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Introduction and Objectives

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1 Introduction

Our current knowledge of neuroanatomy and neurophysiology shows that functional neuronal networks in the brain are essential for the existence of 'mind'. State of the art techniques allow assessing whether a specific function can be ascribed to a neuronal network. It is however essential to realize that the knowledge about the brain that we have today is based on a long history of discoveries in neuroscience. Hence, I will start the general introduction of my thesis with the title "Layer Specific Integrative Properties of Entorhinal Principal Neurons" with an historical perspective on the function of the brain, focusing on memory formation. This will be followed by an introduction to the entorhinal cortex (EC), and the related parahippocampal-hippocampal system to which EC belongs. The overall structural and connectional description will be supplemented with a number of functional notions, introducing the relationships between the four studies that form the experimental core of this thesis.

1.1 History of Neuroscience: Localization of Mind

Already from 500 B.C. some philosophers suggested that the mind was located in the brain (Pythagoras 500 B.C.; Hippocrates 460 B.C.; Plato 427-347 B.C.; Claudius Galen in the 2nd century), while others were questioning this relationship (Aristoteles 348-322 B.C.; Rene Descartes 1596-1650). In the late 18th early 19th century, Gall (1758-1828) developed the pseudoscience called phrenology. He was the first to introduce the idea of cerebral localization of mental function. Gall divided the brain into 35 separate functions based on craniometry and physiognomy. His theory was proven wrong, but he and his students, among which Spurzheim (1776-1832), were the first who showed that lesions of certain parts of the brain result in cognitive changes.

A century later, Brodmann (1909) divided the cerebral cortex into different parts based on cytoarchitectonic characteristics. Jackson (1875), Broca (1861) and Wernicke (1881) provided additional data supporting the so-called localizationalists view by giving evidence that lesions or stimulations in certain cortical areas result in circumscribed functional effects (for a summary see [1]).

"In that same period Ribot proposed that memory loss was a symptom of progressive brain disease and Jackson showed that selective lesions in the brain induce memory loss" [2, 3] as cited in [4].

In the late 19th, early 20th century, Golgi developed a silver stain that permitted the full visualization of neurons [5]. Ramon y Cajal, as well as Fridtjof Nansen used this silver stain to visualize and study neurons and their connections

extensively and they developed the neuron doctrin [6, 7]. They were the first to mention that neurons are anatomical, physiological, genetic and metabolic units of the nervous system. Cajal drew particular attention to the hippocampal formation (HF) highlighting the structural organization and diverse afferent connections, as well as the striking convergence of inputs onto single dendrites [6]. He was the first to depict the direction of the information flow with dendrites receiving information and axons sending information. In his studies on the cortex, Cajal mentioned a particular part of the posterior temporal cortex that he considered to be strongly connected to HF with fibers that merged in the angular bundle and perforated the subiculum. This part is nowadays known as the entorhinal cortex (EC). Cajal was so impressed by the massive connections that he suggested that the physiological significance of HF had to be related to that of EC. He stated that if this part of the posterior temporal cortex, which he called the sphenoidal cortex/angular ganglion, was visual, so would be the hippocampus.

1.2 History of Memory Localization

It was around 1900, the time of Cajal, Nansen and Golgi, that the first indications for a link between memory disturbances and (para)hippocampal damage were made public. Bekhterev reported specific memory impairments in patients with softening of the hippocampus and the areas surrounding it [8]. Yet until the 1950th HF and the parahippocampal region (PHR) were considered to be most important for olfactory functions [3, 9, 10], emotions [11] and attention [12, 13].

In 1949 Donald Hebb published 'The Organization of Behavior: A Neuropsychological Theory' [14]. Today's understanding of memory formation at the cellular network level dates back to Hebb's work. He was the first to suggest that a cell assembly that is activated together repetitively by one common stimulus will show an increase in synaptic strength due to physical growth. Hebb inferred that repetitive associative stimulation together with physical growth leads to an increase in synaptic weight and long-term memory formation. The cell assembly reactivates when a stimulus related to an already formed memory is represented. Moreover, the order in which cell assemblies activate corresponds to a chain of thoughts. Later studies have shown that Hebb was right in that synaptic plasticity exists as an activity-dependent change of synaptic weights. Bliss and Lømo (1973) provided evidence for Hebb's theory and showed in HF that repeated stimulation of cell assemblies results in long-term strengthening of synapses [15] and called this mechanism long-term potentiation (LTP) [16]. Nowadays it is commonly accepted that LTP is the synaptic change underlying long-term memory formation [17-23].

Bliss and Lømo (1973) choose HF to perform their groundbreaking studies, underpinning Hebb's ideas on cellular memory formation, because in 1957 Scoville and Milner directly linked HF, with conscious or declarative memory [24]. Scoville and Milner described a patient called H.M. and nine other patients, who, after bilateral removal of HF and surrounding medial temporal lobe areas to treat their pharmacologically resistant epilepsy, suffered from severe anterograde amnesia, that is, the inability to consciously remember episodes. They further showed that HF and the adjacent PHR play a time-limited role in learning, since most long-term memories from before surgery were still existent. These authors also suggested that a difference between long-term and short-term memory exists, since their patients were still able to memorize for a short period of time. Finally, they demonstrated that other cognitive abilities were functional and not impaired in these patients. This research provided the foundation for some of today's most influential theories on the conscious memory system and did put HF and PHR in the focus of research on learning and memory processes. Subsequent studies have further detailed our understanding by showing that more selective lesions to HF and/or PHR also result in anterograde amnesia in humans [25-29] but in a weaker form to what has been found in H.M. who had a more extensive lesion of medial temporal lobe structures [30].

1.3 Differentiation of Memory

Memory is not uniform but can be divided into multiple types [31] mediated by different loci in the brain. A well-accepted differentiation is between declarative or explicit memory and non-declarative or implicit memory (Fig. 1). Non-declarative or non-conscious long-term memory includes processes as procedural memory (habits and skills), priming, non-associative learning, and classical conditioning. Declarative or conscious memory can be subdivided into the memory for events (episodic memory) and for facts (semantic memory). As already mentioned, HF and PHR are key structures involved in declarative memory, and more specifically episodic memory [32].

Episodic memories include information on at least three different components relevant to represent particular episodes. These components contain information on what has happened, where it happened and when it happened (three w-questions). Over the last decades, research has focused on how HF and PHR mediate information storage and retrieval for each of these information components. In addition it has been studied what the individual roles are of the various subdivisions of HF and PHR that are characterized by specific neuronal networks and sets of connections [33]. Although understanding is far from complete, there is a general acceptance that individual components

of HF and PHR contribute in a unique complementary way, to the final outcome, referred to as episodic memory.

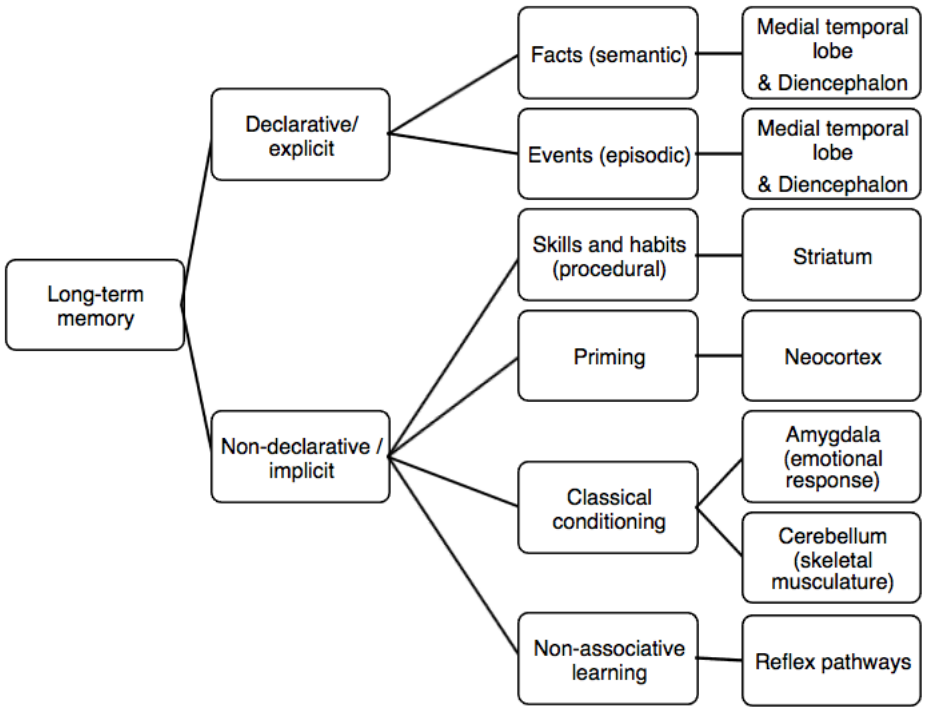

Figure 1: Taxonomy of long-term memory systems together with specific brain structures involved. (Adapted from [34]).

1.4 The Hippocampal Formation

The hippocampal formation (HF) is a cortical structure, which in the rat is C-curved with the rostrodorsal part reaching the septal nuclei of the basal forebrain and the ventral part touching the amygdala (Fig. 2A). The long axis of the hippocampus is called the septotemporal or dorsoventral axis. The axis that runs perpendicular to the long axis is generally referred to as the transverse or proximodistal axis.

Cytoarchitectonic and connectional data support a by now generally accepted subdivisional scheme for HF. It is subdivided into the hippocampus (proper), the dentate gyrus (DG) and the subiculum. The hippocampus in turn is further subdivided into three fields of the Ammon' horn or *cornus ammonis* 1-3 (CA 1-3; Fig. 2B) [35]. In all subareas of HF, three cortical layers are differentiated, in line with the typical organization of the allocortex [36].

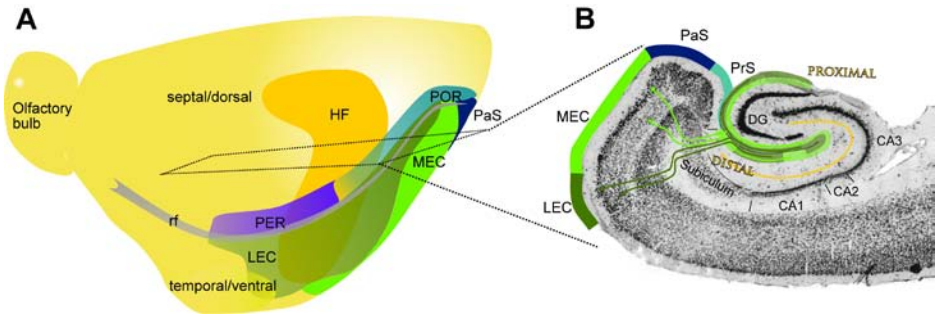

Figure 2: Representation of HF and PHR in the rat brain. **(A)** Schematic drawing of a slightly tilted lateral view of the left hemisphere of a rat brain showing position and extend of MEC (light green), LEC (dark green), perirhinal cortex (PER; light purple), postrhinal cortex (POR; light blue), parasubiculum (PaS; dark blue), rhinal fissure (rf; grey) and HF (yellow). Indicated with stippled lines is the approximate position of a representative horizontal section illustrated in (B). **(B)** Horizontal section illustrating entorhinal-hippocampal connectivity. (Adapted from [37]).

Within HF, granule cells of DG project massively to CA3 by way of so-called mossy fibers. This projection also targets mossy cells in the inner layer of DG, also referred to as the hilus of DG. The latter cells give rise to a strong ipsilateral association and contralateral commissural projection. The pyramidal cells in CA3 form recurrent, associational connections and send the Schaffer collaterals to CA1. Region CA1 projects to the subiculum and deep layers of EC. The subiculum also projects to deep layers of EC. CA1 and the subiculum are the main output regions of HF, although CA3 does contribute extensively to the projections to the septal complex [38], see also [39]. The most extensive cortical input to HF originates in EC. Initially this projection was thought to be almost exclusive to DG, forming the main excitatory input to the intrinsic chain of excitatory connections described above, also known as the trisynaptic circuit [40, 41]. More recently it was firmly established that the projections from EC actually target neurons in all subdivisions of HF (Fig. 2B; for review see [42]).

1.5 The Parahippocampal Region

The parahippocampal region (PHR) is part of the cortical mantle positioned in between HF and the isocortex. It consists of EC, presubiculum (PrS), parasubiculum (PaS), perirhinal cortex and postrhinal cortex, referred to as parahippocampal cortex in primates (Fig. 2A). All these areas are strongly connected and share a number of other cortical and subcortical connections. However, differences are also apparent. For example, whereas the perirhinal and postrhinal cortex are reciprocally connected with EC, PrS and PaS provide main inputs, but receive substantially weaker return projections. Together PHR

is considered as a series of cortical structures that mediate extensive contacts between HF and the rest of the cortex [33, 36, 37, 43, 44]. EC (Brodmann area 28) can be further subdivided into a lateral (LEC; area 28a) and a medial (MEC; area 28b) subdivision based on cytoarchitectonic criteria [45]. Later it has been shown that these cytoarchitectonically different divisions issue two components of the perforant path projection to DG, which show both organizational and functional differences (Fig. 2B) [36, 46-50]. Additionally, LEC and MEC differ in their cortical and subcortical connectivity, and have different connections with other parts of PHR (Fig. 4) [36, 51-54].

To summarize, convergent cytoarchitectonic, connectional and functional criteria indicate that within EC at least two subdivisions can be differentiated, LEC and MEC. Such a division into two connectionally and functionally different parts in EC apparently holds true in non-human primates and in a large number of other mammalian species [52, 55, 56].

1.6 Declarative Memory, the Hippocampal Formation and Entorhinal Cortex

Already Marr suggested that a functional differentiation exists between hippocampal subareas [57]. Today it is well established that within HF and PHR different subareas play a distinct role in answering the three w-questions: where, when and what. Due to differences in the afferent, the efferent and the intrinsic network, within each subarea of PHR and HF a differentiation may exist [53, 58-61]. In short, within HF the following functional differentiations have been proposed. DG neurons, which receive convergent monosynaptic inputs from MEC and LEC [36] are thought to play a major role in pattern separation of spatial (via MEC) and non-spatial (via LEC) inputs [62-66].

Field CA3, receiving convergent inputs from MEC and LEC and mossy fiber input from DG, displays multiple recurrent excitatory connections, and is believed to play a role in pattern completion and associating multimodal information [20, 67-71]. These two structures together may thus provide the system with a memory module capable of efficiently storing and recalling memories.

CA1, the endpoint of the tri-synaptic loop, is supposed to form the basis for association of different stimuli and memory formation within hippocampus [40, 41]. This process depends on the unique position of CA1 receiving direct EC input and indirect inputs via the DG/CA3 system [72]. CA1 is in a position to compare memorized input coming in via CA3 with that of direct input from EC and thus to evaluate whether learned (memorized) and unlearned (new) information match [73].

It is important to note that the above is a simplified description of one view of the functional organization of HF and that many other accounts have been put

forward that include different and much more detailed descriptions. One such example is that in order to learn or remember the temporal order of events, CA1 and CA3 are important in case of spatial tasks [74, 75], whereas for non-spatial tasks only CA1 is needed [76, 77].

1.6.1 Localization of Spatial Memory ('where')

Spatial memory is a form of episodic memory that answers the where question, which can be tested relatively easily in humans and animals. It has been shown that the right hippocampus of taxi drivers, who train and use their spatial memory a lot, shows an increase in size and activity, specifically while the taxi drivers had to recall spatial information [78-80]. A taxi driver with lesioned hippocampi could still recall previously learned major streets of the map but not small streets. This indicates that retrieval of at least details of spatial memories learned in the past remain dependent on the hippocampus [81].

The contributions of individual subareas of HF and PHR to spatial memory processes are primarily studied in animals since the resolution of human imaging is still insufficient. Although it has to be said that recent developments are very promising, providing indications that in the near future the contributions of identified areas to memory processes can be studied reliably [82-85]. Fortunately it appears feasible to compare and extrapolate findings of animal studies with those of human studies because the neuroanatomy of the parahippocampal-hippocampal system in human, monkeys and rodents is strikingly comparable [36, 56]. Moreover, there is extensive knowledge on the importance of HF in particular and PHR to a somewhat lesser extent in spatial memory in rodents.

Spatial memory in rodents is commonly studied by evaluating performance on specific HF and PHR dependent task, such as the radial arm maze [86] or Morris water maze [87]. The radial arm maze consists of a platform from which a certain number of arms extend. The rodent has to remember which arm it visited during the trial or during a second exposure, to efficiently find a food reward at the end of the arms. The Morris water maze tests whether a rat can navigate and remember the location of a rescuing hidden platform in a water bath, using distal landmarks. Lesions of EC or HF in rodents leads to impairments in both tests [88-95].

A fundamentally different way to probe the functional relevance of HF and PHR is to record single neurons while a rat is exploring an environment. In initial studies it was observed that individual neurons in HF fire action potentials only when a rat is at a certain location in space and thus code for distinct spatial fields within an allocentric reference frame. These neurons became known as place cells [96, 97]. More recently, recordings using similar

behavioral designs have been made in almost all PHR components with striking results.

In MEC, neurons have been observed that code information about position (grid cells), head direction (head direction cells), borders (border cells), and also neurons that code for certain conjunctive properties such as grid and directionality [98-101]. In MEC position coding neurons are called grid cells since one neuron codes for multiple positions with regular distances forming a hexagonal grid tessellating the whole environment (Fig. 3).

No such spatially modulated cells have been observed in LEC [102]. In contrast, neurons in LEC tend to fire throughout the environment and react stronger to object identity, context, odor-place or object-context associations [103-108].

Other PHR regions, such as PrS and PaS, which are important input structures for MEC [51], also contain spatially modulated neurons like grid cells, head direction cells, border cells and various forms of conjunctive cells [109, 110].

Much less is known about the functional cell types in the remaining components of PHR, the perirhinal and postrhinal cortices. In humans the perirhinal cortex, is thought to be involved in polymodal sensory processing, particularly when a visual stimulus is highest in complexity [111-113]. The human homologue of the postrhinal cortex, generally referred to as parahippocampal cortex, has been strongly implicated in processing of spatial information [114], see [53]. In rats, strikingly comparable findings have been reported using activity measures based on changes in immediate early gene expression, indicating increased activity in perirhinal cortex when rats were exposed to object discrimination versus increased activity in postrhinal cortex when tasks had a spatial demand [115-118].

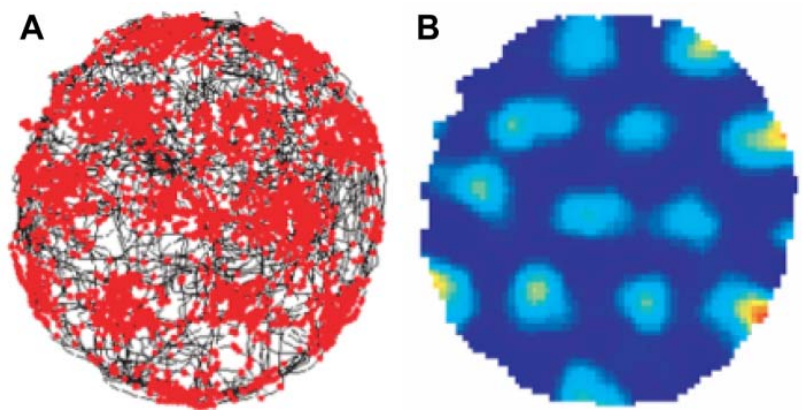

Figure 3: Firing fields of a grid cell in MEC of the rat. **(A)** Black line indicates the running path of a rat in a circular environment with a 200 cm diameter. Red dots represent action potentials of a single neuron in MEC clustering at multiple locations forming a hexagonal grid. **(B)** Firing rate plot of the same neuron as presented in (A). (Figure courtesy of E.I. Moser and M-B. Moser).

1.6.2 Entorhinal Cortex Bands

Connectional studies have demonstrated that EC can be subdivided in a way that is very different from the more traditional differentiation between LEC and MEC. The organization of EC projections to HF, corroborated by the data on intrinsic EC connectivity led to the notion that within EC a lateral, intermediate, and medial band could be differentiated [119-121] (Fig. 4).

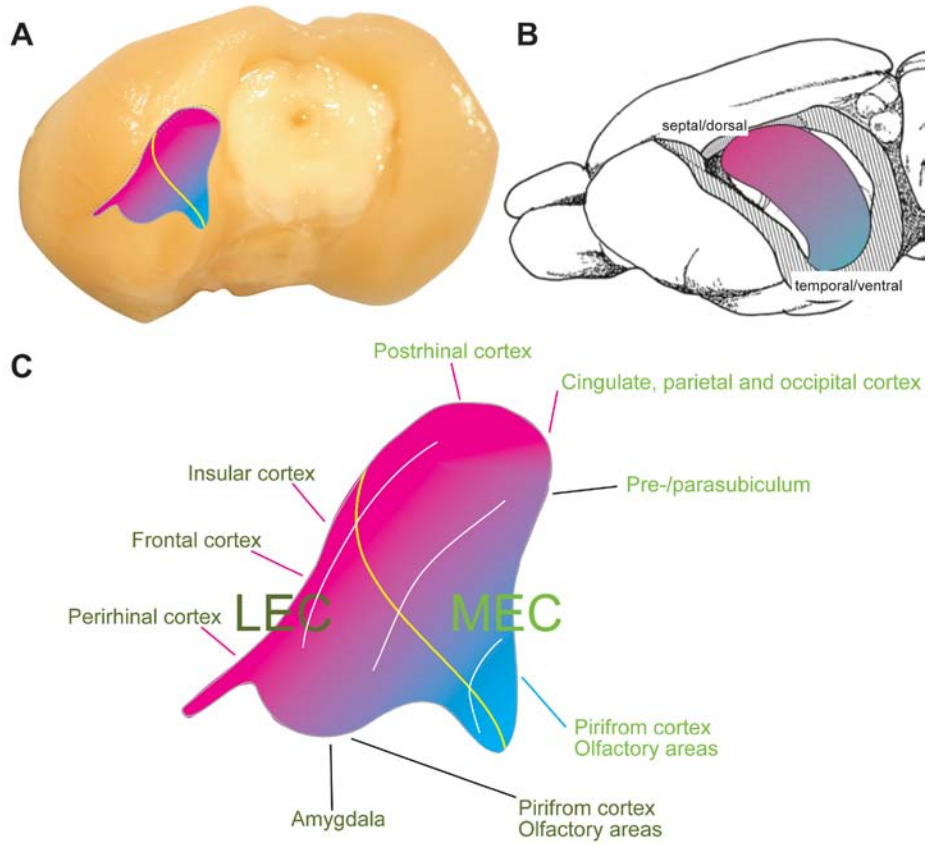

Figure 4: Connectivity of EC with the hippocampus and other cortical areas. **(A)** Picture taken from the caudal part of the rat brain with the cerebellum removed. **(B)** Schematic drawing of the rat brain with part of the cortex removed so that the hippocampus located below the cortex is exposed. (A-B) Representation of the topographic arrangement of entorhinal-hippocampal reciprocal connections. (A) A dorsolateral band of EC (magenta) is preferentially connected to the (B) dorsal hippocampus. Increasingly, more ventral and medial bands of EC (purple to blue) are connected to increasingly more ventral levels of the hippocampus. Yellow line in (A and C) indicates the border between LEC and MEC. **(C)** Enlarged EC, taken from (A), indicating the main connectivity of different portions of EC. Brain areas preferentially connected to MEC are in light green and those to LEC are in dark green. The color of the lines indicates preferential connectivity to the dorsolateral-to-ventromedial bands (magenta to blue) of EC or that no preferential gradient is present (black). (Adapted from [37]).

In rats interconnected portions of LEC and MEC close to the rhinal fissure, referred to as the dorsolateral band of EC, are connected to the dorsal part of HF. Interconnected neurons in the intermediate EC band connect to the intermediate HF, whereas the most medially interconnected band of EC is primarily connected to the ventral HF (Fig. 4) [48, 120, 122-125]. All three bands thus comprise portions of both MEC and LEC that are interconnected within the confines of the band. Connections that link different transverse (or mediolateral) regions of EC, thus providing connectivity between these bands, are rather sparse [120, 122, 123, 126, 127]. A similar organization has been observed in cats [48] and in non-human primates [126, 128].

The associational connections within the bands originate in both superficial and deep layers. Projections originating from layer II (**L**II) and layer III (**L**III) tend to terminate mainly in the superficial layers, whereas projections originating from the deep layers terminate both in the deep and superficial layers. Whether these projections contact excitatory or inhibitory neurons is not unraveled, yet [120, 122, 123, 129-131].

The overall organization of the longitudinal intrinsic connections thus implies that at the level of EC integration across input modalities may occur within each band and this is in line with reports that in the monkey and rodent EC, laterally positioned single neurons respond to different types of sensory inputs [103, 105, 106, 132]. Whether the anatomical border between LEC and MEC defines the morphology and physiology of neurons or whether it is more likely that the bands define the properties is one of the questions studied in Chapter 2 and Chapter 3.

1.6.3 Entorhinal-Hippocampal Topographic Interactions and Memory

As mentioned in the previous section, the bands in EC were initially defined based on the organization of the reciprocal connections with HF. These will be described in more detail in this section, followed by an account of the potential functional relevance of this topographical organization.

Both LEC and MEC project to all parts of HF [36], and although these projections show different termination patterns (Fig. 2B), they also share a number of features. Entorhinal fibers synapse most often onto the dendrites of principal neurons, i.e. on spines, where they form asymmetrical, excitatory synapses. Entorhinal fibers also terminate on inhibitory interneurons, forming both putative excitatory as well as inhibitory synapses with their dendrites [133-136].

In DG, entorhinal axons distribute largely to the outer two-thirds of the molecular layer. It has been noted that differences between species may exist with respect to the precise terminal distribution in relation to the origin of these projections in either LEC or MEC [128, 133]. The projections to DG arise largely from neurons in **L**II. However, projections that arise from deep layers have

been systematically observed, and it is likely that these deep originating fibers show a differential terminal distribution, largely innervating the inner molecular layer and the hilus of DG [137], see also [133]. The same cells in **L**II also form the main origin of the projection that distributes to the outer portions of stratum lacunosum-moleculare of CA3 and CA2 [39, 133]. The fact that inputs from LEC and MEC converge on apical dendrites of DG and CA3 neurons indicates that the same neuron can receive both types of inputs, likely representing non-spatial and spatial information, respectively [49, 138].

The organization of EC-CA1 and EC-subiculum projections is very different from that described above in case of DG and CA3/2. In all species studied, the projections to CA1 and the subiculum originate from principal neurons in **L**III of both MEC and LEC. The terminal distributions of both projection systems exhibit a transverse topography. The rostral EC in the monkey and LEC in the rat projects to the region around the border between CA1 and subiculum (distal CA1, furthest away from DG, and proximal subiculum, closest to DG) whereas caudal EC in the monkey, and MEC in the rat projects to proximal CA1 (close to DG) and distal subiculum (far from DG) [128, 139, 140]. The CA1-subicular projections are topographically organized along the transverse or proximodistal axis as well, such that parts of CA1 and subiculum that receive comparable inputs, that is either from LEC or from MEC, are connected to each other [141-143]. Finally, the projections from CA1 and subiculum back to deep layers of LEC and MEC grossly reciprocate the incoming projections [124, 144]. These data thus indicate that the entorhinal-CA1-subiculum circuitry exhibits a high degree of fidelity and suggest that this circuitry may permit a highly ordered processing of information. Although the functional relevance of this precise organization needs yet to be established, in CA1 differences in spatial properties along the proximodistal axis have been found related to the differential distribution of inputs from MEC and LEC to proximal and distal CA1, respectively. Place cells in the proximal part, receiving input preferentially from MEC carry more spatial information and are more specific than place cells in distal CA1, receiving input selectively from LEC [128, 139, 145]. The fact that place cells are present along the transverse extent of CA1 likely results from the fact that CA3 provides an evenly distributed input that converges with inputs from EC [136]. It is thus conceivable, as has been reported, that place cells in CA1 also react to the physical characteristics, the odor, or the task that needs to be performed at the places visited [138, 146-154].

Additional research has addressed the functional relevance of the topographical organization of the reciprocal entorhinal-hippocampal connections along the long axis of HF. Although the orientation of the hippocampus in various species is quite different [48, 55] the structure has an impressive length in all species, measuring from about 7 mm in mice, through 9-11 mm in rats, all the way up to 4.5-5 cm in human. It has now been

established that in all species studied, entorhinal-hippocampal connectivity is present as described above, the striking difference being that different portions along the long axis of the HF are connected to the different bands of EC (Figs. 4A and 4B). This thus suggests that functional differences in bands of EC will be mapped along the hippocampal long axis or that the opposite mapping may take place. This prediction has been substantiated in that in MEC of the rat changes in firing properties of grid cells in **L**II along a dorsolateral to ventromedial axis are related to corresponding changes in firing properties of place cells in HF. In dorsolateral parts of MEC, the distances between grid fields are smaller and show a higher specificity compared to ventromedial MEC [155]. Within region CA3 a gradient in the scale of representation exists with the scale increasing almost linearly from dorsal to ventral hippocampus [156].

Along similar lines, it has been argued that since the three bands of EC are characterized by very different input-output connectivity (see below; Fig. 4C) they will likely be involved in functionally different behaviors and likely so the different parts of HF. Dorsal lesion of HF, like lesions of the dorsolateral band of EC, lead to impaired spatial learning and memory whereas lesions of ventral HF or the ventromedial band of EC do not affect such behavior [74, 91, 157-163]. Lesions of ventral HF or the connectionally related ventromedial band of EC instead lead to reduced fear related responses in a variety of different behavioral paradigms [157, 164-167]. This strong reaction to emotional stimuli might be due to the strong amygdaloid inputs to the intermediate and ventromedial bands of EC and the ventral two-thirds of HF [161, 168].

Of course it has to be taken into account that each band of EC comprises both a LEC and a MEC component, and in view of the striking connectional and functional differences it can be predicted that the effects of lesions that selectively impact one of the entorhinal subdivisions will be different when compared to those following lesions of the other. In line with this it has been shown that blocking lateral perforant path plasticity disrupts novel object recognition but not spatial object recognition in the hippocampus [49].

1.7 More on Entorhinal Cortex Organization

1.7.1 Entorhinal-Cortical Connectivity

Cortical afferents are dominated by piriform input, but input also arises in frontal, cingular, retrosplenial, insular, parietal, and even visual areas (Fig. 4C) [51]. MEC receives major inputs from piriform, cingulate, occipital and parietal cortex [51]. Similar to what was reported for the monkey, in rat projections from the cingulate and retrosplenial cortices preferentially project to

the more caudal portions of the lateral, intermediate and medial bands of EC [36, 169-172]. Cortical efferents are widespread, largely reciprocating the cortical afferents [56, 173-175]. LEC receives mainly olfactory/piriform, insular and frontal input [51, 176, 177].

Regarding entorhinal afferents, it is clear that most show a distribution largely confined to superficial layer I (**L**I)-**L**III with the exception of inputs from medial prefrontal infralimbic and prelimbic areas together with the cingular and retrosplenial inputs that show a striking preference for deep layers of EC [169]. The entorhinal-cortical projections largely arise from deep EC layers, primarily from layer V (**L**V) pyramidal neurons. Possible exceptions are the entorhinal-infralimbic and entorhinal-olfactory projections, which appear to arise in **L**II and **L**III [173, 178], see [37].

1.7.2 Entorhinal-Subcortical Connectivity

EC sends projections to the nucleus accumbens [179-182] and receives inputs from the ventral tegmental area [183]. The entire EC has strong reciprocal connections with the claustrum [184-188]. Additional connections exist with basal forebrain structures, in particular the medial septal nucleus, the nucleus of the diagonal band, and the substantia innominata [183, 184, 189-191]. It is most likely that entorhinal projections to basal forebrain structures arise in **L**II and **L**V.

Entorhinal-amygdala connectivity has been studied in detail in both monkey and rat. Although all parts of EC are connected with the amygdala, the rostral subfields are more strongly interconnected with the amygdala than the caudal subfields. Whereas in the monkey, the primary afferents originate in the lateral and accessory basal nuclei [192, 193], in the rat, the most prominent inputs arise from the lateral, basal, and accessory basal nuclei [194, 195]. Amygdala input terminates primarily in **L**III of the more ventral and rostral portions of EC. The return projection originates predominantly from cells in **L**V of the corresponding entorhinal domain.

EC is connected with thalamic and hypothalamic structures. Major thalamic input arises in midline nuclei, particularly the reuniens, paratenial, and periventricular nuclei [183, 196-199]. Additional but weaker inputs have been described from the anteromedial thalamic nucleus [200], and the ventromedial nucleus of the hypothalamus [201]. In the rat, it has been shown that EC reciprocates the reuniens input [202]. In the monkey, additional projections have been reported to the magnocellular portion of dorsal medial nucleus, the medial pulvinar, and the dorsolateral nucleus [203, 204]. EC also receives inputs from brainstem structures such as the ventral tegmental area, the dorsal and medial raphe nucleus, and the locus coeruleus, [183, 205-207], see [37].

1.7.3 Entorhinal Cortex Lamination

The lamination of EC is considered the prototype of the transition between the three-layered allocortex, such as the hippocampus, and the six-layered isocortex [208]. These layers show cytoarchitectonic and functional differences and main entorhinal afferents and efferents show a layer specific organization, see [209]. The superficial plexiform or molecular layer (**L**I) contains a dense band of transversely oriented fibers and it varies considerably in appearance among the rostrocaudal and lateromedial extent. **L**I contains some interneurons with small cell bodies and a low number of so-called 'stellate' or 'modified pyramidal neurons'. The majority of neurons in **L**II are fairly large principal neurons, making them distinctly different from neurons in **L**II in the adjacent cortical regions with the exception of PaS. In the latter area, neurons of **L**II are as large, or even slightly larger than those of EC, but entorhinal cells stain darker with a Nissl stain. **L**III is a wide layer of loosely arranged, large to medium sized neurons that are predominantly of the pyramidal type. The deep border of **L**III is the cell-sparse fiber layer called lamina dissecans (sometimes referred to as layer IV). The lamina dissecans is generally better developed in MEC although differences between species exist. The adjacent cell layer (**L**V) is clearly stratified and sometimes subdivided into a superficial and deep layer. Superficial **L**V of large to medium-sized, darkly stained pyramidal cells, is sometimes referred to as **L**Va. Subsequent deeper portions of **L**V (**L**Vb/Vc) have an overall stratified appearance and mainly consist of rather small pyramidal cells with moderately dense packing. In the deepest cell layer VI (**L**VI), which is delineated by the white matter, multiple layers can be distinguished, more in particular in primates, see [37].

1.7.4 Neuron types in the Entorhinal Cortex

Within EC many different types of neurons are present [35]. The majority of these neurons are so called principal neurons, which are excitatory, release glutamate at the synaptic terminals and send an axon to structures outside EC [6, 35, 210-216]. The others are inhibitory interneurons that release γ-aminobutyric acid (GABA) at their synapse and have a local axon confined to EC (for review see [209]), although GABA-ergic neurons with long-range projections have been described [217].

Individual neurons differ with respect to their physiology, morphology and interconnectivity and *in vitro* studies in EC differentiated between neurons with respect to the layer the soma resides in, morphological and chemical properties, intrinsic membrane properties and the integration of synaptic inputs [129, 211-216, 218-225]. All these properties are not unique to neurons in EC but

essentially apply to any other part of the cortex, and the detailed understanding of the relevance of these properties is based on studies in many cortical areas (for summaries and selected reviews of articles read [6, 7, 35, 226-234]). These have all been applied successfully in a number of previously published studies on neurons in EC and provide the basis for the extensive description of principal neurons in MEC and LEC as presented in Chapter 2 and Chapter 3 of this thesis. To compliment these chapters a short summary of the morphology and physiology of interneurons of EC is provided.

1.7.4.1 Morphology of Interneurons in the Entorhinal Cortex

Lorente de Nó initially described two cell types in **L**I, horizontal cells and short axis cylinder cells, nowadays known as multipolar neurons (MPNs; Fig. 5) [35]. This latter category constitutes the majority of cells in **L**I, and generally, they are non- or sparsely spiny. MPNs are quite often positive for calretinin (CR), GABAergic, and two types have been described. Small CR positive MPNs [131] and CR positive MPNs with a laterally extending dendritic tree, both mainly located deep in **L**I [131]. From the perikaryon of MPNs three to five short, curved smooth dendrites arise that branch after a short distance and radiate within **L**I, sometimes extending into **L**II [131, 235]. The diameter of the dendritic tree is around 100 µm in small or 150 µm in the other MPNs, respectively. A minority of CR positive **L**I neurons can be glutamatergic or contain calbindin D28K (CB) or neuropeptide-Y (NPY) [236]. Horizontal cells are also located in the transitional zone between **L**I and **L**II [35, 237, 238]. They have a spherical to elongated soma of 13–15 µm. Almost spine-free dendrites extend laterally and spread horizontally within **L**I and superficial **L**II. The non-collateralizing axon travels towards the deep layers to the hippocampus [237, 238]. Horizontal neurons are GABAergic, in LEC some are positive for vasoactive intestinal polypeptide (VIP), whereas in MEC, the dendritic terminals can stain positive for cholecystokinin (CCK) [238-240].

LII mainly comprises excitatory, glutamatergic cells [221, 241-244] staining positively for CB [209, 245] or reelin [245]. Interneurons within **L**II are MPNs, bipolar, basket, and chandelier cells (Fig. 5). MPNs have polygonal, fusiform, or round cell bodies with multiple, sparsely spiny dendrites, extending in all directions, reaching **L**I and deep into **L**III. The axons of local MPNs travel to the white matter but also form local synapses within **L**II [246]. The family of MPNs contains GABA, VIP, substance-P, CCK, somatostatin (SOM), enkephalin (ENK), or in LEC also NPY [240]. The short-axis cylinder cells described by Lorente de Nó are comparable to these MPNs [35]. Sparsely spiny horizontal bipolar cells, although classified as local/interneurons, project to the hippocampus [238, 246, 247]. The soma is located in **L**II at the border to **L**I. The dendrites are oriented horizontally along the border between **L**I and **L**II [246]. Vertically orientated

bipolar cells have a spindle shaped perikaryon continuing into one smooth thin ascending and one descending primary dendrite that branch into thinner dendrites more distally [131]. CR, VIP, and the corticotrophin releasing factor (CRF) have been found in subpopulations of bipolar cells. In LEC also ENK, CCK and NPY has been observed in this class of neurons [238, 240, 246, 247]. Basket cells have small spherical cell bodies with sparsely spiny dendrites that often ramify into **L**I. The extensive axonal arbor is mainly confined to **L**II and forms basket-like complexes mainly around the soma of stellate and pyramidal cells, preferably forming symmetric, inhibitory synapses [218, 240]. Basket cells are likely GABAergic since they stain positively either for GABA or for Glutamic acid decarboxylase (GAD). The most common type is the one that also stains from the presence of parvalbumin (PV), although CCK may also be present. [218, 240, 248]. Chandelier or axo-axonic cells are characterized by vertical aggregations of axonal boutons, called candles, which preferably are located superficial to the cell body. The somata of chandelier cells are medium sized and have very different shapes. The almost non-spiny, poorly ramifying dendrites originate from the basal and apical poles of the somata, displaying a bipolar or bitufted arbor that often stays within **L**II/III. They come in two types, vertical and horizontal chandelier cells. Vertical chandelier cells are restricted to MEC **L**II-**L**III and they issue a vertically oriented axonal tree that is around 200–300 μm wide and 300–450 μm high with the main axonal branch dividing into several collaterals that form the characteristic vertical aggregations within the upper portion of **L**II/**L**III [249]. Horizontally organized chandelier cells are present in both MEC and LEC **L**II-**L**III, and their axonal plexi are smaller (250–350 μm wide and 100–200 μm high) than that of vertically oriented chandelier cells. Chandelier cells are GABAergic, often PV-positive and form symmetric contacts with initial axon segments of principal cells [250-253].

In **L**III, multipolar local circuit neurons, mainly described in MEC, are characterized by wide-ranging apical dendrites that reach the cortical surface, multiple compact basal dendrites, and a prominent axonal arbarization. The axon distributes into **L**I to **L**III but rarely extends into the lamina dissecans or superficial **L**V [211]. Subgroups of this type of MPN contain GABA, CCK, SOM, substance-P and very rarely somatotropin release-inhibiting factor (SRIF), VIP or ENK (Fig. 5) [236, 239, 254]. Another subgroup of inhibitory MPNs has sparsely spiny dendrites that extend towards deep instead of superficial layers. In addition, these neurons have an axon extending locally with some collaterals projecting to and reaching **L**I [129]. Interneurons resembling pyramidal cells, the so-called pyramidal looking interneurons have also been described as Type 3-(Gloveli) or Type I-(Kumar) cells (Fig. 5) [129, 211]. Pyramidal looking interneurons have non-spiny basal and apical dendrites that branch extensively, forming a dense local network in superficial layers with a circular appearance [211]. The apical dendrites often do not reach the pia. The basal dendrites extend

horizontally comparable to the apical dendrites. Pyramidal looking interneurons have a dense axonal plexus in the local vicinity surrounding the cell body, and extending superficially into **L**II [129]. Bipolar cells have been described in **L**III of MEC and LEC (Fig. 5). They have a spindle-like perikaryon with one ascending and one descending smooth, thin and sometimes long dendrite. The ascending dendritic collaterals traverse throughout **L**II, reaching **L**I. The extent of the descending dendrites has not been described yet. The axon arises from the primary descending dendrite and extends into **L**III and the lamina dissecans, deep to the parent cell body [131]. At least a subpopulation of bipolar cells is known to contain VIP or CR. The latter are more common in LEC than in MEC [131, 235].

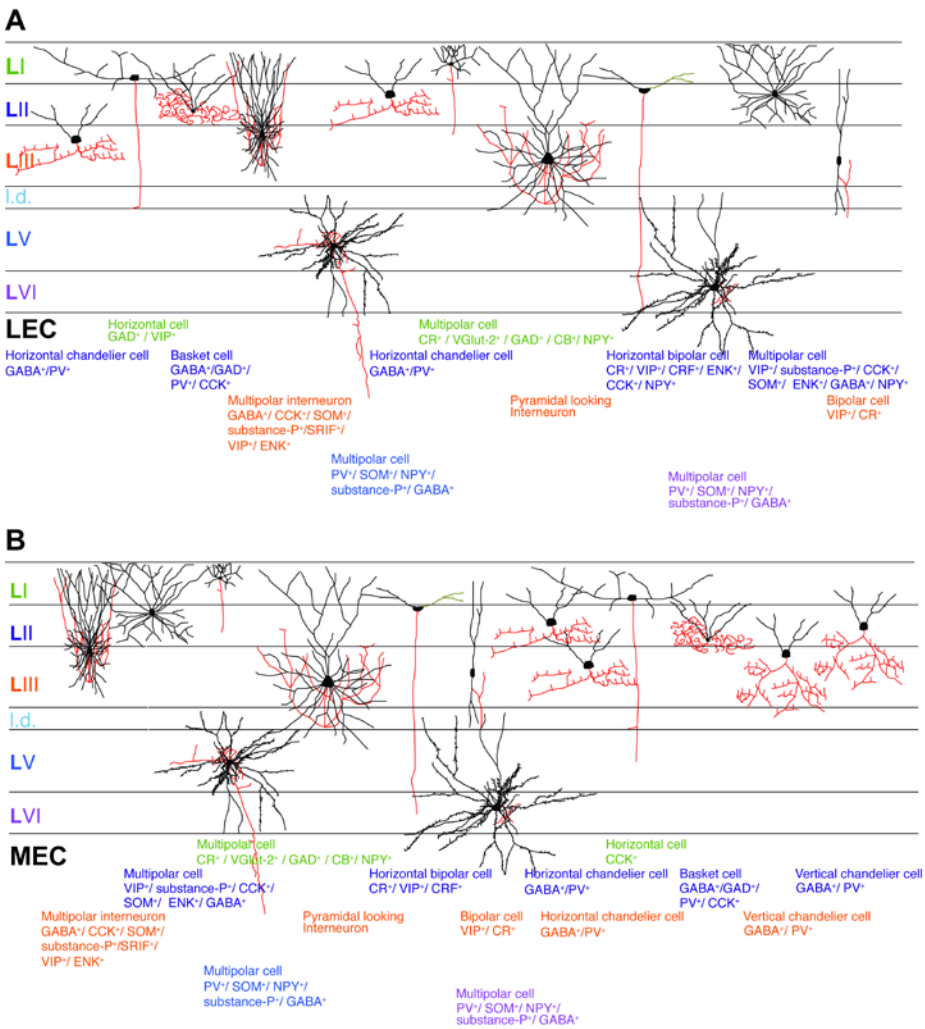

Figure 5: Summary diagrams of the morphology of **(A)** LEC and **(B)** MEC interneurons. (Adapted from [37]).

For **L**V and **L**VI it has been described that multipolar cells might contain PV, SOM, substance-P, NPY, and GABA (Fig. 5) [240, 248]. In **L**VI MPNs have a spherical soma with a diameter of approximately 14 µm. The spiny dendrites have multiple swellings and extend mainly within deep **L**VI, parallel to the layering. The dendrites also extend towards the angular bundle and rarely to **L**III [220]. In addition CR negative and GABA immunopositive morphologically not further identified neurons are located in **L**V [131, 235], see [37].

1.7.4.2. Physiology of Interneurons in the Entorhinal Cortex

Information on the physiological properties of interneurons in EC is rather limited to some cells in **L**II, **L**III and potentially **L**V. Information for interneurons in **L**I and **L**VI is completely lacking. Electrophysiologically, interneurons are generally equaled to fast spiking neurons, which differentiates them from principal neurons [231].

In **L**II fast spiking basket cells spike with a frequency up to 200 Hz and a spike width of 0.51 ± 0.05 ms [218]. They show pronounced afterhyperpolarizations. Stimulating deep EC induces a strong excitatory long duration synaptic input mediated by N-methyl D-aspartate (NMDA) receptors. Also a tonic activation of basket cells through NMDA receptors has been suggested since basket cells often display spontaneous excitatory postsynaptic potentials (EPSPs). Additionally these neurons show fast α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)/kainate events after deep EC stimulation [218] and they receive GABAergic input [254]. Information on the physiology of other morphologically characterized interneurons is not available.

Within **L**III, two types of interneurons have been described [129]. So called type I and type II interneurons. Type I interneurons fire with up to 300 Hz, whereas type II up to 90 Hz. Type I interneurons have a more hyperpolarized membrane compared to type II interneurons. Type I interneurons also have a lower input resistance compared to type II interneurons. The spike afterhyperpolarization amplitude of both interneurons is larger compared to that of principal neurons in **L**III. Type II interneurons have sag currents and rebound bursts, whereas type I have not. The amplitude of spontaneous EPSPs is larger in type I compared to type II interneurons. Type I interneurons show delayed firing after current injections and make synaptic contacts with **L**II principal neurons [129]. Morphologically, type I interneurons are likely PV containing pyramidal looking interneurons, whereas type II interneurons resemble SOM positive multipolar interneurons. Gloveli-type 3 cells, which resemble Kumar type I interneurons, cannot be antidromatic activated by deep EC layer stimulation and they do not develop a prolonged hyperpolarization upon repetitive stimulation of LEC and MEC. Stimulation of deep MEC, PrS and LEC evokes pure EPSPs [211]. Multipolar interneurons, described by Gloveli

as type 4 cells, are similar to the type II Kumar interneurons. Multipolar interneurons have a small rebound potential, and a depolarizing afterpotential (DAP), a fast time-constant and lower input resistance than type 3 Gloveli cells. Interneurons fire highly regularly and do not show a strong spike accommodation as seen in principal neurons. After deep layer stimulation type 4 cells react with a prominent fast inhibitory postsynaptic potential (IPSP) not followed by a slow IPSP. Excitation of LEC leads to a prominent excitation, whereas repetitive stimulation of deep EC layers leads to hyperpolarization. Both stimulations do not lead to a prolonged hyperpolarization [211].
In **L**V fast spiking neurons have been described but a detailed morphological description is missing, which makes it hard to tell whether the neurons described are interneurons or principal neurons [255].

1.7.5 Intralaminar Entorhinal Connectivity

As already mentioned, neurons in different layers have very different inter- and intralaminar connectional patterns varying from axon collaterals confined to the parent cell layer or spanning several layers. But not only the axonal distribution is of importance, the dendritic trees may also play an essential role in that they either span several layers or are more restricted to the parent cell layer [6, 35, 37, 120, 122, 123, 210, 214, 215, 217-219, 237, 246, 256-259]. For EC though, this second level of intrinsic organization has not yet been seriously incorporated into the working concept. This is essential however in order to properly understand how inputs to EC will be processed by the entorhinal network and what the eventual information is that will be conveyed to HF on the one hand and to other cortical and subcortical areas on the other hand. Therefore we studied in Chapter 2 and Chapter 3 the intrinsic network of MEC and LEC and discussed the influence of neuron morphology on the intrinsic network.

1.7.6 Entorhinal-Parahippocampal Connectivity

The perirhinal and postrhinal cortices form the major cortical links of EC. Reciprocal connectivity exists between the perirhinal cortex and LEC, whereas the postrhinal cortex is reciprocally connected to MEC preferentially. Some crosstalk between these two input-output pathways occurs since these respective reciprocal connections do not completely obey the LEC-MEC border. In addition, as described above, the intrinsic connections of EC likely mediate crosstalk [42, 51, 260, 261]. A strikingly similar overall organization of these connections has been reported in other species [48, 56].
PrS and PaS are major input structures of MEC [51]. PrS projections show a complex topographical organization likely reflecting both dorsoventral as well as proximodistal patterns [262]. Superficial PrS layers project bilateral mainly to

superficial MEC **L**I and **L**III [262-264]. Deep PrS layers project ipsilaterally and much weaker mainly to deep layers of MEC [263-265]. PaS projects predominantly to ipsilateral MEC **L**II [262, 266-268]. These projections show an overall similar topographical organization as those from PrS. [262]. MEC projects back to PrS and PaS but relatively weak [123, 266].

In the rat, neurons in superficial layers of PrS and PaS *in vivo* show a typical spatial modulation in that they code specifically for the head direction of the animal [110, 269]. The firing properties of these head direction cells depend among others on inputs from the vestibular system that reach both structures by way of the anterodorsal nucleus of the thamalus [266, 270], which in turn receives its input from the lateral mammilary nuclei [271]. Both these diencephalic nuclei contain neurons that are modulated by head direction [272-275]. Since all layers of MEC contain comparable head direction cells [100, 110] and MEC does not receive direct vestibular inputs it has been postulated that PrS and PaS are the major structures to convey head directional information to MEC. Head directional input has further been postulated to be a relevant input for grid cells in **L**II of MEC to emerge [276, 277]. Head direction cells in PrS and PaS are present and stable already early during development, before stable grid cells are present in **L**II of MEC, strengthening the possible functional relationships between neuronal properties in PrS and PaS on the one hand and those in MEC on the other hand [269, 278, 279]. Therefore we examined the functional interactions between the layer specific inputs from PrS and PaS and neurons in all layers of MEC (Chapter 4) and assessed when during early development these inputs become functional (Chapter 5).

1.8 Objectives

1. To study and compare the intrinsic network of MEC and LEC with a focus on the morphology and physiology of individual principal neurons to discuss their potential role in defining the known functional differences *in vivo* [33, 58].
2. To investigate the inputs of PrS and PaS on MEC principal neurons since MEC is known as a structure receiving layer specific inputs [36].

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