the local infusion of drugs. Both these techniques have very global effects and the ability to specifically activate or inhibit identified neuron types using optogenetics therefore enables a more detailed study of the functional properties of the axon projections.

It has already been shown that thick and slender tufted neurons L5 can be labeled independently with fluorescent proteins (Groh et al., 2010b). It would therefore be very interesting to use optogenetic manipulation of these neuron types to study their postsynaptic targets *in vitro*. Selective expression of channelrhodopsin in slender tufted neurons could be used to investigate the exact postsynaptic targets of these neurons. Light stimulation of the axons from slender tufted neurons in brain slices can then be used to measure the postsynaptic responses of interneurons and pyramidal cells in L2/3, as well as L5 thick tufted neurons. If indeed a connection exists between slender tufted L5 neurons and the apical dendrites of thick tufted L5 neurons, as predicted on the basis of anatomical data (Oberlaender et al., 2011a), it could be studied whether somatic action potentials in thick tufted L5 neurons combined with activation of axons of slender tufted neurons is sufficient to generate calcium spikes in thick tufted neurons. In addition, this technique will also allow more detailed analysis of projections from other cortical areas that might connect to the apical tufts of thick tufted L5 neurons, such as motor cortex (Xu et al., 2012), by selectively expressing channelrhodopsin in specific brain regions using virus injections.

In vivo, cell-type specific expression of the inhibitory halorhodopsin could be used to study the involvement of the two L5 neuron types in behavior. Mice can localize an object using active whisking and can be trained to respond to specific object locations (O'Connor et al., 2010b; O'Connor et al., 2010a). Object touch in this paradigm has been shown to induce calcium spikes (Xu et al., 2012) in the apical dendrites of L5 neurons. Selectively expressing halorhodopsin in slender tufted neurons and using light to inhibit them during behavior could study the involvement of these neurons in this behavior. The potential role of slender tufted neurons in driving L2/3 neurons can also be further investigated using halorhodopsin. Slender tufted neurons have been shown to increase their activity during active whisking (de Kock and Sakmann, 2009b) and the membrane potential of L2/3 neurons has been shown to correlate with whisker position during whisking (Crochet and Petersen, 2006a). Given the strong innervation of supragranular layers by slender tufted neurons (Oberlaender et al., 2011a) and the previously reported connections between neurons in L5A and L2/3 neurons (Petreanu et al., 2009a) it would be interesting to investigate whether the membrane fluctuations during whisking in L2/3 neurons depend on input from slender tufted L5 neurons. Selectively inhibiting slender tufted neurons during whisking using halorhodopsin while performing whole cell recordings of L2/3 neurons could reveal such a mechanism. Together, optogenetic techniques provide a highly valuable tool for cortical circuit analysis as they permit cell-type specific activation and inhibition of neurons, both *in vitro* as well as *in vivo*.

In conclusion, the semi-automated axon reconstruction technique can be used to perform large-scale reconstructions of every neuron type at a relatively low cost, a development that is invaluable for full reconstructions and models of cortical flow. The ability to reveal the precise laminar distribution of axons provides vital insight in the anatomy of cortical circuits and will guide functional studies to reveal signal flow within these cortical circuits during behavior.

Action potential bursts are commonly observed in PFC neurons during wakefulness

As discussed earlier, so far studies into cortical architecture have primarily focused on primary sensory cortices. There is an obvious advantage to using these areas, as delivering stimuli to activate them is relatively straightforward. However, primary sensory areas are perhaps less appealing in terms of their involvement in cognition and the study of brain diseases. The

prefrontal cortex (PFC) has been implicated in a wide range of cognitive functions (Miller and Cohen, 2001) and highly prevalent psychiatric disorders (Goldstein and Volkow, 2011; Arnsten and Rubia, 2012; Myers-Schulz and Koenigs, 2012) but little is known about its cortical microcircuit and physiological properties *in vivo*. In part this is due because PFC research has been performed primarily in non-human primates and humans because of the more complex cognitive tasks the PFC is thought to be involved in. Some authors have even argued that rats do not possess a real PFC (Preuss, 1995), but this argument has been refuted on the basis of both anatomical and functional data (Uylings et al., 2003). In fact, a number of rodent tasks have been designed to probe cognitive functions associated with the PFC in humans such as attention and these tasks have been shown to also depend on the PFC in rodents (Dalley et al., 2004). These results are important, as studies in rodents enable much more detailed analysis of the cortical circuits and electrophysiological properties involved in cognition.

The experiments described in **Chapter 4** are a step in a more layer-based *in vivo* approach of the cortex. First, using whole cell recordings in brain slices, the critical frequency of action potential spiking for the generation of calcium spikes was determined. Next, identified neurons were recorded in the PFC of awake rats to determine the frequency of burst spiking in the PFC, as has previously been shown for the somatosensory cortex (Larkum et al., 1999b). Among L5 neurons, the frequency of action potential spiking at which a calcium-dependent increase in the afterdepolarization can be observed is lower compared to L2/3 neurons. *In vivo*, action potential bursts at the critical frequency are more prevalent among L5 neurons compared to L2/3 neurons. The experimental design is a perfect example of a unique combination of *in vitro* and *in vivo* approach that enabled a direct assessment of cellular properties determined *in vitro* and their presence in awake animals. Juxtasomal recordings ensure that data is unquestionably derived from single neurons, which is vital when determining bursting rate since conventional electrodes often record multiple neurons simultaneously. In addition, juxtasomal recordings enable biocytin filling of recorded neurons for subsequent reconstructions of the dendritic structure. Unfortunately, axons from PFC neurons could not be reconstructed since they were never sufficiently filled with biocytin. Proper biocytin filling for axon reconstruction requires multiple filling sessions over a prolonged period of time, something that cannot be achieved in awake animals since the time to record from neurons is often short due to unstable recordings as a result of animal movement.

During the experiments, rats were allowed to wake up after a short period of anesthesia. During the recordings, the rats were awake as judged by the presence of whisking and body movements but otherwise their behavior can be described as quiescent. In general, it is desirable to relate neuronal activity to behavior, which is why primary sensory areas are often used to study cortical processing as it is relatively easy to control the incoming sensory stimuli thereby making it easy to correlate brain activity with external stimuli. In the PFC such an approach is more problematic because of the wide variety of inputs and the variation of cognitive processes the area is involved in. In the experimental setup employed in this thesis, the behavioral state of the animal is difficult to control for. For example, rats were trained to get accustomed to the head fixation but it is conceivable that the procedure still evokes stress. Because of the connections with structures involved in emotional processing, it cannot be excluded that activity in the PFC was affected by the head-fixation procedure. Conversely, given the involvement of the PFC in cognitive behaviors such as decision-making and attention, the absence of cognitive demand could lead to an underestimation of activity. For example, tetrode recordings have shown that bursts are involved in PFC-dependent emotional behavior and plasticity (Burgos-Robles et al., 2007) and that activity in the PFC is increased in a subset of neurons during an attention task (Totah et al., 2009). Therefore, it is desirable to study PFC activity during relevant behavior in future studies. Only in this way can the involvement of bursts in cortical processing be fully appreciated.

Somatically induced bursts of action potentials can backpropagate and cause calcium spikes in the apical tuft (Larkum et al., 1999b), a mechanism that has been shown in a large number of brain areas *in vitro* (Seamans et al., 1997; Williams and Stuart, 2000; Takahashi and Magee, 2009; Medinilla et al., 2013). The rat PFC is situated at more than two millimeters below the skull and it is therefore impossible to directly assess the presence of dendritic calcium spikes in PFC neurons *in vivo* using calcium imaging. Instead, the critical action potential frequency for the generation of calcium spikes was determined on the basis of the depolarization succeeding the last action potential of a burst, an event that has previously been directly related to the presence of a calcium spike *in vitro* (Seamans et al., 1997; Larkum et al., 1999b; Perez-Garci et al., 2006) and also in the present study could be blocked by the non-specific calcium channel blocker cadmium (Boudewijns et al., 2013). It is important to note however that *in vivo*, it has been shown that action potential bursts do not always translate directly to

calcium spikes (Hill et al., 2013). The indirect measurement of calcium spikes that was used in this thesis may therefore lead to an overestimation of the actual presence of dendritic spikes. However, the results described in this thesis do show the presence of high frequency bursts in the PFC and thus warrant further investigation of action potential bursting and dendritic spikes in the PFC *in vivo*.

In addition to calcium spikes evoked by high frequency action potential bursts, single backpropagating action potentials paired with depolarization of the apical dendrite can also cause calcium spikes, which then can lead to bursts of action potentials (Larkum et al., 1999a). Therefore, to fully understand the functional significance of action potential bursting it is crucial to reveal what the underlying mechanism is *in vivo*. Calcium spikes can be observed during active behavior in the somatosensory cortex as the result of feed forward sensory input from the whiskers combined with feedback activity from the primary motor cortex (Xu et al., 2012). Unfortunately, the presence of somatic action potential bursting was not directly observed in the study by Xu et al. but on the basis of *in vitro* studies such burst spiking is predicted (Larkum, 2012). In this scenario, bursts of action potential signal the coincident activation of feed forward and feedback signals arriving at separate anatomical locations and would therefore convey a different message to postsynaptic targets compared to single action potentials. Since action potential bursts would then depend on calcium spikes, blocking calcium channels would lead to a strong reduction of bursting *in vivo* if this mechanism is the primary cause for bursting. In conclusion, these observations suggest that to understand how the cortex encodes information, it is necessary to understand both the anatomical architecture as well as the cellular coding mechanisms of individual neurons.

Further understanding of the precise anatomical connections of the PFC is required

The PFC is a multimodal association cortex that receives a wide array of information, ranging from (secondary) sensor areas to areas involved in processing of memory, valence and emotions (Groenewegen and Uylings, 2000). Unfortunately, the precise layer-specific pattern of afferent projections to the PFC has not been studied in nearly as much detail as the circuit of primary sensory areas and little is therefore known about a possible segregated input to pyramidal neurons in the PFC. There are some studies however that suggest a laminar organization of afferent input to the PFC. For instance, projections from the

basolateral amygdala target the medial agranular cortex in two bands, one in L1 and L2 and one in L5/L6 (Kita and Kitai, 1990). A more recent study has shown that inputs to the PFC from the thalamus, contralateral PFC, basolateral amygdala and ventral hippocampus do not target L1 and L2/3 uniformly (Little and Carter, 2012). However, this study only focused on L1 and L2/3 and therefore does not give a comprehensive view on the distribution of inputs to the PFC. Further investigation of the precise targets of PFC afferents is necessary to understand the flow of activity within the PFC cortical circuit and ultimately how cognitive processes are encoded in this area. Given the large variety of brain areas that innervate the PFC this will be a major challenge but the involvement of the PFC in higher cognitive processes and a variety of psychiatric disorders of this brain area necessitates such studies.

The majority of what is understood about the function of the PFC is the result of lesion studies or neuronal recordings that lack identification of recorded cell-types and layer. Based on the strong reciprocal connections with a large number of brain areas (Groenewegen and Uylings, 2000; Vertes, 2004, 2006) and its effect on neuromodulatory systems (Vertes, 2004), it is likely that part of the involvement of the PFC lies in the modulation of activity in other brain areas. Attention-related changes in neuromodulatory activity in primary visual cortex (Dalley et al., 2001) could for instance be driven by activity in the PFC. All of the cognitive processes that the PFC is considered to be involved in will depend on interactions between the PFC and other areas. The studies that have revealed the complexity of the cortical circuit of the somatosensory cortex show that it does not suffice to simply speak of a connection between two areas. As shown for coincidence detection in the somatosensory cortex, segregated anatomical inputs are an important factor in the formation of a conceptual experience from different modalities. Therefore, detailed analysis of the precise anatomical connectivity scheme within and between brain regions is required.

More detailed understanding of PFC function can be achieved through (at least) three different approaches. *In vivo* juxtasomal labeling and reconstruction of axons of PFC neurons are needed to reveal the precise anatomical circuit within the PFC, similar to what has been achieved for the somatosensory cortex. The juxtasomal technique is ideally suited to perform these experiments, as it can be used to characterize neurons both anatomically and electrophysiologically. Next, given the myriad of inputs to the PFC, it is crucial to study the precise anatomical locations of these inputs to be able to understand how segregated inputs in the

PFC are integrated at the level of single neurons and thereby affect downstream areas. Again, optogenetic techniques are very suitable to study these anatomical connections. Viral expression of channelrhodopsin coupled to fluorescent proteins in regions that are known to connect to the PFC will label axons in the PFC that originate in the region of infection. The anatomical location of the afferent axons can then be determined on the basis of the fluorescent signal but more importantly, the physiological properties of the connections can be studied by optically stimulating the axons while recording from neurons in different layers of the PFC. This is a major advantage compared to simply describing the layer-specificity of the axon projections, as within each layer basal and apical dendrites of multiple cell types can typically be found. Restricted expression of fluorescent proteins and channelrhodopsin in neurons with a specific molecular profile, as has recently been shown for cholinergic neurons (Wouterlood et al., 2014), can enhance the specificity of these studies even more. Finally, efferent connections from the PFC to target areas should be studied in more detail in order to reveal the precise anatomical targets of PFC axons. Because connections from the PFC to lower cortical areas constitute an example of a feedback projection, it is likely that PFC axons terminate in the upper layers. These connections could therefore modulate activity in these areas depending on the requirements of cognitive tasks. Together, a more detailed analysis of the PFC circuit and its reciprocal connectivity will rapidly increase the understanding of the involvement of the PFC in cognitive processes.

Progress in the functional understanding of the PFC

The complexity of the cortical circuit in the primary somatosensory cortex shows that the function of a brain area cannot be understood through lesion studies. As cognition involves interactions between different brain areas, the complexity of the cortical circuit shows that activity in one area cannot be translated into activity in another area as is often assumed in models. For instance, the differential effect of L2/3 neurons on neighboring L2/3 neurons and L5 neurons (Adesnik and Scanziani, 2010) and the observation described in this thesis regarding processing among different types of L5 neurons (Oberlaender et al., 2011a) illustrate that the behavioral effect of activity in a cortical area is highly complex and both layer and cell-type specific. Thick and slender tufted L5 neurons also have different subcortical targets (Morishima and Kawaguchi, 2006) which shows that also the effects on other cortical areas depend on which neurons are

recruited in a given area. It is therefore of vital importance to strive towards identifying neurons when they are recorded. Because recording from individual identified neurons is time consuming and has a relatively low success rate, these experiments are risky but this is compensated by the more advanced insights that can be achieved.

The experiments that have been performed using tetrodes further illustrate this point. During an attention task, for instance, the proportion of neurons in the prelimbic area of the PFC that increase action potential spiking in anticipation of stimulus presentation during correct trials is roughly equal (slightly lower) to the proportion of neurons that decrease their activity (Totah et al., 2009). Such an observation could indicate that these neurons are part of separate systems and perform a different role during attention behavior, similar to the segregated circuits and different functional properties of L5 neurons in the somatosensory cortex (de Kock et al., 2007a; de Kock and Sakmann, 2009b; Oberlaender et al., 2011a). *In vitro* work has shown that thick tufted corticopontine neurons in the PFC are more likely to show persistent firing in response to acetylcholine administration compared to slender tufted commissural L5 neurons (Dembrow et al., 2010). Given the involvement of acetylcholine in attention behavior (Parikh et al., 2007; Poorthuis and Mansvelder, 2013; Bloem et al., 2014), it would be interesting to investigate whether the different responses of neurons during an attention task can be explained by their sensitivity to acetylcholine.

Performing juxtasomal recordings in moving animals to subsequently identify neurons is possible (Burgalossi et al., 2011; Herfst et al., 2012) but these experiments are tedious, especially when combined with the months of training that are required for rats to perform attention tasks. Therefore, in this thesis the immediately early gene c-fos, which is expressed after neuronal activity (Schoenenberger et al., 2009), was used to study the involvement of brain areas in behavior. The major advantage of the technique is the ability to study activity in any area of interest with layer-specific precision. In addition, all neurons that show activity during a certain task will express c-fos so that layer-specific activity can be quantified. The experiments described in this thesis could not be used to make strong conclusions about which layers are involved in the attention task used, as c-fos expression did not differ significantly between layers in any of the brain regions that were analyzed. In part this could be due to the small group sizes and the resulting lack of statistical power. It would therefore be interesting to repeat

the experiment with a larger group size as the method provides a relatively easy way to asses the location of neuronal activity and can provide a starting point for an understanding of the role of the PFC during attention behavior at the level of cortical layers.

A newly developed technique that utilizes the expression of c-fos enables tagging neurons that are active during a specific task (Reijmers et al., 2007). It has been shown that channelrhodopsin, the light activated ion channel that can be used to activate neurons, can also be expressed using the promoter of the c-fos gene (Garner et al., 2012; Ramirez et al., 2013). Importantly, the expression can be timed so only the neurons that become activated during a specific phase of behavior can be labeled and later reactivated. In order to study the coding mechanisms that underlie cognition, action potential spiking could be measured using electrodes combined with optical fibers to deliver light stimulation. Using this technique, neurons that express channelrhodopsin can be identified as they will show an increase in activity after light stimulation (Lima et al., 2009). Since multiple neurons are recorded simultaneously, the activity of neurons responsive to light stimulation can be compared to other neurons *post hoc*. This provides an alternative way of identifying neurons based on their involvement in a task and can be used to study the amount and timing of action potential spiking during behavior. Alternatively, this technique can also be used in combination with restricted expression of channelrhodopsin in specific types of neurons.

Together, the use of channelrhodopsin can be used to study the complete pathway and the interactions between brain areas that are involved in cognitive tasks. Using simultaneous expression of channelrhodopsin and fluorescent proteins under the c-fos promoter, the layer-specific axonal projections of neurons that become activated during a behavioral task can be revealed. Utilizing the channelrhodopsin present in axon terminals also enables electrophysiological characterization of activated neurons and an assessment of the postsynaptic effects of these specific neurons *in vitro* (Nonaka et al., 2014). Technically it should be possible to express the inhibitory halorhodopsin using the same method in order to inhibit these neurons during precisely timed periods of a behavioral task. Selective inhibition or ablation of activated neurons has been shown previously (Han et al., 2009; Koya et al., 2009), but with techniques that have a lower temporal resolution compared to halorhodopsin. In an attention task, combining these techniques would enable specific labeling of neurons that become active during an attention task in the PFC and further identification in terms of their

morphological and electrophysiological properties using whole cell recordings *in vitro*. Halorhodopsin expression can then be used to inhibit these neurons during specific phases of the attention task to untangle their precise role in behavior. In a separate experiment, channelrhodopsin and fluorescent protein expression can then be used to study the postsynaptic effects of neurons that are involved in this task, for instance in the visual cortex in case of a visual attention task.

Conclusion

The cortex has been the subject of intense studies for over a century and although significant progress has been made, many of its properties remain obscure to this date. In this thesis, experiments were performed that aimed at understanding morphological and electrophysiological properties of the cortex *in vivo*. The development of a semi-automated axon reconstruction technique described in this thesis is a major advancement since it enables realistic, full 3D reconstruction of axonal morphology at a micrometer precision at a drastically reduced cost. This opens the door to large-scale reconstruction of axon morphology, a crucial step in unraveling the cortical circuit. Next, the focus was moved to the PFC, of which the cortical circuit is still poorly understood. The experiments performed revealed that action potential bursts occur commonly but layer-specific in the PFC, thereby providing an important step in a more layer and cell-type specific approach of the PFC. Together, the experiments described in this thesis clearly show the necessity to study the cortex at the level of layers and specific types of neurons. Naturally, the findings have raised new questions that future studies should answer. The development of new techniques has greatly accelerated the ability to study the cortex at a very high level of detail. Yet, the most important step forward is to translate what is known about cellular properties of neurons to the function of the cortex by studying these phenomena during relevant behavior.