

# 6

## **Synopsis of Results and Discussion**

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## 6 Synopsis of results and Discussion

The main objective of this thesis was to systematically study the intrinsic and extrinsic network of the entorhinal cortex (EC). The experimental data contribute to new insights in how the intrinsic network of EC processes incoming inputs and how these may contribute to our understanding of the role of EC in learning and memory processes. In this chapter I will provide a synopsis of the results and a general discussion of the methodology used. Subsequently, the significance of the data presented in this thesis will be discussed, and ideas for future research presented, followed by some concluding remarks.

### 6.1 Synopsis of results

#### 6.1.1 Chapter 2: Cellular properties of principal neurons in the rat entorhinal cortex. I. The lateral entorhinal cortex.

The lateral entorhinal cortex (LEC) provides a major cortical input to the hippocampal formation and receives multimodal sensory inputs from cortical and subcortical areas<sup>1, 2</sup>. We know that neurons in LEC are not strongly spatially modulated<sup>3</sup> and react stronger to sensory contextual information<sup>4</sup>. Earlier studies describing the morphological or physiological properties of neurons in LEC focused on single cell types or single layers. These studies are far from complete in that layer I (LI) and layer VI (LVI) principal neurons were never studied before. We recorded from post-hoc morphologically identified LEC principal neurons in all layers *in vitro* by using standardized whole cell current-clamp recordings from up to four principal neurons in the same or in different layers. This way we controlled for possible interslice/interexperimental bias with respect to comparing properties of principal neurons within the same or different layers along the mediolateral and deep-to-superficial axes. We found that principal neurons in LI, layer II (LII) and layer III (LIII) are the only ones that have the majority of their dendritic and axonal collaterals only in superficial layers. Layer V (LV) contains mainly pyramidal neurons with dendrites and axons extending throughout all layers, and pyramidal neurons with dendrites confined to deep layers. This latter feature also holds true for all LVI principal neurons. Physiologically, input resistances and time constants of LII principal neurons are lower and slower respectively than those observed in LV principal neurons. Fifty-four percent of LII principal neurons show sag potentials, resonance properties, and rebounds at the offset of hyperpolarizing current injection, whereas LIII and LVI principal

neurons do not show any of these LII characteristics. LV principal neurons are different from all other layers in that they show prominent spike frequency adaptation (SFA) and a decrease in spike amplitude in response to strong depolarization, whereas the other layers do not. LV principal neurons also have an inward rectification in response to hyperpolarizing currents, which is less prominent than the sag of LII principal neurons. Despite the well-developed intralaminar communication in LEC, the laminar differences in the biophysical and morphological properties of principal neurons suggest that the *in vivo* firing patterns and functions differ as well, similar to what is known for principal neurons in different medial entorhinal cortex (MEC) layers.

### 6.1.2 Chapter 3: Cellular properties of principal neurons in the rat entorhinal cortex. II. The medial entorhinal cortex.

*In vivo* experiments have revealed that in MEC position coding grid cells, direction coding head direction cells, border defining border cells and various sorts of conjunctive cells (coding for a combination of the previous) exist<sup>5-7</sup>. Within MEC there is even a functional differentiation with LII containing mainly grid and border cells, whereas the other layers contain all cell types<sup>6, 8</sup>. Whether this difference in functionality is supported by layer specific properties was studied in this chapter. As in Chapter 2, multi-patch clamp recordings of up to four post-hoc morphologically identified neurons were performed. Similar to what was observed in case of neurons in LEC, neurons in LI-LIII have their dendritic and axonal arbor in superficial layers, whereas dendrites and axons of LVI neurons are generally confined to deep layers. The dendritic and axonal tree of LV neurons diverges either throughout all, or only in deep layers. Physiologically, MEC and LEC neurons resemble each other, except for LII. In MEC LII, most neurons have a prominent sag potential, a preferred resonance frequency and membrane oscillations, which are not present or less prominent in LEC LII neurons. However, within LII physiological properties do not change abruptly at the anatomical border of MEC and LEC. Instead in the border region there is a gradual change from typical MEC properties towards an absence of these. Taken together with the already reported dorsoventral gradual change in MEC LII properties this suggests that connectionally defined bands that span across both MEC and LEC<sup>9</sup> may relate, at least partially, to the nature of physiological properties of LII neurons.

### 6.1.3 Chapter 4: Monosynaptic inputs from pre- and parasubiculum converge on medial entorhinal cortex principal neurons.

The MEC, presubiculum (PrS) and parasubiculum (PaS) contribute to spatial navigation through the presence of grid cells, head direction cells, border cells and conjunctive cells<sup>6,8</sup>. Which relation these functional neuron types have is unknown. It is known that thalamic nuclei send directional information to PrS and PaS<sup>10-12</sup>. Superficial layers of PrS and PaS likely convey directional information to MEC by way of their efferents that terminate within different MEC superficial layers. For superficial pre- and parasubicular layers it is known that they make synaptic contacts with MEC neurons of superficial layers. The influence of pre- and parasubicular inputs on deep MEC layers is unknown. It is also not known whether MEC neurons receive distinct information from PrS and PaS or whether the same neuron can receive convergent inputs from both cortical brain areas. Recordings made from individual neurons, while extracellularly stimulating PrS and PaS electrically with an electrode or using the uncaging of caged glutamate with ultraviolet (UV) light, show that principal neurons in all layers of MEC receive convergent monosynaptic inputs from PrS and PaS. Responses are layer specific and depend on input frequency. These results suggest that principal neurons in different MEC layers uniquely contribute to information processing and may play a different functional role *in vivo*.

### 6.1.4 Chapter 5: Development of functional projections from pre- and parasubiculum to medial entorhinal cortex in the rat.

It has recently been reported that the directional information in PaS and PrS is already present before grid cells emerge in MEC during postnatal development<sup>13,14</sup>. These data suggest that inputs from PaS and PrS are of importance for the development and maintenance of MEC functionality. Therefore we determined when the pathways from PrS and PaS to MEC become functional. We compared different developmental stages from the beginning of functional connectivity until adulthood using voltage-sensitive dye (VSD) imaging in rat brain slices from postnatal day 5 (P5) to P61 old rats. In a next step we used multi-patch clamp recordings of MEC neurons, while stimulating PrS and PaS extracellularly to support our network data. We found immature mono- and polysynaptic connections between PrS and PaS and superficial MEC around P9. Optical signals and synaptic events in deep MEC layers were observed a little later. From P14/15 the pattern of activity between PrS, PaS and MEC became adultlike, which is when immature grid cell appear in MEC but much earlier than the emergence of adultlike grid cells in MEC.

## 6.2 General discussion methodology

In Chapter 2 and Chapter 3 the physiological and morphological properties of LEC and MEC rat principal neuron properties were compared by performing *in vitro* current-clamp recordings of up to four principal neurons simultaneously, followed by a morphological characterization. In Chapter 4 and Chapter 5 we also used *in vitro* approaches to examine connectivity of two important input structures of MEC, the PrS and PaS<sup>2</sup>, by using extracellular stimulation in PrS and PaS, together with synchronous multi-patch recordings in MEC in rat brain slices. The chosen methods raise two possible technological questions that will be addressed subsequently.

### 6.2.1 *In vitro* versus *in vivo* patch clamp experiments

An important question to address is whether the applied *in vitro* approaches provide relevant information and how this compares to *in vivo* approaches.

The *in vitro* approach makes it feasible to select and identify specific neurons easily, such as selecting a neuron due to a particular position in the cortex under study, as well as size and shape of the soma. In addition, in a number of experiments a newly developed approach was applied that enabled selecting neurons identified as projection neurons to a specific target by retrograde labeling. Alternatively, we visualized inputs to neurons to be patched through anterograde labeling. This approach allowed us to optimize the angle at which slices were cut such as to observe as much of the connectivity under study.

*In vivo* patch-clamp recordings enable characterizations of neurons by way of either basic fundamental properties<sup>15</sup> or by way of behavioral correlates<sup>16-18</sup>. This approach yields very valuable data, but essentially the number of cells obtained per animal is limited to one. Moreover recordings deep in the brain, such as in ventral LEC are notoriously difficult. *In vitro* techniques overcome both difficulties and were thus the method of choice. This is further supported by the fact that intrinsic properties, as reported in Chapter 2 and Chapter 3 for EC show a strong similarity to the sparse *in vivo* data<sup>15</sup>.

*In vitro* experiments as used by us have an obvious problem in that it is likely that sizable portions of dendrites and axons are cut, as clearly illustrated when comparing our data on MEC LII principal neurons with the available single neurons described as a result of an *in vivo* approach<sup>15</sup>. This may seriously hamper connectional studies. To partially circumvent this problem, the slices used for Chapter 4 and Chapter 5 were optimized using anatomical labeling to check for intact connectivity before embarking into large scale slice studies. In an additional approach VSD imaging was used. VSD imaging allows a faithful and efficient readout of monosynaptic and polysynaptic connections in a slice,

although this is essentially restricted to measures of overall depolarizing phenomena<sup>19</sup>. It nevertheless allows to efficiently select slices that maintained connectivity of interest and to obtain a first analysis of evoked responses.

### 6.2.2 Problems of extracellular stimulation

For connectivity studies included in this thesis, evoked responses were recorded following extracellular stimulation. Three different stimulation protocols were applied for the following reasons. Extracellular stimulation with a bipolar electrode facilitates finding long-range connectivity. Bipolar stimulation enabled recording from up to three monosynaptically connected postsynaptic neurons while stimulating PrS or PaS. With this technique it is possible to assess how one common input influences several neurons simultaneously and to reduce the between experiment variability. Pipette stimulation excites a smaller volume of tissue, with minimum stimulation trying to mimic single cell stimulation. It allowed making observations about how single neurons in MEC reacted to stimulation from a local cluster of neurons in PrS or PaS.

The disadvantages of using an extracellular stimulation electrode are twofold. Firstly, one is not able to control for the number of neurons one is stimulating in the stimulation area. Second, even minimum stimulation cannot rule out that passing fibers are activated and when stimulating repetitively different fiber sets can be activated every time one stimulates. These passing fibers may result in antidromic activation in case they originate in the area one is recording from, or in false positive evoked potentials in case they are input fibers passing through the area of stimulation on their way to the recording site.

The observed characteristics of the evoked responses, in particular the latency, minimal jitter in the latency for any particular neuron, the ability of the neurons to follow 100 Hz stimulation and the fact that the neurons respond to minimum stimulation of 0.9 mV with an eEPSP with a relatively low failure rate make antidromic stimulation as a cause for responses unlikely. These parameters also speak in favor of our interpretation that the observed membrane potential changes were caused by monosynaptic, and not by polysynaptic activation<sup>20</sup>. In one study (Chapter 4) glutamate uncaging was used as a final approach to avoid stimulation of passing fibers<sup>21, 22</sup>. Since the results obtained with this approach corroborated our extracellular stimulation data, the conclusion is that extracellular stimulation in a carefully controlled way is still a valid procedure to study connectivity in *in vitro* preparations. Therefore in the study on the development of inputs to MEC only the latter method was applied.

### **6.3 General discussion**

Results presented in this thesis lead to some major conclusions that potentially challenge current notions on the functional organization of EC. Firstly, the intrinsic excitatory networks of MEC and LEC do not differ significantly except for the physiological properties of principal neurons in LII. This brings up the issue which other factors are relevant contributors to the striking functional differences between the two parts of EC. Secondly, principal neurons in all layers of MEC receive inputs that anatomically are confined to superficial MEC layers, raising the question on how to interpret laminar input specificity. Thirdly, early in development, before eye opening, PrS and PaS already send functional inputs to MEC. Therefore PrS and PaS may be of importance in the development of functionality of MEC and this may point to an early relevance of the head directional/vestibular system to functionally organize the adult navigational system in the rat.

#### **6.3.1 Intrinsic network of the lateral- and medial entorhinal cortex**

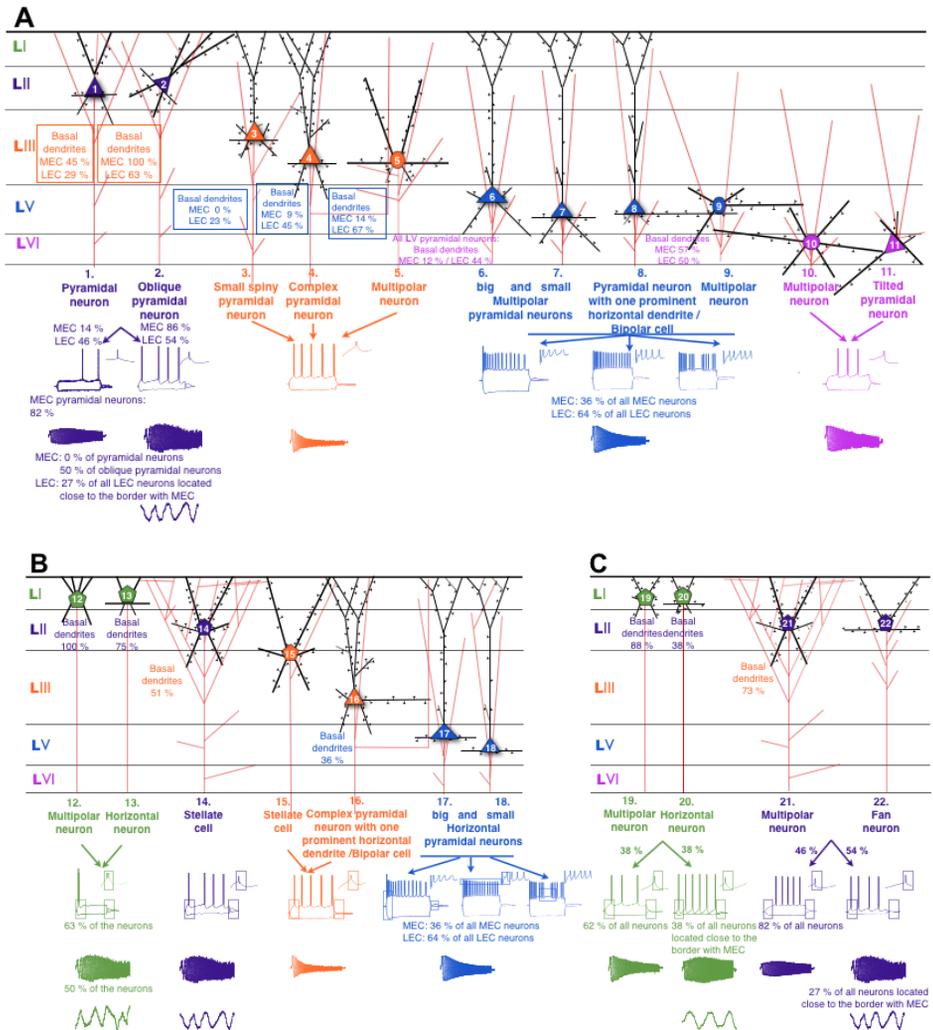
In Chapter 2 and Chapter 3 morphological and physiological properties of single neurons are described in order to assess whether differences in properties can help explaining the differences in function of MEC and LEC. Based on our findings we hypothesize that morphological and physiological properties on their own cannot explain the functional differences between MEC and LEC.

## **6**

##### **6.3.1.1 Differences in principal neuronal properties cannot explain the full extent of the reported functional differences between the two entorhinal subdivisions**

All data show that the majority of principal neurons do not differ between LEC and MEC, except for LII neurons (Fig. 1).

This is different from what was expected from functional data. Since in addition, both input and output projections are different for MEC and LEC our prediction was that the respective intrinsic networks might differ as well. The fact that except for LII, the morphological and examined physiological properties of MEC and LEC principal neurons show only minor differences (Fig. 1), suggests that differences in functionality are more likely due to the differences in afferent and efferent connections. However, differences in the overall organization of intrinsic inhibitory networks cannot be excluded as relevant contributors.



**Figure 1:** Schemes summarizing similarities and differences in MEC and LEC morphological and physiological properties. **(A-C)** Every color represents another layer. LI is in green, purple LII, orange LIII, blue LV and pink LVI. The percentages of basal dendrites of a specific neuron type reaching into the deeper cell layer are written on the left side of the corresponding schematic neuron drawing in the color of the layer the dendrite is reaching. **(A)** Scheme shows schematic drawings of neurons located in different cell layers of MEC and LEC with major differences in findings indicated by a colored rectangle around the text. **(B)** Schematic drawings of neurons being specific for MEC and **(C)** LEC. The corresponding name and physiological properties **(A-C)** of the morphological neurons types drawn are written and presented below the schemes. Below the written name, the responses of a neuron to a positive and negative current step are shown. Middle, a typical voltage response to a ZAP protocol, reaching from 0-12 Hz. Bottom, membrane potential oscillations just below firing threshold if existent in the corresponding cell type. Regions of interest, which define the corresponding layer/ cell type are encircled.

Differences in physiological properties in LII may of course explain parts of the functional differentiation between MEC and LEC but it is relevant to reiterate that the major physiological properties within LII do not change abruptly at the border between MEC and LEC.

### 6.3.1.2 Neuron properties and the implications for information processing

The majority of neurons located in MEC and LEC LI-LV have their apical dendrites traveling to the pial surface, having a dendritic tuft in LI and LII. Therefore, LI-LV neurons are able to receive inputs entering LI and LII. LI neurons do not have dendrites deeper than LII and consequently they cannot receive inputs entering deep to LII. Most LII neurons have basal dendrites in LIII and likely will receive projections entering LIII in addition to those that enter in LI and LII. LIII neurons can potentially either receive inputs entering purely in superficial layers or those that enter in LI-LV. The data presented in Chapter 4 support these conclusions since principal neurons in all three MEC layers are recipients of inputs that originate from PrS and PaS. LV neurons seem to have a special role in the entorhinal network since they can receive inputs entering all EC layers. In case of neurons in LV of MEC, it has been shown that they are also among the postsynaptic targets of projections from PrS and PaS<sup>23</sup> (Chapter 4). The tuft of LV apical dendrites may signal temporal coincidence between inputs<sup>24</sup> and when combined with a back-propagated action potential<sup>25</sup> these inputs may result in a back-propagation-activated calcium spike. This way, EC LV pyramidal neurons may integrate PrS and PaS inputs with those terminating on their basal dendrites that represent hippocampal and retrosplenial information<sup>26-30</sup> and can work as a mismatch system comparing learned with unlearned stimuli as CA1 does in the hippocampus<sup>31, 32</sup>. Within LIII, LV and LVI there are so called multipolar principal neurons located, with their postsynaptic sites remaining solely in the layer the soma resides in, or one layer above and below. It is likely that these principal neurons are local integrators that process local EC inputs of the same layer and project it to whole EC. Multipolar neurons are therefore different from pyramidal neurons which process inputs of several layers, up to LI, and may be global integrators. Special for LV, but also LVI, is that there are principal neurons with their dendrites confined to deep MEC layers, the angular bundle, subiculum, PrS or PaS. These neurons are special in that they may process information passing the angular bundle, deep subiculum, PrS, PaS and integrate it with ongoing activity in EC<sup>33</sup>. Therefore they may play a specific role in the network as inter-area communicators<sup>34</sup>.

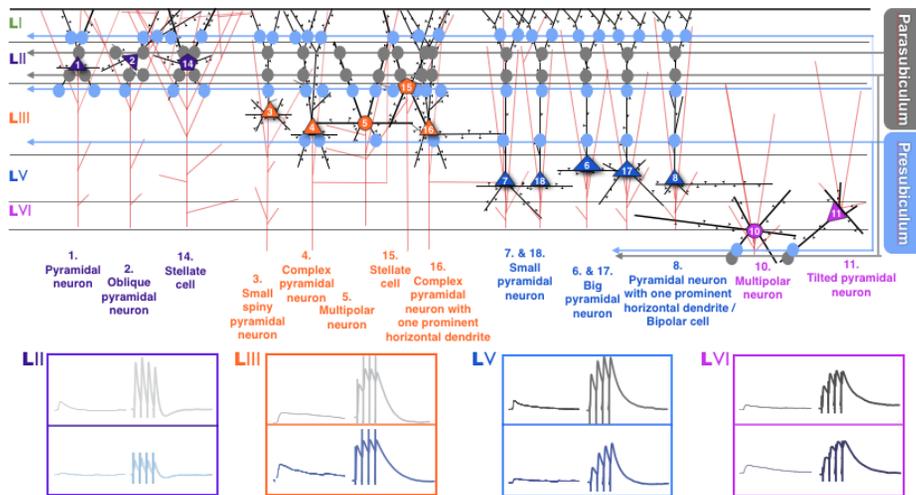
The local intrinsic projections of EC principal neurons are layer specific. Axonal projections suggest that principal neurons in superficial layers are mainly

connected to neurons in superficial layers, whereas LV principal neurons send their axons to deep and superficial layers. The possible postsynaptic targets for these intrinsic connections have only been sparsely addressed experimentally. LII principal cells are more likely to connect with interneurons<sup>15, 35, 36</sup>, whereas LIII neurons have been shown to connect with peer principal neurons<sup>36</sup>. LV neurons are able to connect to superficial principal neurons<sup>37-40</sup> and with peer LV neurons<sup>36</sup>. The axons of LVI principal neurons remain within deep MEC but also reach LII. Communication with LV principal neurons has been confirmed<sup>34</sup>.

With the exception of MEC LII principal neurons, the morphology of principal neurons does not correlate with their physiology even though a diversity of morphological and physiological neuron types exists, as shown in Chapter 2 and Chapter 3. This indicates that besides neuron morphology other factors, such as specificity in inputs and outputs, and most likely specific interactions with neuromodulatory systems and differences in genetic makeup resulting in striking differences in expression and distribution of certain classes of receptors, may constitute an important aspect in defining neuronal physiology and function<sup>41-48</sup>. A few striking examples of these intricate and complicated interactions are seen in EC. One example is the reported correlation between resonance and sag properties of LII stellate cells, and the presence of particular types of h-channels, together with the dependence of these cell type specific characteristics on modulatory factors<sup>46, 49, 50</sup>. Furthermore within LIII, principal neurons exist that have these h-channels on their dendrites but do not show the h-channel specific characteristics with somatic recordings, as LII stellate cells do. This might be explained by differences in the location of the receptors on dendrites<sup>51</sup>. Principal neurons in LVI are unique in that they are the only neurons in whole EC reacting to nicotine application<sup>34</sup>. As a last example, principal neurons in different layers of EC show differences in response properties to muscarinic cholinergic modulation<sup>45, 52-54</sup>.

Irrespective of this obvious lack of correlation between neuron morphology and physiology, the layer specific morphology and physiology as present in the different EC layers (Chapter 2 and Chapter 3) suggest layer specific integration of incoming information<sup>43, 55</sup> since kinetics of evoked excitatory postsynaptic potentials (eEPSPs) measured in the soma are influenced by intrinsic membrane properties<sup>55</sup>, by the dendritic electrotonic properties<sup>56, 57</sup> and differences in the receptors activated<sup>58-60</sup>.

In Chapter 4 we showed that the integration of inputs for the different MEC cell layers is layer- and frequency-dependent in response to single and repetitive PrS and PaS activation (Fig. 2).



**Figure 2:** Monosynaptic inputs from PrS and PaS converge on principal neurons in MEC. Schematic drawings of principal neurons located in different layers of MEC. Every layer is colored differently, LII purple, LIII orange, LV blue and LVI pink. Every dot represents a potential synapse formed with (blue) PrS or (grey) PaS. The name of the corresponding neurons is written below the schematic drawing. In boxes, the average voltage responses of LII-LVI principal neurons in response to a 1 Hz (left) and 20 Hz (right) stimulation of PaS (grey colors, top box) and PrS (blue colors, bottom box). These data indicate that principal neurons located in different layers are influenced differently by incoming stimuli. For example, for LII principal neurons, which showed mainly depression in response to all stimulation frequencies except for 100 Hz, the data suggest that only a summation of synaptic inputs within a very precise time window may induce action potential firing and subsequent information flow from LII. For all other layers stimulation with almost all frequencies lead to facilitation, indicating that high frequencies bursts may be required to trigger an action potential at the end of a burst and thus transfer of information to postsynaptic targets.

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These data indicate that principal neurons located in different layers are influenced differently by incoming stimuli. For example, for LII principal neurons, which showed mainly depression in response to all stimulation frequencies except for 100 Hz, the data suggest that only a summation of synaptic inputs within a very precise time window may induce action potential firing and subsequent information flow from LII. For all other layers stimulation with almost all frequencies lead to facilitation, indicating that high frequencies bursts may be required to trigger an action potential at the end of a burst and thus transfer of information to postsynaptic targets.

These data thus imply for example that the efferent copy of inputs from the head directional system, as represented by PrS and PaS, which is transmitted by LII neurons to DG and CA3, will differ from the copy transmitted to CA1 and

subiculum by neurons in LIII and to the hilus by neurons in LV. The latter may transmit a similar copy to a number of cortical and subcortical targets including the amygdala and the nucleus accumbens, whereas LVI neurons will transmit yet another efferent copy to the midline thalamus<sup>33</sup>. The functional relevance of this scheme and whether this may hold true for LEC as well remains to be studied. The latter possibility though is very likely in view of the striking similarities between neuron properties in both LEC and MEC, except for LII.

### **6.3.1.3 Entorhinal bands may define the properties of individual LII neurons better than the medial and lateral entorhinal cortex border**

The morphology of principal neuron types in LII is rather similar in MEC and LEC, while physiological differences exist. Analyzing the changes in typical physiological properties of MEC such as the sag ratio, membrane potential oscillations, resonance frequency, time constant, and resistance along the mediolateral axis of MEC and LEC, revealed that these properties change consistently from medial MEC to lateral LEC and not abruptly at the border between MEC and LEC. Thus the MEC/LEC border seems not to be the defining factor for individual neuron properties. Instead, it may be that these individual properties are better defined based on existing gradients in the organization of EC. One obvious candidate feature is the presence of three bands in entorhinal cortex, defined on gradients in connectivity with the hippocampal formation and preferential intrinsic wiring may define individual neuron properties better<sup>9, 61</sup>. Functionally similar neurons or neurons processing inputs in a similar way can potentially be found in medial LEC and lateral MEC. In combination with band specific inputs this can lead to differences in functionality between bands<sup>2, 62-66</sup>.

### **6.3.2 The role of pre- and parasubicular monosynaptic inputs on medial entorhinal cortex principal neurons in all cell layers**

For Chapter 4 and Chapter 5 we choose to examine pre- and parasubicular inputs to MEC because neurons in all MEC layers code for head direction. Directional information in turn is likely transferred to MEC LI-LIII via the PrS and PaS. We investigated whether principal neurons in deep layers, that have their postsynaptic sites in superficial layers (Chapter 3), receive PrS and PaS inputs. In Chapter 2 and Chapter 3 we showed that principal neurons in different EC layers show layer specific membrane properties, which may lead to layer specific integration abilities<sup>55</sup>. Therefore we also studied, whether these inputs lead to layer specific integration.

Monosynaptic inputs from PrS and PaS converge on principal neurons in all layers of MEC. Principal neurons in all layers of this multilayered cortex are functionally coupled through at least two inputs common to all layers, which is different to other cortical areas receiving layer specific inputs<sup>67, 68</sup>. It is also different from the classical view of the EC extrinsic connectivity suggesting that superficial MEC layers receive the inputs from cortical and subcortical areas, whereas the deep MEC layers receive the hippocampal output<sup>1</sup>. In light of these findings, the role of individual EC layers, especially of LV, needs to be reevaluated.

### 6.3.2.1 The role of convergence of inputs from PrS and PaS on MEC principal neurons

The inputs of PrS and PaS converge on single neurons. Convergence of inputs of different areas on one principal neuron has been described earlier<sup>69-72</sup>. The role of convergence of inputs from PrS and PaS is not unraveled in this thesis. Up till now it is also not known what the functional difference of PrS and PaS is. Consequently, it is difficult to hypothesize about the role of convergent inputs of PrS and PaS on single MEC neurons. So far it is only known that PrS and PaS have similar spatial coding neurons but that a difference in theta coupling exists<sup>8</sup>. Whether this has an influence on how different layers can integrate inputs from PrS and PaS differently is speculative. In general, convergence of inputs can for example lead to potentiation of inputs as has been shown by McNaughton for MEC and LEC inputs entering DG<sup>73</sup>.

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### 6.3.2.2 Development of functional connections from PrS and PaS to MEC principal neurons

In Chapter 5 we described that the inputs from PrS and PaS start around P9 and become fully functional and adultlike around P15, just around the moment that rat pups have their eyes fully open and which is also way before MEC grid cells are adultlike around P30<sup>13, 14</sup>. This makes it likely that PrS and PaS may play an important role in the development and maintenance of MEC functionality even before eye opening<sup>74</sup>. Interestingly, the development of functional connectivity aligns with the development of single neuron properties, suggesting that the membrane properties may influence integration abilities as we have shown in Chapter 4.

It is likely that axonal projections from PrS and PaS do exist before P9 when we start to see the first weak and instable functional connectivity since

preliminary tracing data show that for example projections from PrS and PaS can be anterogradely labeled from P3 onwards (O'Reilly, personal communication). The lack of functional connectivity at the early developmental stages may be due to lack of synaptic transmission or it could signal that the postsynaptic network is not functional yet. However, the fact that local MEC stimulation can lead to intrinsic MEC activity before P9 (Chapter 5) may indicate that the intrinsic network is already functional at these early ages, which allows us to argue that a lack of synaptic release in PrS and PaS axons is the reason that we do not see functional effects of inputs that are physically present as indicated by anatomical data. It has been well established that the wiring of the brain may be structured normally without the presence of any synaptic release as shown in mutant mice that lack the expression of Munc-18, a protein that is obligatory in vesicle exocytosis<sup>75, 76</sup>. Alternatively, it may be that the inputs from PaS and PrS are different from local connections in that they may depend more on the development of distal portions of the dendrite of their target cells. The latter suggestion may be in line with observations that the spine density on P5 EC LII neurons is much less than the spine density on P14 neurons. Also the neuron morphology in such young animals seems to be immature in that there is an overall lack of well-developed distal dendrites (O'Reilly, personal communication).

#### 6.4 Future directions

The aim of my thesis was to provide a systematic study on the organizational principles that govern the intrinsic networks of LEC and MEC such as to contribute to possible new insights in how the intrinsic network may process incoming inputs and how these may help in understanding the role of EC in learning and memory<sup>77, 78</sup>. My work focused on a systematic comparison of properties of principal neurons present in all layers of LEC and MEC. In addition I have looked at the relevance of one of the most striking organizational features of EC, the laminar terminal distribution of most of the major inputs. For the latter aspect I selected the well-described and functionally understood inputs from PrS and PaS.

With respect to the first aspect it is clear that I neglected a major second class of neurons, the inhibitory interneurons. The inhibitory network likely plays a crucial role in balancing the excitatory network<sup>79, 80</sup>. Moreover, the detailed studies in the hippocampal formation as well as in some neocortical domains point to the high specificity of the interneuronal system. Interneurons may be targeted selectively by inputs and they may themselves only innervate certain classes and components of neurons, like specific dendritic domains, the soma,

or axonal portions<sup>79, 81-86</sup>. Therefore there is a clear need for a comprehensive study for both LEC and MEC on which types of interneurons exist, and the specifics on the related input and output relationships.

Interconnectivity between neurons was also not studied. Interconnectivity between entorhinal principal neurons in LII-LV has been examined in few studies<sup>15, 36, 87</sup>. These studies are not exhaustive and unfortunately also produced contradictory results as far as LII neurons are concerned. Moreover, they did not address the potential role of interneurons in intralaminar connectivity. Information on interlaminar connectivity is also still largely lacking except the established connectivity from neurons in LV to LIII and LII<sup>38, 40</sup> and LVI to LV principal neurons<sup>34</sup>. We propose that our data on local axon distributions of principal neurons in superficial layers suggest the potential of interactions of superficial principal neurons with apical dendrites of deep neurons, in line with some evidence obtained with optical imaging<sup>39</sup>. Information on connectivity will be instrumental for the construction of more reliable conceptualizations on how cingulate, retrosplenial and hippocampal inputs entering deep EC layers<sup>29, 88-90</sup> are transferred to superficial layers and the same holds for the inputs entering purely superficial layers<sup>62, 91-94</sup>.

With respect to inputs entering superficial EC, a further set of inferences can be made that as yet lack experimental support. It would be of interest to establish, whether the observations of PrS and PaS afferents also are valid for input to MEC from the postrhinal cortex, which shows a preferential superficial distribution as well<sup>2, 91, 92</sup>. Likewise in LEC, superficially distributing inputs from the perirhinal cortex, the amygdala and certain olfactory domains likely target principal cells in all layers and not only those in the preferred layer of terminal distribution<sup>62, 92-95</sup>.

Having the full set of data on the intrinsic networks of both LEC and MEC available would enable the generation of biologically plausible models for entorhinal functioning<sup>96-100</sup>. Current models mainly focus on the emergent properties of grid cells in MEC and they strongly depend on a number of required properties of the network. The attractor network class models require the presence of a hidden layer that receives inputs from the 'ring-attractor' network (here grid cell LII) and which projects back to the ring attractor network too, but asymmetrically<sup>96-98</sup>. Our data with neurons in LIII and LV having one axon leaving the soma traveling to superficial layers next to the soma and another one innervating more distant portions of MEC support the notion that principal neurons in LIII and LV may innervate neurons in LII through asymmetric connections and may provide the anatomical underpinning for a similar asymmetric pattern recently reported in an *in vitro* study<sup>22</sup>.

The oscillatory interference models require next to intrinsic and extrinsic oscillations with a certain difference in frequency, at least inputs to one grid

cell from three neurons with a specific directional code<sup>99, 100</sup>. Traditionally, inputs from PrS and to a lesser extent those from PaS have been implicated as relevant players for directional input to MEC<sup>101</sup>. Our connectional and developmental data certainly provide corroborative support for the notion that PrS and PaS play a role in maintaining MEC functionality but obviously also lead to two questions. First, what makes neurons in LII of MEC so specific, that these inputs result in a preferred grid cell *in vivo* phenotype in LII. Second, how come that neurons in all other layers show a mixture of *in vivo* phenotypes that is rather layer aspecific and can also be observed in PrS and PaS<sup>6, 8</sup>.

Regarding the first question, properties of principal LII neurons such as membrane potential oscillations, that are largely absent in neurons in other layers have been implicated as relevant factors for grid cell formation<sup>50, 96, 100, 102, 103</sup>. If so, the here reported gradual changes in properties strongly suggest a change in grid cell properties along the mediolateral axis of MEC, comparable to that reported along the dorsoventral axis<sup>104</sup>. The reported layer specific physiological properties may further contribute to this observed *in vivo* difference but this remains to be established. The absence of a grid cell phenotype in LEC strongly favors the relevance of the contribution made by the inputs from PrS and PaS. This receives further support from the fact that inputs from PrS and PaS to MEC are already functional providing input from adultlike head directional cells when grid cells develop in MEC<sup>13, 14</sup>. Vestibular inputs, known to be crucial for head directional firing<sup>105</sup> do reach brain stem regions upstream of the vestibular nuclei early in embryonic development<sup>106</sup> but whether functional vestibular inputs reach the anterior thalamic complex and the PrS and PaS at an early postnatal stage in rodents is currently not known.

## 6.5 Concluding remarks

This thesis provides an extensive description of lateral and medial entorhinal principal neuron types. The most striking observations are that principal neurons in both entorhinal subdivisions have layer specific physiological and morphological characteristics and that these layer specific properties are remarkably similar, both in morphological and elementary electrophysiological properties between LEC and MEC. The only striking exceptions are found in LII, where the neurons in both subdivisions are markedly different. We further show that neurons in all layers within MEC can receive convergent inputs from PrS and PaS, and that these inputs are functional and stable very early in development. The integration of these inputs is layer- and frequency-

dependent. The layer specific integrative properties likely underlie the functional differences between layers reported *in vivo*<sup>6</sup>. These findings on layer specific integration in MEC can potentially be extrapolated to LEC, except for LII, which is the only layer of these two entorhinal subdivisions harboring principal neurons that show clear differences in physiological profiles and consequently integrative functional abilities. This thus suggests that functional differences between LEC and MEC as observed in the *in vivo* situation most likely are related to differences in physiology of LII principal neurons, complemented by network specific processing of incoming information and possible dependence on different interneuronal circuits as well as differences in the distribution of main modulatory systems. In order to understand the striking functionally different contributions made by both entorhinal subdivisions to the role of the parahippocampal-hippocampal system in learning and memory<sup>107</sup> we need to increase our knowledge on the role of inputs, interneurons and modulatory systems specific for both entorhinal subdivisions.

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