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THE HUMAN ENTORHINAL CORTEX

Anatomic Organization and Its Alteration in Alzheimer's Disease and Temporal Lobe Epilepsy

Doctoral dissertation

To be presented with assent of the Medical Faculty of the University of Kuopio for public examination in the Auditorium L3 of Canthia Building of the University of Kuopio, on November 12, 1999, at 12 noon.

Department of Neuroscience and Neurology and AI Virtanen Institute

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ABSTRACT

The human entorhinal cortex is located in the ventromedial portion of the temporal lobe and consists of eight subfields. It has reciprocal connections with the hippocampus and various other cortical and subcortical structures, and thus forms an integral component of the medial temporal lobe memory system. The entorhinal cortex is damaged in the early stages of Alzheimer's disease (AD) and also in temporal lobe epilepsy (TLE).

The present studies characterize the normal neuronal organization of the human entorhinal cortex using calcium-binding protein immunohistochemistry (*e.g.*, parvalbumin, calretinin, and calbindin D28k, hereafter referred to as calbindin) and lucifer yellow microinjections. Moreover, the changes in the entorhinal neuronal populations containing the three calcium-binding proteins are described for AD. The reorganization processes in the entorhinal cortex and in its hippocampal target areas in normal and diseased brain were also studied with the aid of highly polysialylated neural cell adhesion molecule (PSA-NCAM), which is abundantly expressed in the developing brain, as well as in brain areas that undergo continuous remodeling.

The major findings of these studies were: 1A) The distribution of calcium-binding proteins largely followed the cytoarchitectonic borders of the eight subfields of the entorhinal cortex. Parvalbumin neurons were morphologically interneurons. Although calretinin and calbindin were localized in non-pyramidal cells, they also labeled some pyramidal-like neurons. The high density of non-pyramidal neurons containing these calcium-binding proteins in layers II and III suggests they form a critical network that controls entorhinal outputs to the hippocampus. In addition, their largely non-overlapping distribution suggests that each neuron type modulates a different subset of topographically-organized entorhinal outputs. 1B) Based on the shape of the somata and primary dendritic trees, spiny neurons were divided into classical pyramidal, stellate, modified stellate, and horizontal tripolar cells. Vertical extension of the dendritic branches to adjacent layers supports the idea that inputs terminating in specific layers influence target cells located in various entorhinal laminae. There is more overlap in the dendritic fields of layers II and III than between the superficial and deep layers, which supports the idea of segregation of information flow targeted to superficial or deep layers of the human entorhinal cortex. 2) In AD, the most profound neuronal loss and neurofibrillary tangle formation was observed in the intermediate, lateral, and caudal subfields of the entorhinal cortex. Parvalbumin- and calbindin-containing non-pyramidal neurons were morphologically altered early in the entorhinal pathology, whereas calretinin-containing non-pyramidal cells were morphologically better preserved. Our findings suggest that the specific subfields and layers of the entorhinal cortex that contain distinct calcium-binding proteins are differentially vulnerable. This might impact the topographically-organized inputs and outputs of the entorhinal cortex. 3) In the entorhinal cortex, the major changes in PSA-NCAM immunoreactivity were observed in layers II and III of AD and TLE patients when compared to controls. In hippocampus, the PSA-NCAM immunoreactivity in the outer molecular layer of the dentate gyrus was increased in AD, whereas the inner third of the molecular layer had major changes in TLE. In TLE cases with mild overall neuronal loss in the hippocampus and in AD, the number of PSA-NCAM positive infragranule cells was increased whereas no PSA-NCAM positive infragranule cells were observed in cases with severe hippocampal damage in TLE. Whether the loss of granule cells in TLE is related to the reduced capacity for granule cells to differentiate remains to be explored.

Taken together, the human entorhinal cortex is formed of neurochemically heterogeneous subfields in which information flow is presumably differentially modulated by local interneurons. The pathologic changes occuring in AD and TLE do not involve the entire entorhinal cortex to the same extent, and some neuronal types are more vulnerable than others. In addition, the reorganization of neuronal circuitries takes place in the entorhinal cortex and in its hippocampal target areas in AD and TLE.

National Library of Medicine Classification: WL 359, WL 385
Medical Subject Headings: Alzheimer's disease; entorhinal cortex; hippocampus; epilepsy, temporal lobe; neuronal plasticity; calcium-binding protein/analysis; nerve fibers; calretinin/analysis; calbindin/analysis; parvalbumin/analysis; NCAM/analysis

Nec scire fas est omnia.

Horatius (65-8 B.C.)

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Kuopio, September 1999

Mia Mikkonen

ABBREVIATIONS

AD	Alzheimer's disease
CA	Cornu Ammonis
CAM	cell adhesion molecule
CERAD	The Consortium to Establish a Registry for Alzheimer's Disease
CNS	central nervous system
EC	entorhinal cortex, caudal subfield
ECL	entorhinal cortex, caudal limiting subfield
EI	entorhinal cortex, intermediate subfield
ELc	entorhinal cortex, lateral caudal subfield
ELr	entorhinal cortex, lateral rostral subfield
EMI	entorhinal cortex, medial intermediate subfield
EO	entorhinal cortex, olfactory subfield
ER	entorhinal cortex, rostral subfield
GABA	gamma amino butyric acid
GAD	glutamate decarboxylase
GFAP	glial fibrillary acidic protein
Ig	immunoglobulin
NCAM	neural cell adhesion molecule
PSA	polysialic acid
TLE	temporal lobe epilepsy

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications that are referred to in the text by the Roman numerals **I-V**.

I Mikkonen M, Soininen H, Pitkänen A: Distribution of parvalbumin-, calretinin-, and calbindin-D28k-immunoreactive neurons and fibers in the human entorhinal cortex. *Journal of Comparative Neurology, 388: 1: 64-88, 1997.*

II Mikkonen M, Pitkänen A, Soininen H, Alafuzoff I, Miettinen R: Morphology of spiny neurons in the human entorhinal cortex: Intracellular filling with lucifer yellow. *(submitted)*

III Mikkonen M, Alafuzoff I, Tapiola T, Soininen H, Miettinen R: Subfield- and layer-specific changes in parvalbumin, calretinin and calbindin-D28k immunoreactivity in the entorhinal cortex in Alzheimer's disease. *Neuroscience, 92: 2: 515-532, 1999.*

IV Mikkonen M, Soininen H, Tapiola T, Alafuzoff I, Miettinen R: Hippocampal plasticity in Alzheimer's disease: Changes in highly polysialylated NCAM immunoreactivity in the hippocampal formation. *European Journal of Neuroscience, 11: 5:1754-64, 1999.*

V Mikkonen M, Soininen H, Kälviäinen R, Tapiola T, Ylinen A, Vapalahti M, Paljärvi L, Pitkänen A: Remodeling of neuronal circuitries in human temporal lobe epilepsy: Increased expression of highly polysialylated neural cell adhesion molecule in the hippocampus and the entorhinal cortex. *Annals of Neurology, 44: 6: 923-934, 1998.*

CONTENTS

1. INTRODUCTION

The study of medial temporal lobe structures has been revolutionized during the last half century. The beginning of this revolution was due partly to patient called HM, who underwent surgery for epilepsy during which the medial portions of the temporal lobes were removed bilaterally (Scoville and Milner 1957). After this operation, HM was unable to store and retrieve new information about events. The magnetic resonance imaging data of HM describing the exact areas that were removed was only recently published (Corkin et al. 1997). The resection extended approximately 5 cm posteriorly in both hemispheres including the amygdala, perirhinal and entorhinal cortex, and the anterior hippocampus. Knowledge of the structures necessary for memory formation is increasing due to the contributions of HM and other amnestic patients (Rempel-Clower et al. 1996), monkey models of amnesia (Alvarez et al. 1995, Leonard et al. 1995, Zola-Morgan et al. 1989a, Zola-Morgan et al. 1993), and new functional magnetic resonance imaging techniques (Fernandez et al. 1999, Fernandez et al. 1998).

The entorhinal cortex (Brodmann's area 28), a structure involved in memory, is located on the ventromedial surface of the temporal lobe (Garey 1994, Insausti et al. 1995). The length of the entorhinal cortex from the most rostral tip to its caudal end is 2.5 to 3 cm. Rostrally, the entorhinal cortex is located beneath the amygdaloid complex and more caudally beneath the hippocampus. Recent studies indicate that the rodent, monkey, and the human entorhinal cortex are chemoarchitectonically, cytoarchitectonically, and connectionally heterogeneous structures (Amaral et al. 1987, Insausti 1993, Insausti et al. 1997, Insausti et al. 1995). The human entorhinal cortex can be divided into eight subfields based on these differences (Insausti et al. 1995). The contribution of the entorhinal cortex to memory can be understood in terms of its cortical and subcortical connectivity. First, neuroanatomic studies in monkey indicate that this cortical area receives a strong convergence of information from cortical and subcortical areas (Insausti et al. 1987a, Insausti et al. 1987b, Van Hoesen and Pandya 1975a, Van Hoesen et al. 1975, Van Hoesen and Pandya 1975b, Van Hoesen et al. 1972). Second, the entorhinal cortex has robust projections from layers II and III to the hippocampus (Witter and Amaral 1991). Third, the hippocampus, in turn, has connections to layer V of the entorhinal cortex, which projects to the neocortex (Witter 1993). Thus, the entorhinal cortex communicates not only with the hippocampus, but also with the rest of the cortex. In addition to funneling information from the neocortex to the hippocampus, the entorhinal cortex has active properties that contribute to the signal processing. The local interneurons have critical role in controlling this information flow.

The entorhinal cortex is affected in Alzheimer's disease (AD) (Braak and Braak 1991, Gomez Isla et al. 1996, Hyman et al. 1984) and in temporal lobe epilepsy (TLE) (Du et al. 1995b, Du et al. 1993). In these diseases, the pathologic changes do not involve the entire entorhinal cortex to the same extent, but some neuronal types are more vulnerable than others. For example, certain neuronal subpopulations of layers II and V contain pathologic neurofibrillary tangles in early AD, whereas neurons in layers III and VI rarely have tangles (Hyman et al. 1984). In TLE, instead, certain cell populations of layer III are lost (Du et al. 1993). Relatively little is known about the normal morphology of the neurons of the human entorhinal cortex. Calcium-binding proteins, a group of cytosolic proteins, are primarily localized in inhibitory interneurons in rat, monkey and, human, and thus provide a marker for local interneurons (Andressen et al. 1993, Baimbridge et al. 1992). Very few specific markers for principal cells are available, and thus various intracellular filling techniques

are important for morphologic studies of the principal cells. Investigation of the laminar organization and distribution of interneurons and principal cells in the human entorhinal cortex would provide valuable baseline information about the circuitry of the human entorhinal cortex. Furthermore, the study of cell fate and the morphologic changes of various neuronal populations, as well as the possibility of neuronal reorganization in neurologic disorders, would extend our understanding of the pathologic processes occuring in this brain area.

The reorganization and plasticity of the central nervous system are controversial issues. Are the structures and connections between neurons in the central nervous system formed only during development? Ramon y Cajal expressed his view in 1928 (Ramon y Cajal 1928): "...once the development was ended, the fonts of growth and regeneration of the axons and dendrites dried up irrevocably. In adult centers, the nerve paths are something fixed, ended, immutable. Everything may die, nothing may regenerate." Or, is the central nervous system always changing its structure to adapt to the demands of the outer world? The hippocampus and adjacent structures which are important for learning and memory, are the major candidates for continuous remodeling that occurs throughout life. Indeed, there are new findings suggesting that neurogenesis continues throughout the human life in the dentate gyrus (Eriksson et al. 1998). Lesion studies in which known connections are lesioned and changes in projection brain area are examined are used to explore structural plasticity in animal models. The impact of entorhinal cortex lesions on the function of the hippocampal loop is widely studied (Deller and Frotscher 1997). Neural cell adhesion molecules, which are important in neural migration, neurite outgrowth, and axonal branching during the development of the nervous system, are also involved in structural rearrangement and lesion-induced plasticity (Doherty et al. 1995, Rutishauser and Landmesser 1996). For example, the expression of immature highly polysialylated form of the neural cell adhesion molecule (PSA-NCAM) increases in the denervated regions of the rat brain (Miller et al. 1994, Styren et al. 1994). Plasticity of human hippocampus can be studied in AD and TLE. Essential questions are related to the ability of the central nervous system to modify its structure following epileptic insults or in AD, and to the region-specific differences in plasticity inside the central nervous system. One crucial question that remains is whether plasticity is always beneficial.

In the present series of studies, the intrinsic organization of the human entorhinal cortex was characterized using calcium-binding proteins (*e.g.* parvalbumin, calretinin and calbindin D28k; hereafter referred to as calbindin) which are known to label different types of interneurons and certain types of pyramidal neurons in the mammalian brain. Furthermore, the organization of the entorhinal cortex was studied using intracellular microinjection techniques in lightly fixed human entorhinal cortex. The distribution and the morphology of the cells and fibers immunoreactive for these three calcium-binding proteins were compared in the normal adult human entorhinal cortex. Moreover, the changes in those neuronal populations were described in Alzheimer's disease. The reorganization processes in the entorhinal cortex and in its hippocampal target areas in normal and diseased brain were evaluated using PSA-NCAM, which is expressed abundantly in the developing brain and in brain regions that undergo continuous remodeling.

2. REVIEW OF THE LITERATURE

2.1. Location and histologic appearance of the human entorhinal cortex

2.1.1 Location and the adjacent structures of the human entorhinal cortex

The entorhinal cortex is a part of the hippocampal formation, the other areas of which are the hippocampus proper, dentate gyrus, and subicular complex (Amaral and Insausti 1990). The entorhinal cortex is located in the rostral half of the ventromedial surface of the temporal lobe. The name "entorhinal" refers to its location inside the rhinal sulcus, in the olfactory area. The entorhinal cortex is spatially associated with the amygdaloid complex rostrally and hippocampal formation caudally. The entorhinal cortex extends from approximately 5 mm rostral to the beginning of the amygdala and caudally it extends to the mid-levels of the hippocampus. The structures that align with the entorhinal cortex medially vary at different rostrocaudal levels. At its rostral portion, the entorhinal cortex is medially attached to the periamygdaloid cortex and more caudally it is attached the subiculum. The lateral border is located in the medial or lateral bank of the collateral sulcus, and the entorhinal cortex is near the perirhinal cortex. The strict border between these cortices is not easily determined and the transition area is often called transentorhinal cortex (Braak and Braak 1985). The entorhinal cortex is also near several arteries, such as the internal carotid, posterior communicating, anterior choroidal, and posterior cerebral arteries (Insausti et al. 1995).

2.1.2 Histologic appearance of the human entorhinal cortex

The human entorhinal cortex was identified as a separate region since early in the 20th century (Insausti 1993). Ramon y Cajal illustrated it in 15-day old human infants, where he was able to differentiate seven layers (Ramon y Cajal 1995). Brodmann (1909) called this region area 28 or entorhinal cortex (Garey 1994). The human entorhinal cortex consists of six different layers, although different laminar definitions also exist (Amaral et al. 1987, Braak 1980, Insausti et al. 1995, Lorente de No 1933, Ramon y Cajal 1995) (See Table 1).

Table 1. Nomenclature of the entorhinal laminae (modified from Schmidt et al. 1993).

Braak, 1980, human	mol	pre-α	pre-β	pre-γ	Ld	pri-α	pri-β	pri-γ	lcp
Ramon y Cajal, rabbit, mouse, human	1	2	3		4	5	6	7	
Lorente de No, 1933, mouse	I	II	III		IIIa	IV	V	VI	
Amaral et al., 1987, monkey	I	II	III		IV	V		VI	
Insausti et al., 1995, human	I	II	III		IV	V		VI	

(Lcp) = Lamina cellularis profunda; (Ld) =Lamina dissecans; (mol) = molecular layer; (pre-a **, pre-**b **, pre-**g**) = layers of the principal external stratum; (pri-**a **, pri-**b **, pri-**g**) = layers of the principal internal stratum.**

The nomenclature and numbering system described by Insausti et al., (1995) is used in this series of studies. The nomenclature is synonymous with the nomenclature used for monkey by Amaral et al., (1987).

Layer I is composed mainly of fibers and occasional cells. Layer II is the most conspicuous layer, consisting of cell islands of polygonal and often star-shaped cells. The cell islands of layer II are different in shape, depending on the rostrocaudal and mediolateral location. Layer III is composed of the superficial portion where neurons are often clustered together and of the deep portion with homogeneously-arranged neurons. Layer IV is either present or absent depending on the subfield. This layer is a cell-sparse stratum between the smaller pyramidal cells of the layer III and the larger pyramidal cells of layer V. Layer V is formed of darkly-stained pyramidal cells. In the rostral levels, the layer V is largely fused together with layer VI, whereas in more caudal levels it is clearly separable from layer VI. At the rostral level, layer VI invades the white matter, whereas in the more caudal levels it is strictly separated from white matter (Insausti et al. 1995).

Several subfields of the entorhinal cortex have been described based on the cytoarchitectonic differences. Depending on the researcher this brain region has been divided into three (medial, intermediate and lateral) to 23 subfields (Insausti 1993). In 1995, Insausti et al. described eight entorhinal subfields of the human entorhinal cortex according to cytoarchitectonic criteria resembling the nomenclature used for monkey entorhinal cortex by Amaral and colleagues in 1987. These subfields are: Olfactory subfield (EO), Lateral rostral subfield (ELr), Rostral subfield (ER), Medial

intermediate subfield (EMI), Intermediate subfield (EI), Lateral caudal subfield (ELc), Caudal subfield (EC), and Caudal limiting subfield (ECL; Fig. 1).

1) The Olfactory subfield (EO). The EO is the rostral-most area wrapped around the amygdala. It is characterized by a very thin layer II and clumps of neurons in the superficial portion of layer III. The deep portion of layer III gradually changes to layer V without a clear acellular gap. Layers V and VI are fused together.

2) The Lateral Rostral subfield (ELr). The ELr is located laterally to EO and it typically has a thicker layer II and clear cellular clusters of layer II. The acellular zone is more distinct between layers III and V. Furthermore, layers V and VI are more easily identified as separate layers.

3) The Rostral subfield (ER). The ER is interspersed between the EO and ELr in more caudal sections. It also has neurons clustered in the superficial portion of layer III, but they are organized and not scattered as in the EO. Layers V and VI are fused together.

4) The Medial Intermediate subfield (EMI). The EMI is located medially beneath the hippocampus. The typical cytoarchitectonic features are a thin layer III, clearly demarcated layers III and V (layer IV is particularly broad), and also clearly demarcated layers V and VI.

5) The Intermediate (EI). The EI is located near the EMI. The EI has the most typical appearance of the entorhinal cortex with its wart-like elevations of layer II, by which the entorhinal cortex can be macroscopically identified. The EI has clear cellular clusters in layer II. Layer III has scattered neurons, that do not form clear clumps. Layers V and VI are distinguishable, but the acellular layer between them is not as wide as in the EMI.

*6) The Lateral caudal subfield (ELc).*The ELc limits the EI laterally. The cell islands of layer II are smaller and more elongated compared to the adjacent EI. Layers V and VI are fused.

7) The Caudal subfield (EC). The EI is caudally replaced by EC. The EC has rather rounded layer II cell islands. Layer III is rather uniform. The acellular zone between III and V is absent.

8) The Caudal Limiting subfield (ECL). In the ECL, layer III becomes thinner and fuses with layer V. Layers V and VI are clearly demarcated by the acellular zone.

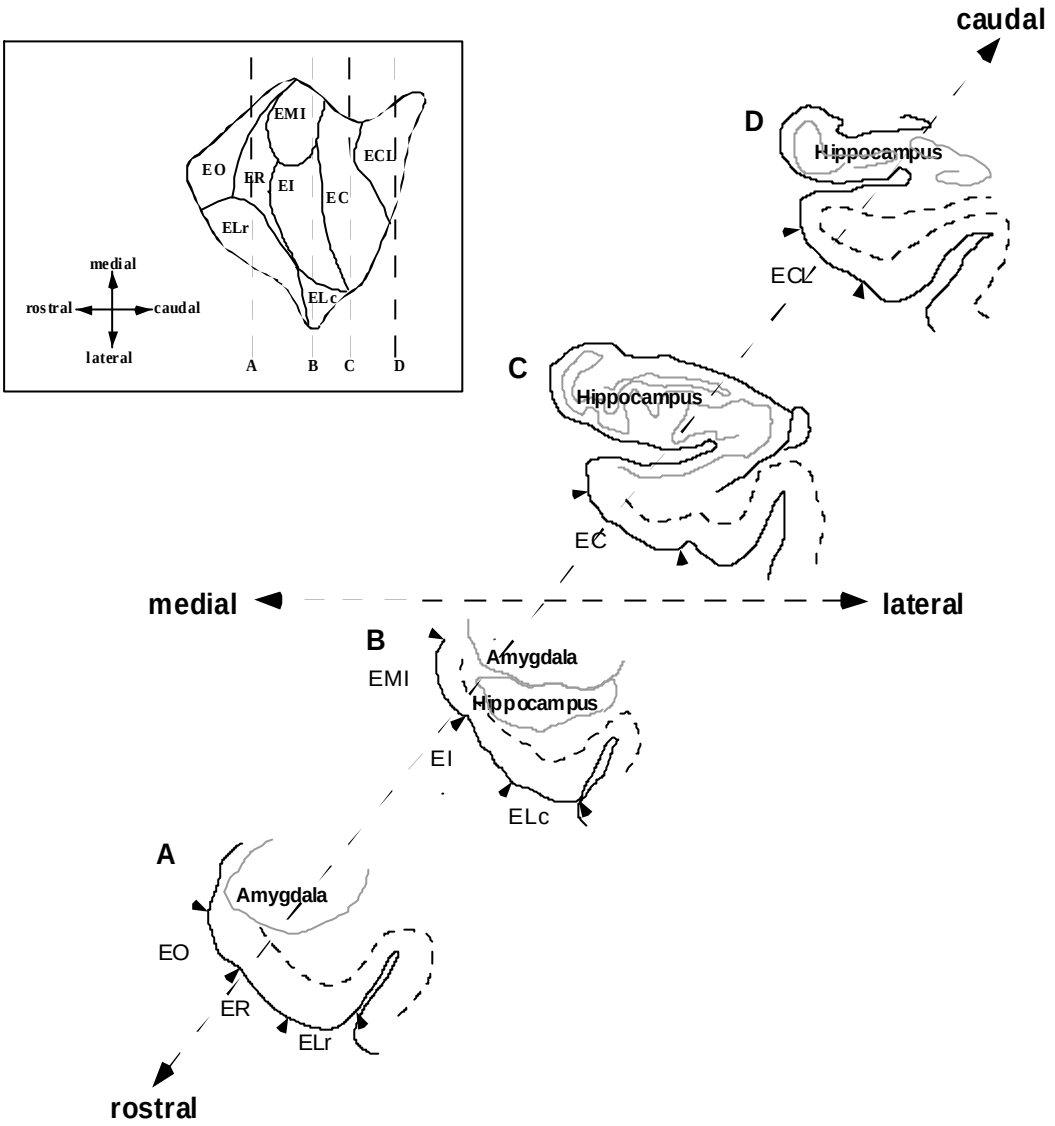

Figure 1. Eight subfields of the human entorhinal cortex from rostral (A) to caudal (D).

2.2 Connections of the entorhinal cortex

2.2.1 Cortical and subcortical connections of the entorhinal cortex

Anatomic tract-tracing studies in primates and rodents reveal widespread connections between the entorhinal cortex and many cortical, subcortical, and hippocampal structures (summarized in Figs. 2 and 3) (Witter 1993). Two-thirds of the neocortical information projecting to the entorhinal cortex is conveyed *via* the perirhinal and parahippocampal cortices (Insausti et al. 1987a). Thus, the perirhinal cortex and the parahippocampal cortex form the great majority of the inputs to the entorhinal cortex (Suzuki and Amaral 1994b). Most of the connections to the entorhinal cortex are reciprocal (Witter 1993) and thus prominent efferents from entorhinal cortex terminate in the perirhinal and parahippocampal cortices (Suzuki and Amaral 1994b). The olfactory structures are the only primary sensory systems that have direct projections to the entorhinal cortex (Insausti et al. 1987a). There are dense anterior cortical projections from the infralimbic and orbitofrontal cortices to entorhinal cortex (Insausti et al. 1987a). Posteriorly, the orbitofrontal cortex is connected with insular cortex,

and the insular cortex, in turn, is connected with the perirhinal cortex and entorhinal cortex (Insausti et al. 1987a). The cingulate cortex also innervates entorhinal cortex (Insausti et al. 1987a).

There are numerous subcortical connections. The cholinergic fibers of the basal forebrain project to the entorhinal cortex (Insausti et al. 1987b). Emotionally-controlled information comes from the amygdala, where projections are derived from the cortical nucleus and the basolateral and lateral nuclei (Insausti et al. 1987b). From the brain stem, serotonergic afferents to the entorhinal cortex originate in the raphe nuclei, and noradrenergic inputs arise from the locus coeruleus and ventral tegmental area (Insausti et al. 1987b). Thalamic afferents from reuniens nuclei and anterior thalamic nuclei also innervate the entorhinal cortex (Insausti et al. 1987b). These numerous connections to the entorhinal cortex project to various layers and various subfields (Figs. 2 and 3).

2.2.2 Hippocampal connections of the entorhinal cortex

The most studied efferent connection of the entorhinal cortex is the route between layer II of the entorhinal cortex and the molecular layer of the dentate gyrus (Witter and Amaral 1991). This connection, also called the perforant pathway, is considered to be the major route for communication between the neocortex and the hippocampus. In rodents and primates this projection connects the rostromedial entorhinal cortex with the rostral hippocampus and the caudolateral entorhinal cortex with the caudal/septal hippocampus (Witter and Amaral 1991) (Fig. 4). The intrinsic circuitry of the hippocampal formation is well-described in rodents and primates (Witter 1993). The axons of neurons in layer II of the entorhinal cortex reach the dendrites of granule cells in the outer portion of the molecular layer of the dentate gyrus. The granule cell axons (mossy fibers) innervate the dendrites of the CA3 pyramidal cells, which in turn project to the CA1 region. From the CA1 region and the subiculum, the network loop is closed when the axons of CA1 and subicular neurons reach the layer V of the entorhinal cortex. Layers V and VI, in turn, project back to the cortical and subcortical structures (Good and Morrison 1995). In addition to the layer II connection, there is also a direct route from layer III to *stratum lacunosum moleculare* of CA1 region. (Fig. 5)

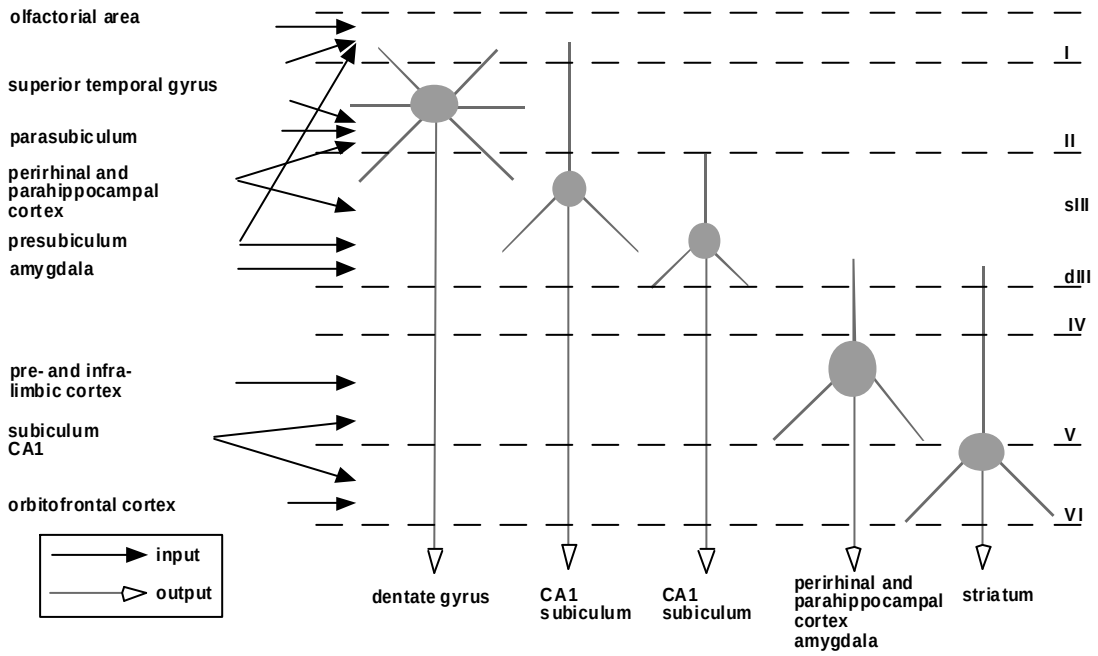

Figure 2. Entorhinal inputs and outputs of different layers.

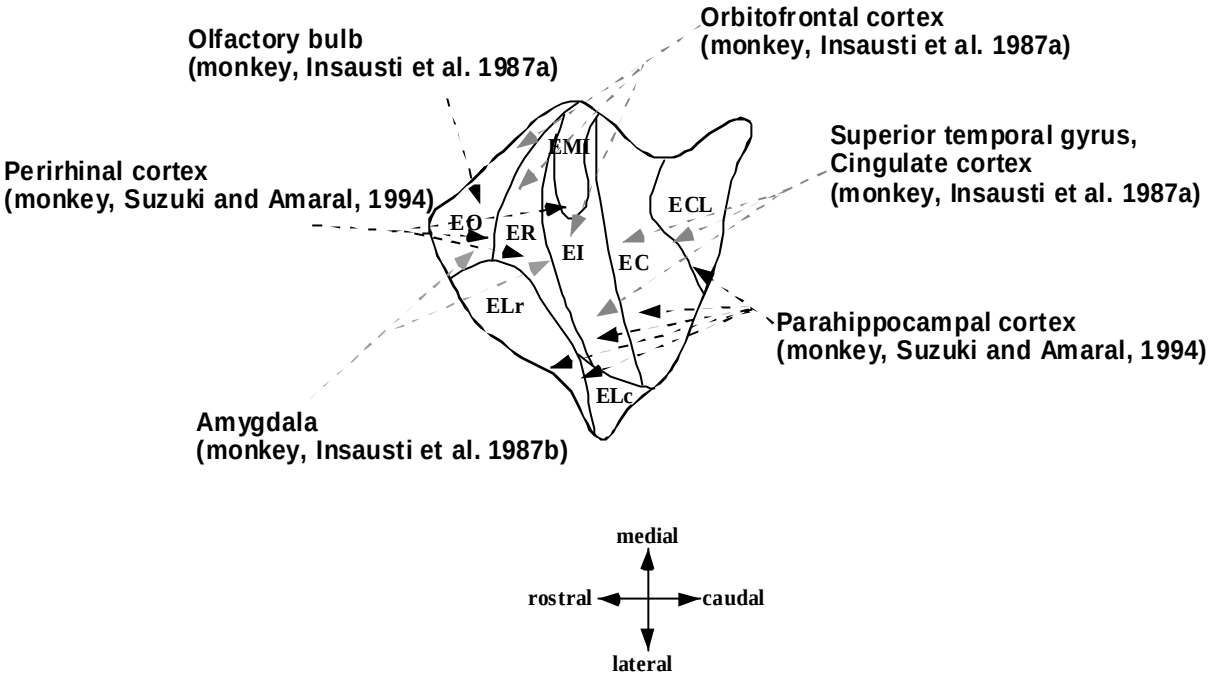

Figure 3. Major afferent connections of the entorhinal cortex.

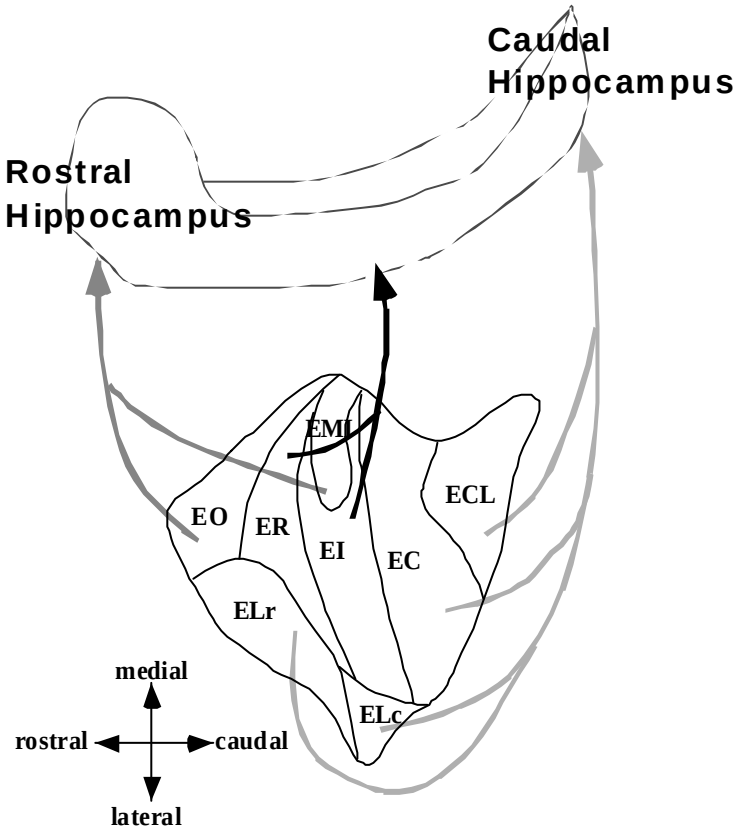

Figure 4. Topography of the entorhinal-hippocampal connectivity. The neurons located in the medial entorhinal cortex project to rostral hippocampus, whereas neurons located in lateral entorhinal cortex project to caudal hippocampus.

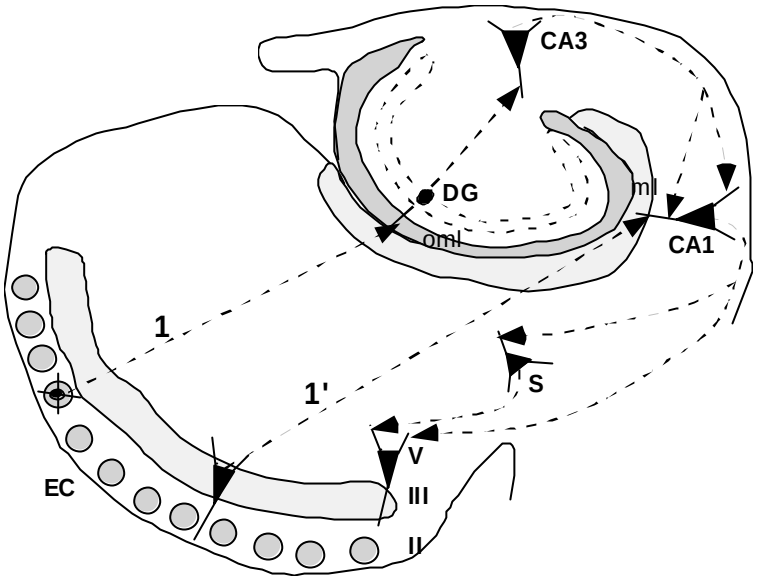

Fig 5. Entorhinal-hippocampal connectivity. The neurons located in the layer II of entorhinal cortex (EC) project to the granule cell dendrites located in the outer two-thirds of the molecular layer (oml) of the dentate gyrus (DG) (1). Layer III neurons of the entorhinal cortex project to stratum lacunosum moleculare (ml) of CA1 (1').

2.3 The role of the entorhinal cortex in memory formation

Long-term cognitive memory, also called declarative memory, is dependent on the medial temporal lobe structures, consisting of the hippocampus, and the entorhinal, perirhinal and parahippocampal cortices (Squire and Zola 1996). The medial temporal lobe structures are functionally responsible for memory involving specific events, i.e. episodic memory, and memory for factual information, *i.e.* semantic memory (Squire and Zola-Morgan 1991). Many studies indicate that these structures are responsible for long-term memory formation. After some period of time, however, the memories become independent of the medial temporal lobe structures (Squire and Zola-Morgan 1991).

The role of entorhinal cortex in this medial temporal lobe memory system can be understood in terms of its connectivity. The anatomic organization of the medial temporal lobe structures is hierarchical, and in this organization the entorhinal cortex forms a bi-directional gateway. Thus, the perirhinal cortex (responsible for quality of visual stimuli) and parahippocampal cortex (responsible for stimulus location) are at the one end of a chain of feedforward projections from the unimodal and polymodal cortical areas to the entorhinal cortex (Suzuki and Amaral 1994a). The entorhinal cortex then forwards this sensory information to the hippocampus *via* the perforant pathway and receives the hippocampal information after processing by the hippocampal loop (Amaral 1993).

Significant memory impairment in monkeys is observed when the hippocampus, dentate gyrus, and subicular complex are lesioned (Alvarez et al. 1995). Entorhinal and perirhinal ablations alone also produce memory deficits (Leonard et al. 1995, Zola-Morgan et al. 1989b). This indicates that multiple areas of the medial temporal lobe contribute to normal memory function. It is still unclear, however, whether each of the medial temporal lobe regions performs essentially similar mnemonic tasks so that the deficits become more severe when more of the processing regions are removed. On the other hand, these regions might have their own function that is independent of the others. Recent

functional magnetic resonance imaging studies indicate that the activation level of the entorhinal cortex predicts whether experiences will be remembered or forgotten (Fernandez et al. 1999).

2.4. Neuronal populations and chemoarchitectonics of the entorhinal cortex

2.4.1 General

There are numerous methods for differentiating neurons into various subclasses. Classically, neurons are divided according to (1) location of the somata (*i.e.*, brain area and layer) (2) morphologic characteristics *i.e.*, shape of the somata (*e.g.*, rounded, polygonal), geometry and orientation of dendrites (*e.g.*, multipolar or bipolar; horizontal or vertical), spine density (*e.g.*, no spines, sparsely spiny, numerous spines), (3) axonal projection areas (local or to what brain area the neuron projects) or collateral arborizations of axon. On the other hand, neurotransmitters (*e.g.*, GABA, glutamate, *etc.*), receptor-types (*e.g.*, NMDA-receptor, muscarinic receptor, *etc.*) or certain other markers that the cell contains (*e.g.*, calcium-binding protein) differentiate one type of neuron from another. Furthermore, various neuronal types have differences in their electrophysiologic characters or in the precise stimulus that activates the neuron (*e.g.*, light or darkness, or certain specific place within the environment).

2.4.2 Golgi studies and intracellular filling studies

Classic Golgi stains or intracellular filling techniques have been used for characterizing the morphologically variable cell types of the entorhinal cortex (Carboni et al. 1990, Germroth et al. 1989, Germroth et al. 1991, Lorente de No 1933). In primates and rodents, the neuronal populations of the entorhinal cortex can be divided into projection cells (also called principal cells), the axon of which projects to another brain area, and into locally arborizing non-projection cells (also called interneurons, non-pyramidal cells, or non-principal cells) (Belichenko 1993, Germroth et al. 1991). The projection cells and non-projection cells have been further divided into several classes according to their somal and dendritic morphology.

Recently, the projection neurons of the rat entorhinal cortex were divided into spiny pyramidal, pyramid-like, spiny stellate of various types, and sparsely spinous horizontal and multipolar cells (Germroth et al. 1989, Germroth et al. 1991, Schwerdtfeger et al. 1990). The non-spiny or sparsely spiny multipolar or bipolar neurons are mainly locally arborizing types. In monkey, the neurons of the lateral entorhinal cortex are classified on the basis of their dendrites and spine densities into spinous and sparsely spinous types (Carboni et al. 1990). Golgi methods were used in the early studies of mammalian entorhinal cortex by Ramon y Cajal and Lorente de No Lorente de No (1933). In layer II, they observed star cells and cells with horizontal axis cylinders. In layer III they observed pyramidal cells and spindle cells. In layers V and VI, they described pyramidal and horizontal cells, globular cells, and polygonal cells. Thus far, no specific nomenclature for neurons in the human entorhinal cortex has been presented, although some neuronal populations have been described (Beall and Lewis 1992).

2.4.3 Calcium-binding proteins as neuronal markers and their functional aspects

Certain cell populations of the entorhinal cortex can be characterized with the aid of calcium-binding proteins (*i.e.*, parvalbumin, calretinin and calbindin D28k) immunohistochemistry. There are, however, more than 200 calcium-dependent proteins that are involved in Ca^{2+} buffering and transport, regulation of enzymes, differentiation, and secretion, as well as promoting neurite extension (Heizmann and Braun 1995). The calcium-binding proteins are a group of homologous proteins that contain a characteristic structure, that consists of pouches for the acceptance of Ca^{2+}. The best known calcium-binding protein is calmodulin, the conformation of which changes after binding Ca^{2+}. The binding of Ca^{2+} to calmodulin then triggers the activity of the neighboring enzymes (Heizmann and Braun 1995). Many other calcium-binding proteins are less versatile in their function. Mice with reduced calbindin expression, however, are impaired in memory tasks (Molinari et al. 1996). Furthermore, calbindin or calretinin are involved in long-term potentiation (Molinari et al. 1996, Schurmans et al. 1997). Calretinin and calbindin also promote resistance to degeneration by buffering the intracellular calcium concentrations (Guo et al. 1998, McMahon et al. 1998). Cells containing calretinin and calbindin are resistant to kainate *in vitro*, though parvalbumin-containing neurons are still vulnerable (Heizmann and Braun 1995). Furthermore, there are differences in the ability of these molecules to bind calcium: parvalbumin is a slower Ca^{2+} buffer than calbindin, because Mg^{2+} has to dissociate from parvalbumin before it can bind Ca^{2+} (Heizmann and Braun 1995). In addition, the specific calcium-binding protein content seems to be related to the differential receptor subtype expressed by the neurons (Kondo et al. 1997) or to electrophysiologic properties (Du et al. 1996, Kawaguchi and Kubota 1993). It is also possible that there are differences *in vivo* in the vulnerability of these neuronal subtypes in neurologic diseases. In fact, various calcium-binding protein-containing cells are resistant or decreased in number and size depending on the disease, brain area or calcium-binding protein type studied (Heizmann and Braun 1995).

The main use of the antibodies against calcium-binding proteins is the labeling of specific neuronal pathways, nuclei and brain areas, and neuronal populations. Animal studies indicate that certain calcium-binding proteins are good markers for certain types of non-pyramidal cells, which are mainly GABAergic, but also for certain pyramidal cells, which are non-GABAergic. (Andressen et al. 1993).

Parvalbumin (12 kDa) is a marker for basket cells (Andressen et al. 1993, Katsumaru et al. 1988) *i.e.*, interneurons that form contacts with the somata of other neurons and for chandelier cells that form several contacts with the axon-initial segments (Andressen et al. 1993, DeFelipe et al. 1989b, Katsumaru et al. 1988, Sorvari et al. 1995). In rats, all parvalbumin-immunoreactive neurons of the entorhinal cortex are also GABA-immunoreactive (Miettinen et al. 1996). Thus, parvalbumin-positive neurons are thought to be responsible for inhibition, especially in the perisomatic region. The cell bodies of parvalbumin cells are often described as multipolar and no spines are observed on their dendrites (Schmidt et al. 1993). The parvalbumin-positive neurons comprise less than 10% of the total neuronal population (Andressen et al. 1993).

Calretinin (29 kDa) is located in bipolar and vertically-oriented multipolar neurons that form contacts with pyramidal and non-pyramidal cell dendrites (Andressen et al. 1993, Freund and Buzsaki 1996). Furthermore, calretinin-containing cells that have the morphologic appearance of Cajal-Retzius neurons are observed in layer I in the human neocortex (Belichenko et al. 1995, Gabbott et al. 1997). On the other hand, certain pyramidal cells in the human prefrontal cortex are also calretinin-positive (Gabbott et al. 1997).

Calbindin (28kDa) is located in non-pyramidal cells that form contacts mainly with dendrites of other neurons (Andressen et al. 1993). The calbindin positive neurons are often radially-oriented bipolar neurons that form a population of double bouquet neurons (Andressen et al. 1993). Calbindin is also found in faintly-stained pyramidal neurons (Ferrer et al. 1992). It is estimated that calbindin-positive neurons form less than 5% of all neuronal populations (Andressen et al. 1993). Approximately, 70% of the calretinin or calbindin neurons in the human temporal lobe are GABAergic (del Rio and DeFelipe 1996). Taken together, certain calcium-binding proteins label morphologically, connectionally, and presumably electrophysiologically variable cell populations, which makes them important tools for anatomic studies.

2.4.4 Chemoarchitectonic heterogeneity of the entorhinal cortex

Neurotransmitters and various peptides have a distinct distribution in the various layers of the entorhinal cortex, as well as in various subfields. The main excitatory output from the entorhinal cortex to the hippocampus utilizes glutamate as a neurotransmitter (Köhler 1986a, Köhler 1986b, Solodkin and Van Hoesen 1996). Some of the neurons of layers II and III are calbindin-immunoreactive or neurofilament protein-immunoreactive (Beall and Lewis 1992). Furthermore, these neurons of layers II and III are cytochrome oxidase-immunoreactive (Hevner and Wong Riley 1992). The main inhibitory transmitter of the entorhinal cortex is GABA and, as in other brain areas, many of the GABAergic interneurons co-localize with calcium-binding proteins in rat, monkey, and human in a lamina-specific manner (Beall and Lewis 1992, Brion and Resibois 1994, Miettinen et al. 1996, Miettinen et al. 1997, Seress et al. 1994, Seress et al. 1993, Tunon et al. 1992). The typical feature of the entorhinal cortex is that the layer II projection neurons are heavily innervated by GABAergic basket-like terminals, which are parvalbumin immunoreactive (Solodkin and Van Hoesen 1996). The inhibitory terminals of layer II in monkey (symmetric synapses) are more numerous in the intermediate, lateral, and caudal subfields and not as common in the rostral portions, similarly to parvalbumin immunoreactivity (Goldenberg et al. 1995, Tunon et al. 1992). Dopaminergic staining is most conspicuous in the rostral part of the monkey entorhinal cortex (Akil and Lewis 1993), whereas the cholinergic fibers in the human are more restricted to lateral and caudal portions (De Lacalle et al. 1994).

In addition, various receptor subunits have lamina-specific localizations in the entorhinal cortex. Labeling of mRNA for a glutamate receptor subunit (GluR2) is distributed in a laminar manner with particularly high levels of labeling in the cellular clusters of layer II of the human entorhinal cortex (Longson et al. 1997). The labeling of mRNA for the different subunits of the GABA(A) receptor are localized in layer II, similarly to glutamatergic neurons, implying a high concentration of inhibitory synapses on the excitatory cells (Longson et al. 1997). Muscarinic and nicotinic-receptors are also localized in the human entorhinal cortex (Court et al. 1997). Furthermore, galanin receptors are observed in layers II and V of the human entorhinal cortex (Deecher et al. 1998). Taken together, the entorhinal cortex is a neurochemically a heterogeneous structure, a characteristic that evidently influences the information processing and also on the vulnerability of the entorhinal cortex.

2.5 Entorhinal damage in neurologic diseases

2.5.1 General

The entorhinal cortex is damaged in various brain diseases, such as temporal lobe epilepsy (Du et al. 1993), schizophrenia (Arnold et al. 1995) and in degenerating disorders, such as frontotemporal dementia (Frisoni et al. 1999) and Alzheimer's disease (Braak and Braak 1992, Braak and Braak 1995, Braak and Braak 1996). The number of neurons of the entorhinal cortex is diminished in temporal lobe epilepsy (Du et al. 1993) and in Alzheimer's disease (Gomez Isla et al. 1996). In schizophrenia, there are controversial findings regarding the damage in the entorhinal cortex. That is, in some studies the cell clusters of layer II are proposed to be malformed, but when the cytoarchitectonic heterogeneity of the entorhinal cortex is taken into account no such malformations are found (Akil and Lewis 1997, Krimer et al. 1997).

2.5.2 The destruction of the hippocampal formation in Alzheimer's disease: Changes in the entorhinal cortex

Alzheimer's disease is the leading cause of dementia in the world. The major risk factor for Alzheimer's disease is age. Thus, the prevalence is 0.3 % for ages 60 to 69 years, 3.2% for ages 70 to 79 years, and 10.8% for ages 80 to 89 years (Rocca et al. 1991). A decline in memory and inability to learn new material are the first cognitive symptoms observed in AD. Also, spatial orientation, word finding, and personality changes are observed in the early stage of the disease. Later on, memory disturbances become more severe, including a worsening of remote memory, and behavioral and psychiatric disturbances. Clinically, the diagnosis of probable Alzheimer's disease is made when symptoms have an insidious onset, and there is progression of dementia, and no other systemic or brain disease can account for the dementia (McKhann et al. 1984). Various genetic factors are related to AD (Hardy et al. 1998, Hardy and Gwinn-Hardy 1998, Yanker 1996). Familial AD can be caused by either missense mutations of presenilin 1 and 2 on chromosomes 14 and 1, respectively, or point mutations of the amyloid precursor gene on chromosome 21. Moreover, genetic risk factors contributing to AD have been described and most consistently apolipoprotein E ε4 is related to early onset of the disease (Roses 1998).

The definite diagnosis of Alzheimer's disease is based on the neuropathologic abnormalities originally reported by Alois Alzheimer in 1907, who noted the occurrence of numerous senile plaques and neurofibrillary tangles in the cerebral cortex of a demented patient (DeArmond et al. 1997, Mirra et al. 1991). Formation of neurofibrillary tangles, neuronal loss, decrease in dendritic extent, and synaptic depletion disturb the communication among various cortical areas, resulting in anatomic isolation and fragmentation of many cortical zones. Different cortical circuits, however, are not equally vulnerable to Alzheimer's pathology. In particular, two cortical systems that appear to be involved in the neural processing of memory are selectively vulnerable to degeneration in Alzheimer's disease. The first one consists of connections between the hippocampus and its neighboring cortical structures within the temporal lobe. The second one is the cortical cholinergic system that originates in neurons within the basal forebrain and innervates the entire cortical mantle (Geula 1998).

According to Braak and Braak, the first neurofibrillary changes occur in the transentorhinal area (transentorhinal stages I-II); they then cover layer II of the entorhinal cortex and parts of the CA1 area and the subiculum (limbic stages III-IV). In the later stages, all isocortical association areas are affected (Braak and Braak 1991). On the other hand, pathologic amyloid deposits first appear in the basal portions of the frontal, temporal, and occipital lobes. Layer III of the entorhinal cortex also has clouds of amyloid. At later stages, medium densities of amyloid deposits are present in almost all isocortical association areas. The primary sensory areas, however, are almost devoid of these changes. The hippocampal formation is usually only mildly involved in amyloid accumulation: CA1 and subiculum and molecular layer of dentate gyrus contain amyloid deposits. Thus, the susceptibility of various neuronal populations for neurodegeneration differs. The reason for this is unknown.

The damage to the entorhinal cortex occurs very early in the disease course; layer II neurons in the transentorhinal region and in the lateral portion of the entorhinal cortex contain neurofibrillary tangles (Braak and Braak 1991). At this stage the dementia is not obvious in the clinical dementia tests, and thus it is considered the preclinical stage of the Alzheimer's disease (Braak and Braak 1991). In addition to the tangle formation, cell loss in the layer II of the entorhinal cortex is observed in mildly-demented AD patients (Gomez Isla et al. 1996). The neurofibrillary tangles are formed in the large pyramidal-shaped or stellate cells of layer II and later also in layer V neurons, whereas the non-pyramidal cells are mainly devoid of neurofibrillary tangles (Braak and Braak 1985, Brion and Resibois 1994, Solodkin et al. 1996). Layer III of the entorhinal cortex is often occupied by amyloid plaques in later stages of Alzheimer's disease (Braak and Braak 1991). Furthermore, the number of synapses is decreased in the entorhinal cortex of AD patients (Heinonen et al. 1995). Also, as revealed by magnetic resonance imaging studies, the entorhinal cortex atrophies very early (Juottonen et al. 1998). Although the general loss of neurons has been previously described, the changes of the specific cell populations of the subfields of the entorhinal cortex and the detailed fate of the various non-pyramidal cells has not yet been described. Furthermore, the consequences of selective neuronal loss in the cellular level are not widely studied.

Although cortical connectivity does not explain the etiology of AD, many pathomorphologic changes and some aspects of the progression of the disease can be explained on an anatomic basis (De Lacoste and White 1993). In AD the cells of origin the entorhino-hippocampal projections are damaged and synaptic loss is observed in the outer molecular layer of the dentate gyrus. Although the etiology and the time course of AD and entorhinal lesions of rat are fundamentally different, there are numerous similar changes in the lesion model and Alzheimer's disease (Geddes et al. 1985). Sprouting of either cholinergic or non-cholinergic cells and neuritic plaques occur in the outer two-thirds of the molecular layer, which suggests that sprouting is related to the pathogenesis in AD (Aubert et al. 1994, Geddes et al. 1986). Furthermore, the growth-related molecules, synaptic-axonal proteins, and cytoskeletal proteins are localized in the regions where plaques are formed (Masliah et al. 1994a, Masliah et al. 1991a, Masliah et al. 1994b, Masliah et al. 1991b).

2.5.3 The destruction of the hippocampal formation in temporal lobe epilepsy: The role of entorhinal cortex

Epilepsy is a syndrome of unpredictable spontaneously recurrent seizures. Its prevalence ranges from 150 to 1950 per 100000 (Keränen 1988). According to the etiologic factor, epilepies are divided into three categories. In symptomatic epilepsies, brain damage or disease is the most probable cause of seizures. In cryptogenic epilepsy, no certain etiologic factor can be determined. In idiopathic epilepsy, certain genetic factors most likely cause the seizures and no other etiology can be determined. In addition, epileptic seizures are divided into either partial or generalized epilepsies. In partial epilepsies, there is a specific brain area that initiates the seizures, whereas in generalized epilepsy no certain brain areas can be determined as an initiator of the seizures. Furthermore, the consciousness of the patient in a simple partial seizure is undisturbed, whereas the consciousness of patient in a complex partial seizure is disturbed (Commission on Classification and Terminology of the International League Against Epilepsy 1989).

The temporal lobe epilepsy is the most common type of the partial epilepsies. TLE usually manifests as complex partial seizures, which account for 40 % of the epileptic seizures in adults (McNamara 1992). Epileptic seizures, abnormal and excessive synchronized electrical activities in a group of neurons of the temporal lobe, present as psychosensory events including taste, smell, fear, sexual, pleasure sensations, and memory disturbances (Engel 1996). Symptomatic TLE is thought to be a consequence of the initial neuronal damage, which propagates reorganization of neuronal circuitries, an imbalance between excitation and inhibition, and causes seizures. Most of the neuropathologic and electrophysiologic knowledge about temporal lobe epilepsy is based on the findings in the hippocampus, although several medial temporal lobe structures are damaged.

Selective neuronal loss and active gliosis are the basic morphologic changes observed in temporal lobe epilepsy (Mathern et al. 1997). These changes are most prominent in CA1 region and in part of the subiculum (the so called Sommer's sector). The cell loss and gliosis are called hippocampal sclerosis (Mathern et al. 1997). Also, the hilus of the dentate gyrus and the CA3 region exhibit cell loss (end folium sclerosis) (Mathern et al. 1997). On the other hand, the CA2 region and granule cells of the dentate gyrus are often less severely diminished (Mathern et al. 1997). The differential vulnerability of the subfields of the hippocampus proper and dentate gyrus raise a question as to which cell populations are susceptible. Differences are suggested to be related to the calcium-binding protein content of the neurons and to differences in the receptor subtypes.

Hippocampal sclerosis occurring in TLE is accompanied by axonal reorganization (Sutula et al. 1989, Sutula et al. 1992). In this sprouting process, the mossy fibers of the granule cells (dynorphin immunoreactive) sprout into the inner portion of the dentate molecular layer (Houser 1992, Houser et al. 1990). In addition to mossy fibers sprouting, axons containing neuropeptide Y, somatostatin, and GABA demonstrate sprouting (Mathern et al. 1995). In addition to neuronal loss, abnormal localization of the granule cells, which is called dispersion, is observed. Dispersed granule cells of the dentate gyrus are localized in the supragranular or inner molecular layer (Houser et al. 1990). The reason and role of the dispersion are not known.

In addition to the hippocampus proper, other medial temporal lobe structures are involved in TLE, such as the amygdala (Kälviäinen et al. 1997, Pitkänen et al. 1998) and the entorhinal cortex (Du et al. 1993). Cell loss is the most profound in layer III of the rostral entorhinal cortex (Du et al. 1993). In experimental studies of status epilepticus of rats, layer III of the medial portion of the entorhinal cortex is suggested to be the most vulnerable (Du et al. 1995a). Evidence is also beginning to point to the entorhinal cortex as one of the primary sites in which temporal lobe seizures propagate and reverberate (Spencer and Spencer 1994). Furthermore, the structural changes in the entorhinal cortex might propagate changes in the hippocampus. On the other hand, there are studies that favor the idea that the presubicular or thalamic connection to the entorhinal cortex is the determining factor of cell loss (Bertram et al. 1998, Eid et al. 1996). In recent magnetic resonance imaging studies, the volume of the entorhinal cortex was also decreased in a subpopulation of patients with temporal lobe epilepsy (Bernasconi et al. 1999, Salmenperä et al. 1998). Very little is known, however, about morphological changes in the entorhinal cortex in TLE.

2.6 Plasticity of the hippocampal formation

2.6.1 Plasticity of the central nervous system: Neural cell adhesion molecule and its polysialylated form

Plasticity and the mechanisms controlling plasticity in the central nervous system are presumably essential for development, but they are also important for learning and memory (Cremer et al. 1997, Cremer et al. 1994). It has become increasingly evident that certain molecules, such as cell adhesion molecules (CAMs), regulate neuronal development. They also regulate maintenance of the neuronal connections and plasticity of these connections in the adult brain.

Adhesion molecules are ligands and receptors that mediate cell-to-cell and cell-to-substratum interactions in a lock and key manner. The numerous adhesion molecules are classified functionally into calcium-dependent or calcium-independent groups (Fields and Itoh 1996). The largest group of calcium-independent cell adhesion molecules (CAMs) is the immunoglobulin (Ig) superfamily, which differs in the number of Ig motifs and fibronectin repeats. The CAMs are located in the cellular membrane so that the COOH-tail is cytosolic and the NH2-terminus is located extracellularly (Fields and Itoh 1996). The three major forms have molecular masses of: 120, 140, and 180 kDa (NCAM-120, NCAM-140, and NCAM-180, respectively), which have similar extracellular domains but differ in their anchoring to the membrane (Jorgensen 1995). All three isoforms of NCAM have five Ig loops and two fibronectin type III domains. Each Ig loop has been implicated in various functions: Ig1 is important for cell adhesion and neurite outgrowth and Ig5 contains the site for polysialylation. NCAM-120 is the predominant form in glial cells, whereas larger forms are expressed in neurons.

Polysialic acid (PSA) is a developmentally regulated carbohydrate composed of a linear homopolymer of α-2,8-linked sialic acid residues (Fryer and Hockfield 1996, Kiss and Rougon 1997, Rutishauser and Landmesser 1996, Seki and Arai 1993a). NCAM is the major, perhaps the only, carrier of PSA in the mammalian brain (Cremer et al. 1994). The polysialylation of NCAM occurs presumably in the Golgi apparatus by sialyltransferases (Kiss and Rougon 1997). The polysialylated form of the neural cell adhesion molecule (PSA-NCAM) is highly expressed in growing axons during embryonic stages when it is involved in neurite extension and synaptogenesis, yet in the adult brain its expression is normally quite low (Muller et al. 1996, Seki and Arai 1993a).

PSA-NCAM is only expressed in the adult brain in areas that retain neuroplastic potential, such the hippocampus, hypothalamus, and olfactory areas (Seki and Arai 1993a). PSA in NCAM attenuates adhesion forces and modulates overall cell surface interactions, thus allowing the dynamic changes in the shape and movement of cells, as well as in their processes (Rutishauser and Landmesser 1996).

The expression of PSA-NCAM is limited to certain areas of the hippocampus. In the adult human hippocampus, the outer portions of the molecular layer of the dentate gyrus and the hilus contain PSA-NCAM–positive profiles (Barbeau et al. 1995, Miller et al. 1993). Furthermore, recent data of PSA immunoreactivity in a 5-month old human infant indicates that PSA is associated mainly with a population of granule-like cells and their mossy fiber axons (Ni Dhuill et al. 1999). The number of granule cells is maximal during the first three years of life but it declines by an order of magnitude between the second and third decades and remains relatively constant and restricted to the granule cell layer/hilar border (Ni Dhuill et al. 1999). In infants the immunostaining has been observed in the inner molecular layer; however, as development advances, immunostaining relocates to the outer molecular layer from 2 years of age onwards (Ni Dhuill et al. 1999). In addition, numerous polysialylated hilar neurons become evident at 2 to 3 years of age and remain constant until the eighth decade of life. These findings suggest that NCAM polysialylation has a crucial developmental role. Thus, an attenuated PSA-NCAM-mediated neuroplasticity continues throughout the human lifespan.

Expression of PSA-NCAM is limited to the areas that are important for memory formation suggesting that these areas undergo continuous remodeling under normal circumstances (Fryer and Hockfield 1996). Indeed, removing PSA with endoneuraminidase, an enzyme that specifically cleaves the alpha-2,8-linked polysialic acid impairs the acquisition and retention of spatial memory of rats in the Morris water maze (Becker et al. 1996). Furthermore, in adult rats the sialylation states of NCAM transiently increase during the acquisition and consolidation of a passive avoidance task (Regan and Fox 1995). The induction of long-term potentiation was also completely prevented by endoneuraminidase treatment in hippocampal organotypic slice cultures (Muller et al. 1996).

The general idea for decades has been that there is no ability to form new neurons in the adult brain. Recent data, however, indicate that in certain brain areas the ability to generate neurons is preserved throughout life *i.e.* a new kind of neuroplasticity has been observed. These brain areas are olfactory areas (Murrell et al. 1996) and the human hippocampus (Eriksson et al. 1998). Some of the newly-formed granule cells in the hippocampus are PSA-NCAM immunoreactive, suggesting that the embryonic form of NCAM is a marker for newly-formed granule cells (Kuhn et al. 1996, Seki and Arai 1993b, Seki and Arai 1995). There is also further interest in the possibilty of regulating neurogenesis and the granule cell changes in various diseases or disease models (Cameron and McKay 1998). Indeed, seizures, injections of kainic acid (Gray and Sundstrom 1998, Parent et al. 1997), or ischemic attack (Liu et al. 1998), increase the formation of dentate granule cells and aberrant networks.

2.6.2 Lesion induced plasticity and changes in PSA-NCAM expression

The mechanisms regulating the development of the nervous system and plasticity in the adult nervous system have traditionally been thought to be separate from those that drive the events following brain injury. Brain plasticity, however, could also be crucial for the recovery from brain damage (Aubert et al. 1995). Following lesions of the perforant pathway in rat, an increase in PSA-NCAM

immunoreactivity is observed in the denervated outer two-thirds of the molecular layer of the dentate gyrus, but also in the inner one-third (Miller et al. 1994, Styren et al. 1994). Twenty-four hours after lesioning, PSA-NCAM immunoreactivity in the inner-third was lost, but remained in the outer two-thirds until the reinnervation was completed. The regulatory mechanisms of PSA-NCAM expression in the dentate gyrus are not known. A decrease in the synaptic activity, regression of presynaptic processes, and reactive gliosis in the denervated area could induce upregulation. This lesion-induced increase in the expression of the embryonic form of NCAM in the adult suggests that the reorganization process that occurs in response to damage in the hippocampus mimics normal developmental processes that occur during synapse formation.

Reorganization of neuronal circuitries might be involved in neurologic disorders. The consequences of selective cell loss in temporal lobe epilepsy are still unknown. One hypothesis is that the depletion of the hilar neurons, which normally form contacts with the granule cell dendrites in the inner molecular layer of the dentate gyrus, leads to free synaptic areas in the inner molecular layer. The granule cells, in turn, might send axon collaterals to these free synaptic sites. This mossy fiber sprouting is thought to be related to hyperexcitability and oversynchronization of the granule cells (Houser 1992, Houser et al. 1990). In neurodegenerative diseases, such as AD, neurons in the entorhinal cortex die sequentially in a process that takes several years. As a consequence, a diffuse loss of axons occurs in the molecular layer and degenerating axons are adjacent to yet unaffected axons from the entorhinal cortex. Thus, the sprouting of homologous fibers is likely to influence the course and duration of the degenerative disease in a functionally beneficial manner and the clinical manifestations of the disease can take years to appear (Cotman and Anderson 1988, Deller and Frotscher 1997, Geddes et al. 1985). During the progression of AD, a continuous but rather slow action of pathogenetic factors might activate compensatory mechanisms, serving to regain a neuronal population much of its synaptic connectivity in the presence of cell loss. Plasticity and sprouting are controversial issues in AD, however, because of the thinking that plasticity might be diminished compared to that in the normal aging adult or that regeneration might fail (DeWitt and Silver 1996).

AD and TLE both involve deterioration of the entorhinal-hippocampal loop. The diseases, however, damage somewhat distinct neuronal populations. In the entorhinal cortex, layer II neurons are filled with tangles in AD, whereas in TLE the layer III neurons are disappeared. On the other hand, the CA1 region is one of the most vulnerable areas in both diseases. Thus, the possible reorganization process in the entorhinal-hippocampal loop between these diseases might differ, but also similarities may exist. Consequences of the reorganization process might be different. Thus, in TLE sprouting is thought to cause more seizures, whereas in AD, sprouting is thought to preserve and slow the clinical manifestations of the disease.

3. AIMS OF THE STUDY

The entorhinal cortex is an important structure in the medial temporal lobe memory system, serving to connect the cortical structures and hippocampus. The entorhinal cortex is particularly vulnerable in AD and TLE. The evaluation of the neuronal organization of the entorhinal cortex and its hippocampal target areas in normal and diseased brain will contribute to a better understanding of this brain area in normal brain and in AD and TLE.

The aims of the present series of studies were:

1) To characterize the neuroanatomic organization of the human entorhinal cortex using calcium-binding protein immunohistochemistry (parvalbumin, calretinin, and calbindin) (A) and intracellular microinjection technique (B). The distribution and the morphology of the neuronal populations were studied in the normal adult human entorhinal cortex. **(I, II)**

2) To evaluate the pathologic changes in a lamina- and subfield-specific manner in Alzheimer's disease with special attention on the calcium-binding protein-containing neurons. **(III)**

3) To evaluate and compare the reorganization processes of the entorhinal cortex and its target areas in hippocampus in controls, and in AD **(IV)** or TLE **(V)** using immunohistochemistry for highly polysialylated neural cell adhesion molecule.

4. MATERIALS AND METHODS

4.1 Subjects

Brain tissue was obtained from 54 subjects. The autopsies (n = 29) were performed at the Department of Pathology in the Kuopio University Hospital. The brain tissue from biopsies (n = 25) were obtained from surgical operations in drug-refractory TLE patients performed at the Department of the Neurosurgery in the Kuopio University Hospital (Professor Matti Vapalahti). All the material used was studied by a neuropathologist to confirm the neuropathologic diagnosis (Docent Irina Alafuzoff or Docent Leo Paljärvi). The studies were conducted with the approval and following guidelines of the Ethics Committee of the University of Kuopio. Permission to obtain the postmortem tissue was provided by the National Board of Medical Legal Affairs.

In the first study, five brains with no evidence of clinically or pathologically observed brain disease were used (I). The second study consisted of three cases that were clinically and pathologically without any neurologic disease (II). The material used in the third study consisted a total of 20 brains (14 from AD patients and 6 from control subjects) (III). The fourth study consisted of 22 brains (12 AD cases and 10 control cases) (IV). The fifth study included 32 brains (25 TLE and 7 controls). In studies I, III, IV, and V the control material was partially overlapping, as was the material from AD patients in studies II and IV.

Neuropathologic Diagnosis of AD. Prior to fixation, the brains were weighed and visually evaluated for gross lesions and vessel abnormalities. Specimens from the right hemisphere were used for histopathologic diagnosis. Samples were taken from six different cortical areas (frontal, temporal, parietal, precentral, and occipital cortices and the gyrus cinguli), from four regions of subcortical gray matter (striatum, amygdala, thalamus, and hippocampus), and from five infratentorial regions (substantia nigra, locus coeruleus, medulla, vermis, and cerebellar cortex). The tissue samples were embedded in paraffin, cut into 7 μm–thick sections, and processed for diagnostic purposes using hematoxylin eosin counterstain combined with a modified version of Bielschowsky's silver impregnation method or thioflavin-S stain. All cases were classified into one of the neuropathologic diagnostic groups described by CERAD (The Consortium to Establish a Registry for Alzheimer's Disease) (Mirra et al. 1991).

TLE patients. Hippocampal specimens were obtained from patients who underwent epilepsy surgery due to drug-refractory TLE. The epileptic focus was on the left side in 10 patients and on the right side in 15 patients. Entorhinal tissue was available from five patients. The duration of epilepsy was 21 ± 14 years. The etiologies associated with epilepsy were asphyxia (n = 3), infection (n = 4), vascular malformation (n = 1), brain tumor (n = 2), head trauma (n = 1), heterotopia (n = 1), and dysplasia (n = 1). No clear etiologic factors associated with epilepsy could be identified in 12 cases (cryptogenic epilepsy). All patients underwent a thorough clinical, electrophysiologic, neuropsychologic, and imaging evaluation prior to surgery for epilepsy.

4.2 Histologic procedures

Fixation and cutting. Coronal slabs (1.5-cm thick) through the hemisphere or the slabs obtained from biopsy specimens were fixed in 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 72 h at 4°C. The slabs were sectioned into smaller pieces and cryoprotected in 10% glycerol with 2% dimethylsulfoxide (DMSO) in 0.1 M sodium phosphate buffer (pH 7.4) for one day at 4°C and thereafter in 20 % glycerol with 2 % DMSO in 0.1 M sodium phosphate buffer (pH 7.4) at 4°C for 3 days. The brain tissue was then frozen and stored at -70°C until immunohistochemical processing. Frozen 50 µm–thick coronal sections were cut with a sliding microtome and collected in a 1:8 series throughout the entire rostrocaudal extent of the entorhinal cortex.

Immunohistochemical stainings. For the immunohistochemical staining of calcium-binding proteins, mouse anti-parvalbumin (monoclonal, 1: 10000, Swant, Bellinzona, Switzerland), rabbit anti-calretinin (polyclonal, 1: 7500, Swant), or rabbit anti-calbindin-D28k (polyclonal, 1: 5000, Swant) or mouse anti-calbindin-28k (monoclonal 1: 5000, Swant) antibodies were used. The PSA-NCAM immunohistochemistry was performed by using a mouse monoclonal antibody, 12E3 (a gift from Dr. T. Seki, Tokyo, Japan; IgM, diluted 1:800), that recognizes the PSA portion of the highly polysialylated form of NCAM (Seki and Arai 1991). Additional stainings were performed for the fifth study: dynorphin-A (R# 367-2; dilution 1:12 000; Peninsula Laboratories Inc., Belmont, CA) and monoclonal antibody raised against glial fibrillary acidic protein (GFAP; # 814 369; dilution 1:500-1:1000; Boehringer Mannheim Biochemica, Mannheim, Germany). For study IV, anti-NCAM (AB 1505, Chemicon International, Temecula, CA, USA) antibody was used in Western blot analysis.

To reduce endogenous peroxidase activity, the sections were pretreated with 3% hydrogen peroxide and 10% methanol in 0.02 M potassium phosphate buffer containing 0.9% sodium chloride (KPBS). Non-specific binding was then blocked by preincubating the sections in a solution containing 10% normal horse serum (for monoclonal parvalbumin and calbindin) or normal goat serum (for polyclonal calretinin and calbindin, and PSA-NCAM) and 0.5% Triton X-100 in 0.02 M KPBS. Sections were incubated 2 to 3 days at 4°C in one of the four primary antibody solutions containing 1% normal horse (parvalbumin and calbindin) or goat (calretinin, calbindin, PSA-NCAM) serum in 0.02 M KPBS and 0.5% Triton X-100. Sections were then rinsed and placed in the appropriate secondary antibody (for parvalbumin biotinylated horse anti-mouse IgG (1: 200), for calretinin and calbindin biotinylated goat anti-rabbit IgG (1:100; Vector, Burlingame, CA), and for PSA-NCAM biotinylated goat anti-mouse IgM (1:300; #BA-2020, Vector)) in 0.02 M KPBS containing 0.3% Triton X-100. After rinsing, sections were further incubated in avidin-biotin complex, rinsed, and reacted with 3,3'-diaminobenzidine tetrahydrochloride (DAB; 0.05% solution containing 0.04% hydrogen peroxide). For the PSA-NCAM immunohistochemistry the peroxidase reaction was developed using 0.0004% hydrogen peroxide as a substrate and 0.03% DAB as a chromogen intensified with 0.1% ammonium nickel sulphate in 0.1 M sodium phosphate buffer (pH 7.4). For electron microscopy of PSA-NCAM, the reaction was developed using DAB as the chromogen with no nickel intensification. The sections were mounted onto gelatin-coated slides, dried overnight at 37°C, and coverslipped. The calcium-binding protein sections were mounted onto glass slides and intensified with osmium tetroxide and thiocarbohydrazide prior to coverslipping.

For electron microscopy, sections were treated with 1% osmium tetroxide (OsO_4) for an hour, dehydrated, and embedded in Durcupan (AMC, Fluka) between a glass microscope slide and coverslip. The coverslip was removed with the aid of a razor blade and the area of interest was re-embedded for further ultrathin sectioning into a flat-bottom embedding capsule. Ultrathin sections were mounted on copper grids.

Other stainings. One series of sections was stained with thionin to identify the cytoarchitectonic subfields of the entorhinal cortex. An adjacent series of sections was stained using the method of Bielschowsky for characterizing any neuropathologic changes in the entorhinal subfields of AD (III).

4.3 Antibody specificities and Western blot procedure

The specificities of the parvalbumin, calretinin, calbindin, and PSA-NCAM antibodies were previously described (Braak et al. 1991, Celio et al. 1988, Schwaller et al. 1993, Seki and Arai 1991). In the present analyses, no notable effect of postmortem delay on the immunoreactivity at the light microscopic level was found. In addition, omitting any of the four antibodies from the primary antibody incubation resulted in a complete absence of immunoreactive elements. To determine the specificities of polyclonal calbindin and calretinin antibodies and PSA-NCAM, a Western blot analysis was performed using normal human or rat hippocampal tissue. Briefly, the tissue was homogenized in phosphate-buffered saline containing 1% Triton X-100. Identical samples were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (Laemmli 1970). After immunoblotting the gel according to the method of Towbin et al. (1979), the membrane was probed with either the polyclonal anti-calretinin, polyclonal anti-calbindin antibody, PSA-NCAM, or with anti-NCAM (AB 1505, Chemicon International, Temecula, CA) antibody, followed by a secondary incubation in horseradish peroxidase–linked anti-rabbit immunoglobulin $F(ab)_2$ fragment (Amersham). Immunoreactive bands were visualized using a chemiluminescence system.

4.4 Lucifer yellow microinjection procedure

Fixation and cutting. The tissue specimen containing the whole rostrocaudal extent of the entorhinal cortex was removed and immediately immersed in cold 4 % paraformaldehyde in 0.1 M PB at pH 7.4 for 1 hour. Subsequently, the entorhinal cortex was divided into small blocks (20 x 10 x 10 mm) and the *pia mater* was removed and the blocks were further fixed in a new batch of cold 4 % paraformaldehyde in 0.1 M PB for 3 to 5 hours in 4° C. Thereafter, the tissue was sectioned into 200-µm-thick sections perpendicular to the pial surface. The sections were collected in cold phosphate buffer, and stored at 4° C until used.

Microinjection. A 200-µm-thick section was floated onto a glass plate held down by a piece of millipore filter with a hole cut out to expose the injection area. The section was immersed in a petri dish with 0.1 M PB and the injection chamber was placed under to a fluorescence microscope (Nikon Optiphot). The glass microelectrodes were filled with 6 % aqueous solution of lucifer yellow (Sigma Chemicals, St. Louis, MO). After penetration of the microelectrode into a cell body, lucifer

yellow was iontophoretically injected with a negative constant current of 2 to 5 nA for 5 to 10 minutes, until the dendritic tree and dendritic spines appeared fluorescent.

4.5 Analyses of the tissue

The sections were analyzed with either a Nikon Optiphot-2 or a Leica DMRB microscope under brightfield, darkfield, and Nomarski Optics. In the first study the parvalbumin immunoreactive neurons in four different levels of the entorhinal cortex were plotted with a computer-aided digitizing system (Minnesota Datametrics, St. Paul, MN). Camera lucida drawings were drawn from the most typical morphologies of the parvalbumin, calretinin, and calbindin immunoreactive neurons. To obtain approximate sizes of the somata, the cross-sectional areas were measured using a Quantimet 570 analyzing system (Cambridge instruments Ltd. Cambridge, UK.) Low power brightfield were taken with a Nikon multiphot 6x9 cm system. High magnification photomicrographs were taken with a Leica DMRB system. **(I)**

With aid of the lipofuscin in most of the cell bodies, it was possible to differentiate the subfields and layers of the entorhinal cortex. The photographs were taken with a Nikon Optiphot fluorescence microscope equipped with a mercury lamp and epifluorescence optics. Lucifer yellow was excited at 450 to 490 nm and emission was detected using a 520-nm barrier filter. The confocal images were prepared with Confocal Laser Scanning microscope (Leica Lasertechnik, Germany) equipped with an argon-krypton laser mounted on a microscope (Leitz Diaphan). In this microscope, Lucifer yellow was excited by 488 nm laser light and detected using a barrier band pass filter (535 nm). Optical images were made in the XY plane at 0.5-1 μm intervals for a total of 70-100 optical sections per scanning sequence using an image size of 512 x 256 pixels. Photographs were obtained through a video printer (Sony Mavigraph, Japan). The size of the somata was measured using stereologic methods (Stereo Investigator, Microbrightfield, Colchester, VT). **(II)**

In addition to the thionin staining, the Bielschowsky stain was used to compare the extent of entorhinal degeneration between control and AD cases. In two of the control cases, occasional neurofibrillary tangles were observed in layer II of the ELr, EI, ELc, EC, and ECL subfields. In contrast, neurofibrillary tangles were abundant in the entorhinal cortex in cases that fulfilled the diagnosis of AD based on CERAD criteria. The material was assigned into groups: mild, moderate, or severe destruction of the entorhinal cortex based on neurofibrillary and amyloid pathology in the EI subfield (see also Solodkin et al. 1996). Mild cases of entorhinal destruction had neurofibrillary tangles only in layer II, moderate cases had neurofibrillary tangles present in both layers II and V, and severe AD cases had neurofibrillary tangles and plaques in layers II, III, V, and VI. Low power brightfield pictures were taken with a Nikon multiphot 6x9 cm system. High magnification photomicrographs were taken with a Leica DMRB system. **(III)**

For quantification of the PSA-NCAM immunostaining, the optical density (OD) in the dentate granule cell layer, and in the inner one-third and outer two-thirds of the molecular layer was measured using the Image-Pro Plus System (Media Cybernetics L.P., Silver Spring, MD) connected to an Olympus Provis microscope (Tokyo, Japan) with a 10x objective. The width of the measured area (150 μm) parallel to the granule cell layer was kept constant. Because the thickness of the molecular layer varied from case to case, the entire thickness of the layer was measured, and thereafter, divided it into the inner one-third and outer two-thirds. The OD of PSA-NCAM

immunoreactivity for each pixel was measured, defining the black level as 0 and the white level as 255. Each case was measured twice and the mean value of the two measurements was used in the analysis. The OD of the granule cell layer did not differ between controls and epilepsy cases or controls and AD cases. Therefore, the OD in the inner and outer molecular layers was expressed as an OD ratio = (OD measured/OD of the granule cell layer). To calculate the density of infragranular PSA-NCAM positive neurons, the number of immunostained neurons was plotted in two successive sections of each case. Thereafter, the length of the granule cell layer was measured by drawing a line along the infragranular portion of granule cell layer using a Quantimet 570 image analyzer (Cambridge Instruments Ltd., Cambridge UK). The density of immunopositive infragranular cells in each case was expressed as the number of neurons/mm. The ultrastructural localization of PSA-NCAM (IV) was examined with a Jeol 1200 EX electron microscope. In TLE patients, the density of neurons in the granule cell layer, hilus, CA3 pyramidal cell layer, CA2 pyramidal cell layer, and CA1 pyramidal cell layer, the density of mossy fiber sprouting (Cavazos et al. 1991) and the severity of gliosis (Watson et al. 1996) were scored to estimate the destruction of the hippocampus. **(IV-V)**

Statistical analysis. The data were analyzed using SPSS for Windows V.6.0.1 software. The Mann-Whitney *U*-test was used to compare differences between the two patient groups. Correlations were calculated using a two-tailed Pearson's correlation test (V) and Spearman's correlation (IV). The data are presented as mean ± standard deviation (SD). A p-value of less than 0.05 was considered statistically significant.

5. RESULTS

5.1 Intrinsic organization of the human entorhinal cortex

5.1.1 Morphology and laminar distribution of the calcium-binding protein-containing neurons (I)

The parvalbumin-, calretinin-, and calbindin-immunoreactive neurons formed morphologically distinct neuronal populations, which also differed in their laminar distribution in the human entorhinal cortex.

Parvalbumin. Parvalbumin-immunoreactive neurons had the typical appearance of non-pyramidal cells. The dendrites of these neurons were aspiny or sparsely spiny. Based on the morphology and size of their somata and the morphology of the dendritic trees they were divided into small, medium, or large multipolar or bipolar neurons. Parvalbumin-immunoreactive fibers and neurons were most densely located in the deep portion of layer III and in the cellular clusters of layer II. In the deepest border of the cellular cluster of layer II, one to four large multipolar neurons typically had thick dendrites reaching the adjacent cluster as well as layers I and III. Typical to parvalbumin were the immunoreactive axonal baskets of layer II around the unstained somata. In the deep portion of layer III, the medium-sized neurons formed the majority of the parvalbumin-positive neurons. In layers V and VI, fewer parvalbumin positive neurons were observed; however, parvalbumin-immunoreactive cartridge-like structures were frequent in these layers.

Calretinin. Calretinin-immunoreactive neurons were aspiny or sparsely spiny small multipolar or bipolar neurons that were remarkably smaller and had rounder somata than the parvalbumin-immunoreactive neurons. These small neurons were located in layers II and III. In layer II, they were located in the cellular clusters, mainly in the superficial regions, but also between the cellular clusters. The dendrites of small calretinin neurons typically ran radially. In the deep layers, layers V and VI, calretinin immunoreactivity was localized in the large neurons, which had typical appearance of pyramidal cells. These large neurons had prominent apical dendrites running towards the more superficial layers.

Calbindin. Calbindin-immunoreactive neurons were multipolar or bipolar non-pyramidal cells that had a more divergent somal size and morphology compared to calretinin-immunoreactive neurons. Furthermore, the staining intensity varied from lightly-stained to darkly-stained cell bodies. Calbindin neurons were more homogeneously distributed throughout the layers, but they also preferred the superficial layers. Lightly-stained multipolar cells with pyramidal shaped somata were typically observed in the layers II and III of EO and EMI.

5.1.2 Distribution of the calcium-binding protein-containing neurons in the entorhinal subfields (I)

The overall distribution of the parvalbumin, calretinin and, calbindin immunoreactive fibers, terminals, neuropil and cells largely followed the cytoarchitectonic borders of the eight subfields of the human entorhinal cortex. The concentrations and distributions were largely dependent on the subfields analyzed. For example, the border between the EMI and EI was easily visualized with aid of

parvalbumin immunoreactivity; the EMI was almost devoid of parvalbumin, and the density of fibers was high in the EI. The regional distribution of the three calcium-binding proteins was segregated rather than overlapping. The highest densities of parvalbumin-immunoreactive fibers and neurons were observed in the caudal and lateral subfields of the entorhinal cortex, whereas calretinin and calbindin immunoreactivity were highest in the rostromedial subfields.

Parvalbumin. The EO, ER, and EMI subfields were almost devoid of parvalbumin immunostaining, except for occasional fibers in layer I as well as in the deep portion of layer III of the ER. The number of parvalbumin-immunoreactive profiles and the intensity of fiber staining was increased in the ELr and EI subfields, especially in layer II and in the deep portion of layer III. The immunostaining was most intense in the caudal subfields, including the EC and ECL where the density of parvalbumin-immunoreactive neurons was the highest throughout the entorhinal cortex and where the fiber staining was also high.

Calretinin. Calretinin immunoreactivity was localized in the somata, dendrites, axons, punctae and neuropil. Calretinin immunoreactivity was more homogeneously distributed than parvalbumin immunoreactivity throughout the entorhinal subfields. The multipolar and bipolar immunoreactive neurons were randomly distributed in the EO, ER, and EMI, more laminated in the ELr, and densely clustered in the EI, ELc, EC, and ECL subfields. Furthermore, large calretinin-immunoreactive pyramidal-shaped neurons with long radially-oriented apical dendrites were numerous in layers V and VI of the EO, ER, and EMI, where immunoreactive fiber and neuropil staining were densest. The pyramidal-shaped calretinin-immunoreactive neurons were also more sparsely distributed in the ELr, EI, ELc, EC, and ECL subfields.

Calbindin. Calbindin immunoreactivity was localized in somata, dendrites, axons, punctae, and neuropil. Calbindin immunoreactivity was homogeneously distributed among the entorhinal subfields with only a few visible differences. The EO, ER, and EMI subfields typically had the densest fiber and neuropil staining. The lightly-stained multipolar and pyramidal-shaped calbindin-immunoreactive neurons of layers II and III were most numerous in the EO and EMI subfields. The smaller multipolar and bipolar immunoreactive neurons were scattered throughout the layers, although they were less numerous in layer II of the EI subfield.

5.1.3 Morphology and distribution of the microinjected spiny neurons of the human entorhinal cortex (II)

The spiny neurons were divided into four major categories according to the shape of the somata and the morphology of the primary dendrites:

A) Stellate cells/Multipolar cells had rounded and often circular cell bodies and multidimensional dendritic trees. Stellate cells often had five or more primary dendrites, which were similar in diameter. The primary dendrites radiated from the somata in a star-like fashion and gave off several secondary and tertiary branches. The secondary and tertiary branches of the dendrites were covered with spines having long necks and small rounded heads, a typical drumstick appearance.

B) Modified stellate/Oval "unipolar" cells had oval cell bodies with one thick primary dendrite. Thus, on the basis of the primary dendrite the neuron had unipolar appearance. The other primary

dendrites were thinner and arborized in a star-like fashion in all directions from the somata. The secondary dendrites and the thin primary dendrites were covered by drumstick-like spines.

C) Horizontal tripolar cells had triangular or fusiform cell bodies that were elongated horizontally and oriented parallel to the pial surface. The dendrites emanated from the three poles of the somata, giving a tripolar appearance. The secondary branches were covered by spines without very prominent heads. These neurons resembled pyramidal cells except that they were horizontally oriented and all the primary dendrites were similar in diameter.

D) Classical pyramidal cells had triangular somata and tripolar primary dendritic trees. These neurons had thick apical dendrites, which were oriented perpendicular to the cortical surface giving a vertical orientation to the neuron. The two to three basal dendrites of the pyramidal cells bifurcated repeatedly. The secondary and tertiary branches were covered with drumstick-like spines.

In layer II, the majority of the neurons were stellate or modified stellate cells. In the rostromedial subfields, the layer II-neurons were more often horizontal tripolar neurons. In layers III, V and, VI the neurons were mainly classical pyramidal cells and were smallest in somal size in layer III. Layer II neurons had the densest ramification of dendritic tree in the cellular cluster of layer II, but also several branches of dendrites reached layers I and III. In the superficial portion of layer III, the dendrites also reached the intercluster space of layer II or to the deep portion of layer III. In the deep layers, the apical dendrites of the pyramidal cells reached up to layer III or remained in the deep layers, V and VI.

5.2 Vulnerability of the subfields and changes in calcium-binding protein containing-neurons in Alzheimer's disease (III)

Thionin and Bielschowsky stains revealed the most dramatic cell loss and neurofibrillary tangle formation in layers II and V of the lateral, intermediate, and caudal subfields in AD cases. Accordingly, immunohistochemical staining revealed that neurons and fibers that contained calcium-binding proteins were also more frequently altered in these subfields than in the rostromedial subfields. Detailed analysis further revealed that non-principal cells containing parvalbumin or calbindin had morphologic alterations early in the entorhinal pathology of AD, whereas non-principal neurons containing calretinin were better preserved even in AD cases with severe entorhinal pathology. The degeneration of parvalbumin-immunoreactive neurons and basket-like networks and calbindin-immunoreactive non-principal neurons were observed mainly in layer II. The calretinin-immunoreactive non-principal neurons, however, were radially organized and morphologically intact longer than parvalbumin and calbindin non-principal cells. In the most severe cases calretinin immunoreactive cells formed aggregates in the border region of layers II and I. The pyramidal-shaped neurons containing either calretinin or calbindin were often preserved, although morphologic alterations were observed.

5.3 PSA-NCAM immunoreactivity in the entorhinal cortex and in its major target areas in the hippocampus (IV-V)

5.3.1 Controls

In all studied areas, PSA-NCAM immunoreactivity appeared as punctuate precipitates in cell bodies, fibers, and neuropil.

Entorhinal cortex. In the entorhinal cortex, only occasional immunopositive cell bodies were seen. In contrast, radially oriented and darkly stained 20- to 50-μm–long segments of fibers were typically observed within the cellular clusters of layer II in all the subfields and in the superficial portion of layer III especially in the EO, ER, and EMI subfields. Layer V had a dense mesh of fiber staining in the EI, EC, and ECL subfields. Other layers also had fiber and punctuate neuropil staining, although not as strong as that in layer V. The PSA-NCAM–positive bundles of layer II of the entorhinal cortex in control cases were characteristic of axon initial segments. Although PSA-NCAM immunoreactivity was mainly attached to membranes, it was also present in the cytoplasm.

Dentate gyrus. Most of the immunopositive neurons in the hilus had multipolar somata with three to five dendrites. A few large immunopositive fusiform neurons were also observed at the crest of the granule cell layer. In all controls, there were immunopositive, medium-sized multipolar neurons that lined up along the infragranular region of the granule cell layer. These cells had an apical dendrite that transgressed the granule cell layer and extended toward the middle and outer thirds of the molecular layer. The rest of the neurons in the granule cell layer were immunonegative. Occasional medium-sized multipolar cells were observed in the molecular layer. The outer two-thirds of the molecular layer was more intensely labeled in PSA-NCAM preparations than the inner third, and thus the molecular layer appeared laminated. Occasionally, a slightly increased intensity of the labeling in the supragranular region was observed. Within the granule cell layer, the immunopositive fibers and punctae surrounded the unstained or lightly-stained somata.

CA1. Immunopositive neurons were observed in the *stratum oriens, stratum pyramidale,* and *stratum lacunosum moleculare* of the CA1 region. These neurons were either multipolar or fusiform. A substantial density of varicose fibers was observed in the *stratum oriens, stratum radiatum,* and *stratum lacunosum moleculare*. The *stratum pyramidale* contained a lower density of punctate fibers and light neuropil labeling that was similar in density throughout the mediolateral extent of CA1 region.

5.3.2 Alzheimer patients

PSA-NCAM immunoreactivity was localized in neuronal cell bodies, fibers, and neuropil.

Entorhinal cortex. In layer II and the superficial portion of layer III of the entorhinal cortex of AD patients, the radially-oriented stained fiber segments observed in control tissue had disappeared and were replaced by non-oriented fiber staining. This was most evident in the EI, ELr, ELc, EC, and ECL subfields. Layer V contained even denser fiber staining than did controls, especially in the EI, EC, and ECL.

Dentate gyrus. PSA-NCAM immunoreactivity was especially high in the outer molecular layer and but also increased in the inner molecular layer in the dentate gyrus of AD patients when compared to controls. The optical density of the granule cell layer in the dentate gyrus did not differ significantly between AD patients and control cases. The number of PSA-NCAM–immunopositive infragranule cells was higher in the AD group, however, compared to controls.

CA1. The major finding in the CA1 region of AD patients was the disorganization of PSA-NCAM–immunoreactive fibers, especially in the *stratum oriens*, *stratum pyramidale* and *stratum radiatum*.

5.3.3 Temporal lobe epilepsy patients

In TLE patients, PSA-NCAM was localized in neuronal cell bodies, fibers, and neuropil. Glial-like staining was observed only in one patient with cortical migrational disorder.

Entorhinal cortex. In PSA-NCAM preparations, the most remarkable abnormality was the appearance of dense immunopositive plexi in layer II of the ER subfield of the entorhinal cortex. These plexi surrounded small immunopositive neurons, a pattern that was never observed in control cases. This finding was present in three of five patients from which entorhinal tissue was available for analysis. In these three cases, substantial neuronal loss in layer III of the ER in thionin preparations was observed.

Dentate gyrus. In patients with temporal lobe epilepsy, the optical density of punctate PSA-NCAM immunoreactivity was increased both in the inner and outer molecular layers of the dentate gyrus, compared to controls. The intensity of PSA-NCAM immunoreactivity in the inner molecular layer correlated with the duration of epilepsy, severity of hippocampal neuronal loss, density of mossy fiber sprouting, and astrogliosis. In TLE patients with only mild neuronal loss in the hippocampus, the density of infragranular immunopositive neurons was increased two-fold compared to controls, whereas in TLE patients with severe neuronal loss, the infragranular PSA-NCAM positive cells were not present. In the hilus, the somata and tortuous dendrites of some surviving neurons were intensely stained in patients with TLE.

CA1. The pattern of neuropil and fiber labeling in the CA1 pyramidal cell layer differed from controls in 15/23 cases. Typically, the loss of pyramidal cells in thionin preparations was most severe in the proximal portion (closest to CA2) of the CA1. The damaged region contained only scattered clusters of thick and tortuous varicose PSA-NCAM-immunopositive fibers that surrounded the darkly stained multipolar cells. At the border region between the damaged proximal and the better preserved distal (closest to the subiculum) CA1, a substantial increase in PSA-NCAM positive varicose fibers and neuropil in the *stratum oriens* and *stratum pyramidale* was observed. This area also contained numerous darkly-stained medium-sized multipolar neurons surrounded by varicose fibers.

6. DISCUSSION

In the present series of studies, the detailed distribution and morphologic types of the calcium-binding protein-containing neurons **(I)** and the spiny neurons **(II)** in the human entorhinal cortex were described. Most of the earlier morphologic studies of spiny neurons were performed in rodents and primates. No previous studies systematically described the distribution of various calcium-binding proteins in human entorhinal subfields, although there are more general descriptions of parvalbumin, calretinin, and calbindin elements in the human entorhinal cortex (Beall and Lewis 1992, Brion and Resibois 1994, Schmidt et al. 1993, Tunon et al. 1992). The changes in the subfields of the entorhinal cortex in AD with thionin and Bielschowsky stains and calcium-binding protein immunohistochemistry **(III)** was also described. The differences in susceptibility to damage of various entorhinal subfields and certain cell populations extend the understanding of the pathologic changes of the entorhinal cortex in AD. In addition, the changes in the cellular organization of the entorhinal cortex and in its hippocampal projection areas in Alzheimer's disease **(IV)** and temporal lobe epilepsy **(V)** were described with aid of PSA-NCAM immunohistochemistry.

6.1 Methodologic considerations

Research Material. The research material consisted of autopsy material from the brain bank system, in which the autolysis time was as short as possible (Table 1 in studies **I**, **III**, and **IV**). Differences in postmortem time did not have a remarkable effect on the calcium-binding protein or PSA-NCAM immunoreactivity (also shown by Ni Dhuill et al. (1999) at the light microscopical level. Thus, the autopsy material was used as control material to the temporal lobe epilepsy specimens obtained from surgeries for epilepsy. On the other hand, for ultrastructural analysis at electronmicroscopic level, brains with a long postmortem delay were not suitable. Furthermore, intracellular filling was not possible to perform in tissue over 16 hour postmortem delay.

Immunohistochemistry. The specificities of antibodies used in the present series of studies were previously described or studied in the context of these studies. Omission of the antibody resulted a blank section without any immunoreactive profiles. The Western-blot analysis revealed the specific bands of staining with certain molecular weights.

Microinjection technique. The microinjection technique is useful for detecting single neurons without disturbing other neuronal or glial elements. Compared to the traditional Golgi method, it is time-consuming in terms of the number of filled neurons per hour, but it is also more specific because one can fill neurons of interest in specific layers or areas. The intracellular filling technique is easier in human material than in rat material, because human neurons contain lipofuscin whereas in rat only old animals have lipofuscin in their cell bodies. Selection between the non-pyramidal and pyramidal neurons can be made even with aid of the lipofuscin of the somata, because in non-pyramidal cells the lipofuscin is rounded and compact, whereas in pyramidal cells lipufuscin is more loosely arranged. Some neuronal types, however, might be easier to fill than others. For example, intracellular filling is biased towards frequently-occurring cell types with large somata. Furthermore, some neurons remain weakly-fluorescent despite filling and they cannot be analyzed. Dendritic staining of a neuron located in the middle of the section might differ from that of neurons located on the edges of the sections. In study **II**, 369 neurons were filled, 17% of which had sparsely spiny or

aspiny dendritic trees and often small somata. Therefore, they resembled interneurons that can be stained with antibodies raised against parvalbumin, calretinin, or calbindin. In the present study, 52 neurons were in layer II, 33 in layer III, 39 in layer V, and 8 in layer VI. Thus, the data provided from layer VI neurons are based on a relatively low number of observations. Furthermore, a large majority of all dye-filled neurons were located in the ER, ELr, and EI subfields of the entorhinal cortex. The morphologic differences were, however, more remarkable between cellular layers than between the various subfields and, therefore, the location of most of the filled neurons was not believed to influence the collection of the data regarding the laminar differences in the morphology of neurons.

6.2 Comments on the entorhinal nomenclature and division of the entorhinal cortex into subfields - Usefulness of the calcium-binding proteins

The medial and lateral borders of the entorhinal cortex are somewhat unclear. The area entorhinal dorsalis (area 34) medial to area entorhinalis (area 28) was described according to Brodmann (1909; see Garey (1994), Insausti et al. (1995). This area corresponds the EMI, and some investigators still use the expression “area 34” instead of the EMI. In the present study the EMI differed from the adjacent EI and medially-located periamygdaloid cortex in parvalbumin as well as in calretinin stainings, and thus parcellation of the area was easy. In the depth of the collateral sulcus, the entorhinal cortex gradually changes to the perirhinal cortex. In this region, which is called the “transentorhinal cortex” (Braak and Braak 1985), the cellular clusters of layer II of the entorhinal cortex slide towards the layer III. This transition area, which is not considered the entorhinal cortex proper, can also be easily visualized with parvalbumin immunostaining, when clusters gradually slide into deeper layers (Fig. 2 in study **I**). Thus, the lateral and medial border can be differentiated with aid of calcium-binding protein staining.

The nomenclature of the entorhinal cortex used in this study is useful largely because it is comparable with monkey and rat nomenclature (Amaral et al. 1987, Insausti et al. 1997). This aids the comparison of the connections of the entorhinal cortex, morphologies of the cell types and distribution of various markers between the species. On the other hand, in AD studies Braak’s nomenclature of entorhinal layers is often used (Braak and Braak 1991). The nomenclature of layers used here can be quite easily compared to that of Braak’s (see Table 1), while the subfield divisions (16) (Braak 1980) are more difficult to compare with the subfields of Insausti. Also simplified divisions have been also used in AD studies (lateral, intermediate, caudal), which might be too simplified, because the location and borders might be difficult to define (Hyman et al. 1986). The eight subfields of the human entorhinal cortex have differences in their calcium-binding protein immunoreactivity, as indicated in Figures 11, 12 and 13 of study **I**. The differential distribution of the calcium-binding protein containing-neurons in the human entorhinal cortex might also have functional relevance.

6.3 Classification of the human entorhinal neurons into various morphologic categories

All of the identified parvalbumin, calretinin, or calbindin immunopositive dendrites were either aspiny or sparsely spiny. As described earlier in the human temporal cortex (del Rio and DeFelipe 1996), these three calcium-binding proteins labeled largely non-overlapping cell populations of the human entorhinal cortex. Morphologically, parvalbumin-immunopositive cell bodies and dendritic

arborizations as well as basket-like plexi and cartridges resembled basket cells and chandelier cells observed in the monkey prefrontal cortex (Lund and Lewis 1993), human amygdala (Sorvari et al. 1995), and human entorhinal cortex (Schmidt et al. 1993). The calretinin-immunoreactive neurons had bi-tufted dendritic trees that resembled the vertically projecting local circuit neurons described in monkey prefrontal cortex (Lund and Lewis 1993), in the human amygdala (Sorvari et al. 1996b), in the rat (Miettinen et al. 1997), and in the human entorhinal cortex (Brion and Resibois 1994). Furthermore, calretinin-immunoreactive neurons in layer I had the morphology of Cajal-Retzius cells (Belichenko et al. 1995). Calbindin-immunoreactive neurons often had vertically oriented dendritic trees that resembled double-bouquet neurons described in monkey neocortex (DeFelipe et al. 1989a) or in the human amygdala (Sorvari et al. 1996a), and in the human entorhinal cortex (Tunon et al. 1992). In calretinin preparations, there were also large pyramidal shaped neurons in layers V and VI with long apical dendrites directed to superficial layers. These cell types resembled those earlier described in monkey prefrontal cortex (Conde et al. 1994), but were not earlier described in the human entorhinal cortex (Brion and Resibois 1994). In the rat entorhinal cortex, the calretinin-immunoreactive neurons located in the deep layers were GABA-immunonegative (Miettinen et al. 1997), which supports the idea that these types of neurons are projection neurons. Furthermore, in calbindin preparations, lightly-stained pyramid- shaped neurons located in layers II and III were observed, similarly as previously described in monkey prefrontal cortex and in human temporal cortex (Conde et al. 1994, Ferrer et al. 1992).

There are only a few studies of the morphology of spiny neurons in the human entorhinal cortex (Beall and Lewis 1992, Belichenko 1993). The data obtained in study **II** was mostly compared to the findings described in rat (Germroth et al. 1989, Germroth et al. 1991, Klink and Alonso 1997, Schwartz and Coleman 1981) and monkey (Carboni et al. 1990) entorhinal cortex. In the present study, stellate or multipolar neurons had multiple spiny primary dendrites of approximately the same thickness, and had no prominent apical dendrites. Neurons with similar morphology in the mammalian entorhinal cortex were termed "star cells" by Lorente de No (1933). Stellate cells resembled "spiny stellate cells" previously described in the entorhinal cortex of the rat (Klink and Alonso 1997) and "spinous multipolar neurons" in the entorhinal cortex of monkey (Carboni et al. 1990). Modified stellate cells or unipolar cells have one primary dendrite and several thinner dendrites arborizing in a star-like fashion. In monkey, a morphologically similar cell is called a pyramidal neuron (Carboni et al. 1990). According to Braak and Braak (Braak and Braak 1985), this cell type could also be considered a transitional type between the pyramidal cell and stellate cell. In our material, however, the primary dendrite of these neurons was shorter than that in neurons classified as pyramidal cells. Moreover, modified stellate cells were typically located in superficial layers whereas pyramidal cells were located in deeper layers. Therefore, the modified stellate cells were classified in their own category. Neurons classified as horizontal tripolar cells had triangular soma and horizontally-oriented dendrites. These neurons resembled the horizontal tripolar cells described previously in the rat (Klink and Alonso 1997, Schwartz and Coleman 1981). Consistent with classic studies by Lorente de No (1933) and more recent studies in the rat (Germroth et al. 1989) and monkey (Carboni et al. 1990) entorhinal cortex, classical pyramidal neurons were found in layers III, V, and VI of the human entorhinal cortex. Taken together, comparison of the present data with previous studies suggests that the morphologies of neurons in the human, monkey, and rat entorhinal cortex are similar.

6.4 Anatomic organization of the neurons in the human entorhinal cortex: Some functional aspects

Spiny neurons in the cellular clusters of layer II were composed of stellate, modified stellate, and horizontal tripolar cells. The spiny neurons had several primary dendrites that branched extensively within the cluster of layer II. Furthermore, they had dendrites extending to layer I or down to layer III. Thus, the present data indicate that entorhinal spiny neurons that are located in layer II and give rise to the perforant pathway to the dentate gyrus have dendritic fields extending through layers I, II, and III. These neurons are in the position to integrate information from the olfactory areas, superior temporal gyrus, presubiculum, and perirhinal and parahippocampal cortices (Amaral et al. 1983, Caballero-Bleda and Witter 1994, Insausti et al. 1987a). The parvalbumin neurons localized in layer II also had extensive dendrites reaching layers I, II, and III. Furthermore, the parvalbumin-immunoreactive axonal baskets were observed around primary neurons. Thus, parvalbumin immunoreactive neurons might influence the synchronization of the primary neurons, which suggests that they form a critical network that controls entorhinal outputs to the hippocampus. The calretinin-immunoreactive neurons formed fiber plexi inside the cellular clusters but also radially organized fiber bands between the cellular clusters. The calretinin-immunoreactive non-pyramidal cells form symmetric synapses with the dendrites and somata of pyramidal cells, but they also form synapses with non-principal cells (del Rio and DeFelipe 1996, del Rio and DeFelipe 1997, Freund and Buzsaki 1996). Thus, they might modulate information of layer II differently compared to parvalbumin neurons, and they might form a disinhibitory network. The largely non-overlapping distribution of parvalbumin, calretinin and calbindin neurons in layer II of the entorhinal subfields indicates that each of the cell populations may modulate a different subset of topographically organized entorhinal outputs *i.e.* the caudolaterally-located parvalbumin neurons regulate the output to caudal hippocampus, whereas calretinin and calbindin neurons control the information more extensively in all rostrocaudal and mediolateral levels.

The apical dendrites of layer V and VI neurons largely remained in deep layers III, IV, V and VI. Thus, layers V and VI are mainly influenced by inputs coming from the CA1 region and subiculum. The parvalbumin-immunoreactive neurons located in the deep layers formed cartridge-like structures around axon-initial segments of pyramidal cells. This eventually has impact on the memory trace transfer from the entorhinal cortex to neocortex. In conclusion, there appears to be more overlap in the dendritic fields in the layers II and III than between the superficial and deep layers, which supports the idea of segregation of information flow targeted to superficial layers (layers projecting to hippocampus) or deep layers (layers projecting to neocortex) of the human entorhinal cortex.

6.5 Alteration of the organization of the entorhinal cortex

6.5.1 Changes in calcium-binding protein containing-neurons in AD patients

Parvalbumin-immunoreactive neurons are characterized as either basket or axo-axonal GABAergic neurons that are responsible for perisomatic inhibition of the principal cells (Freund and Buzsaki 1996). In this study, the parvalbumin-containing neurons and the parvalbumin-immunoreactive basket-like network in layer II were diminished in AD (see also Figs. 3 and 4 in study **III**). The findings are in accordance with those from a previous study (Solodkin et al. 1996). The parvalbumin-immunoreactive neurons in layer III and the fiber staining in this layer were preserved

longer, and changes appeared only in the most severe AD cases. The pyramidal cells of layer III were also better preserved than principal cells of layer II. Therefore, the loss of the parvalbumin-immunoreactive neurons and fibers appeared to parallel the degenerative sequence of the primary neurons in a layer-specific manner.

Calretinin was localized in pyramidal-shaped neurons in layers V and VI, in Cajal-Retzius cells in layer I, and in non-pyramidal neurons all over the human entorhinal cortex. The calretinin-immunoreactive nonpyramidal cells form symmetric synapses with the dendrites and somata of pyramidal cells, but they also form synapses with non-principal cells (del Rio and DeFelipe 1996, del Rio and DeFelipe 1997, Freund and Buzsaki 1996). In the present study, the calretinin-immunoreactive nonprincipal neurons of layers II and III in the entorhinal cortex were preserved in AD cases and the dendritic staining of these neurons was reduced only in Alzheimer cases with severe entorhinal pathology (see Fig. 8 in study **III**). In the previous study, calretinin immunoreactive neurons in the entorhinal cortex of AD patients were also preserved but they did have dendritic changes (Brion and Resibois 1994). Taken together, the findings indicate that calretinin containing neurons react to pathologic changes at least in the later stages of AD, although they are not shown to contain neurofibrillary tangles (Brion and Resibois 1994). The border between layers I and II in the AD cases had abundant aggregates of small calretinin-immunoreactive neurons, giving the impression that these neurons were actually multiplying in the AD subjects relative to controls. Similarly, an increased density of calretinin-immunoreactive neurons was previously found in the frontal cortex of AD patients (Sampson et al. 1997). While these phenomena can be related to the general atrophy of the cortex, the observation that calretinin-immunoreactive non-pyramidal cells had a healthy appearance when the parvalbumin-immunoreactive neurons were almost lost, indicates a differential susceptibility between these two neuronal populations in the entorhinal cortex of AD patients. Curiously, the basal dendrites of the layer V pyramidal cells in AD cases were prominently stained in calretinin preparations whereas the apical dendrites of these neurons were only weakly stained. In control subjects, this pattern of dendritic staining was reversed (see Fig. 9 in study **III**). This might reflect possible plastic changes, either pre- or postsynaptic, occurring in the dendritic regions of the layer V pyramidal cells in patients with Alzheimer's disease.

Calbindin immunohistochemical preparations revealed increased staining intensity in some neurons in AD subjects compared to control subjects. Normally the calbindin-immunoreactive principal neurons of layers II and III of the entorhinal cortex were faintly stained, but in AD cases their cytoplasm was darkly stained (see Fig. 13 in study **III**). On the other hand, calbindin-immunoreactive non-principal cells exhibited several morphologic changes indicating ongoing degenerative processes occurring in these neurons (see Fig. 12, **III**). These changes occured even in early stages of entorhinal pathology, as was also observed in the parvalbumin neurons. In conclusion, particular non-principal cells containing parvalbumin or calbindin undergo pathologic changes in the entorhinal cortex of patients with AD.

The calcium-binding protein containing-neurons rarely contain neurofibrillary tangles (Brion and Resibois 1994, Sampson et al. 1997, Solodkin et al. 1996). In previous reports, these neurons either degenerated or survived different insults in a region-specific manner (Arai et al. 1987, Brady and Mufson 1997, Ferrer et al. 1991, Ferrer et al. 1993, Fonseca and Soriano 1995, Hof et al. 1991, Hof and Morrison 1991, Hof et al. 1993, Morrison et al. 1998). The present study revealed that in the entorhinal cortex these neurons were differentially vulnerable in AD depending on which calcium-

binding protein they contained. However, it is not known what determines the vulnerability of a neuron. The type of calcium-binding protein, but also other conditions, like receptor types (Kondo et al. 1997), might determine the fate of a cell in neurodegenerative diseases. Relating to this, AD pathology of layer II was also most prominent in the caudolateral subfields that are shown to contain cholinergic innervation (De Lacalle et al. 1994), which might be associated with the cholinergic deficits of Alzheimer's disease (Davis et al. 1999).

6.5.1 Reorganization of neuronal circuitries in the entorhinal cortex and in its hippocampal target areas in Alzheimer's disease and in temporal lobe epilepsy

Changes in PSA-NCAM in the dentate gyrus. In TLE, the expression of PSA-NCAM was increased throughout the molecular layer, particularly in its inner third (see Fig. 3 in study **V**). This increase resulted in the disappearance of lamination in immunostaining. The density of PSA-NCAM immunoreactivity correlated with the density of mossy fiber sprouting in the inner molecular layer. This indicates that a proportion of immunoreactivity is located in the growing axons of granule cells. In addition to axonal expression PSA-NCAM might occur, in the granule cell dendrites that extend to the molecular layer and undergo plastic changes in temporal lobe epilepsy (von Campe et al. 1997) and were previously found to express PSA-NCAM as a response to injury in rat (Styren et al. 1994). In contrast, in AD, the lamination was strengthened and the outer portion of the molecular layer was clearly demarcated from the inner portion (see Fig. 2 in study IV). On the other hand, in AD the immunostaining in the inner third of the molecular layer of dentate gyrus was also increased. In AD, the dendrites of granule cells are not degenerated inside the amyloid plaques (Einstein et al. 1994) and also sprouting in molecular layer is thought to occur (Gertz et al. 1987).

Newly generated granule cells in the infragranular region of adult rats transiently express highly polysialylated NCAM (Seki and Arai 1993b). Recently, it was reported that even a few seizures can induce the differentiation of immature infragranular cells into granule cells in the rat (Bengzon et al. 1997, Parent et al. 1997). In our study, there was a two-fold increase in the density of infragranular PSA-NCAM immunopositive cells in eight patients with temporal lobe epilepsy who had only a mild overall neuronal loss in the hippocampus and whose granule cell layer appeared intact. Only a few, if any, infragranular immunopositive neurons were present in patients with severe hippocampal neuronal loss. In these cases, the loss of granule cells was apparent in thionin preparations. No decrease was observed in the number of PSA-NCAM-positive infragranule cells in severe Alzheimer's disease and also the granule cells remain preserved (Braak and Braak 1998). The human granule cell layer is altering its organization also in adults (Eriksson et al. 1998). This might have an impact on endurance although its entorhinal inputs are lost. The disappearance of PSA-NCAM-positive infragranular cells in temporal lobe epilepsy patients with severe hippocampal damage suggests that the capacity for granule cell differentiation is reduced in the sclerotic hippocampus. In the hilus, even in the most sclerotic hippocampus of temporal epilepsy, some of the remaining neurons were darkly PSA-NCAM-immunolabeled and had tortuous thick immunopositive processes emanating from the soma. In AD, however, these hilar neurons had an appearance similar to those of controls. This might indicate differences in reorganization of hilar neurons depending on the disease.

PSA-NCAM changes in CA1. In cases of TLE where neuronal loss primarily involved only the proximal portion of the CA1 region, the border region between the damaged and undamaged cellular layer of CA1 was intensely PSA-NCAM-immunopositive. At the border area, *stratum pyramidale*

and *stratum oriens* contained intensely labeled varicose fibers and punctate neuropil labeling. Also, the somata of immunopositive neurons were darkly stained. In the area where the loss of CA1 pyramidal cells was almost complete, only a few large darkly stained multipolar neurons with tortuous dendrites were found mixed with a lower density of varicose fibers. In AD, the cell loss in CA1 region was enormous. In AD, however, there were darkly-stained PSA-NCAM positive fibers. These data suggest that the remaining neurons in CA1 express plasticity in human TLE and AD, and thus, the plasticity in humans might occur outside the dentate gyrus.

PSA-NCAM in the entorhinal cortex. Layer II of the entorhinal cortex projects to the outer two-thirds of the molecular layer of the dentate gyrus. In three of five cases of temporal lobe epilepsy, increased PSA-NCAM immunostaining in cells and the surrounding neuropil in layer II of the rostromedial portion of the entorhinal cortex (subfield ER) was observed. In all three cases, there was also a clear neuronal loss and increased gliosis in layer III. From two of these cases, hippocampal PSA-NCAM preparations were available for analysis. Both cases had an increase in the density of PSA-NCAM immunoreactivity in the outer two-thirds of the molecular layer compared to the controls. These data indicate that the expression of PSA-NCAM might be increased in the entorhinal cortex in a subpopulation of patients with temporal lobe epilepsy. Even though the number of cases is low, these findings suggest that as in the hippocampus the expression of PSA-NCAM in the entorhinal cortex is associated with neuronal damage and might occur simultaneously with increased PSA-NCAM expression in its monosynaptic projection field. In Alzheimer's disease, the layer II was filled with neurofibrillary tangles and cellular clusters had largely disappeared in the intermediate, lateral, and caudal subfields. In contrast to control tissue in which the PSA immunostaining was localized in the axon-initial segments, in AD tissue the PSA-NCAM staining was mainly localized in non-oriented fiber staining inside the former clusters of layer II. This suggests that the changes are also occurring in the axon initial segments of neurons in Alzheimer's disease. The staining intensity in the outer two-thirds of the dentate gyrus was slightly increased in those control cases in which layer II of the entorhinal cortex contained occasional neurofibrillary tangles. This suggests that PSA-NCAM expression increases with increasing degeneration of perforant pathway.

PSA-NCAM expression is upregulated in regenerating axons during axonal elongation and downregulated upon target innervation (Aubert et al. 1998). On the other hand, PSA-NCAM remains upregulated in areas which do not find appropriate contacts (Aubert et al. 1998). Thus, PSA-NCAM is a marker for ongoing remodeling. Changes in PSA-NCAM in the dentate gyrus, the CA1 region, and the entorhinal cortex support the idea that synaptic remodeling is an ongoing process in TLE and AD. In the present study, the density of immunoreactivity in the molecular layer was the highest in patients with the longest duration of TLE or AD patients with severe Alzheimer pathology. TLE patients also had the most severe hippocampal pathology according to assessments of thionin, dynorphin, and GFAP stained sections. The increase in PSA-NCAM immunoreactivity in the hippocampus and the entorhinal cortex parallels the neuronal cell loss observed in temporal lobe epilepsy and Alzheimer's disease. There is increasing evidence that aberrant sprouting contributes to the neuronal alterations observed in these diseases (Houser et al. 1990, Phinney et al. 1999). The molecular mechanisms underlying sprouting and reorganization remain to be explored, but they might involve a selective reactivation of certain developmental mechanisms. The embryonic form of NCAM is massively re-expressed in hippocampal areas where neurofibrillary tangles, amyloid plaques, and neuronal loss occur or where neurons suffer from a lack of inputs, or are targets of

mossy fiber sprouting. Whether the re-expression of this molecule contributes to the etiology of neuropathologic changes or is beneficial is not known. Negatively charged PSA epitopes alter the adhesive properties of the membranes and might therefore guide the reinnervation of surviving neurons in TLE and AD.

7. CONCLUSIONS

The goals of the thesis were: (1) to characterize the anatomic organization of the human entorhinal cortex with aid of calcium-binding protein-containing neurons (**I**) and using intracellular filling of spiny neurons with lucifer yellow (**II**), (2) to describe changes in the organization of the human entorhinal cortex of AD patients, especially in neurons containing parvalbumin, calretinin, and calbindin (**III**), (3) to study the reorganization process in the entorhinal cortex and its hippocampal target areas in normal brain, in AD and TLE using the polysialylated neural cell adhesion molecule (**IV, V**).

The results can be summarized:

(1A) The distribution of parvalbumin, calretinin, and calbindin largely followed the cytoarchitectonic borders of the eight subfields of the human entorhinal cortex. The regional and laminar distribution of these calcium-binding proteins was segregated rather than overlapping. Thus, the highest density of parvalbumin-positive neurons and fibers was observed in the caudolateral subfields, whereas calretinin and calbindin immunoreactivities were high in rostromedial subfields, although both immunoreactivities were also found in the caudolateral subfields. Parvalbumin neurons were morphologically non-pyramidal cells, forming either basket-like structures around cell somata or cartridge-like structures around the axon-initial segments. Although, calretinin and calbindin were also localized in non-pyramidal cells, they additionally labeled pyramidal-like neurons in layers V and VI (calretinin) and in layers II and III (calbindin) in the entorhinal cortex. The high density of non-pyramidal neurons containing these calcium-binding proteins in layers II and III suggests that they form a critical network that controls entorhinal outputs to the hippocampus. In addition, the largely non-overlapping distribution of the parvalbumin, calretinin, and calbindin neuronal populations in the entorhinal cortex indicates that each of them may modulate a different subset of topographically organized entorhinal outputs.

(1B) Based on the shape of the somata and primary dendritic trees, spiny neurons were divided into four categories: classical pyramidal, stellate, modified stellate and horizontal tripolar cells. The morphologic differences were more varied between the layers than between subfields. In layer II, majority of neurons were stellate or modified stellate. In the rostromedial subfields, however, there were also tripolar cells. In layers III, V, and VI the majority of neurons were classical pyramidal cells. Vertical extension of the dendritic branches to adjacent layers supports the idea that inputs terminating in specific layers influence target cells located in various entorhinal laminae. There is more overlap in the dendritic fields in the layers II and III than between the superficial and deep layers, which supports the idea of segregation of information flow targeted to superficial or deep layers of the human entorhinal cortex.

(2) In AD, the most profound neuronal loss and neurofibrillary tangle formation was found in the intermediate, lateral and, caudal subfields of the entorhinal cortex. Detailed analysis revealed that the parvalbumin- and calbindin-containing non-pyramidal neurons were morphologically altered earlier than calretinin-containing non-pyramidal cells in the entorhinal pathology of AD. Our study suggests that specific subfields and layers of the entorhinal cortex that contain distinct calcium-binding proteins are differentially vulnerable. This might impact the topographically organized inputs and outputs of the entorhinal cortex.

(3) In the entorhinal cortex, the changes in PSA-NCAM immunoreactivity were observed in layers II and III of AD and TLE patients when compared to controls. The PSA-NCAM immunoreactivity in the outer two-thirds of molecular layer of the dentate gyrus was increased in AD, whereas the inner third of the molecular layer had major changes in TLE. The increase in PSA-NCAM immunoreactivity in the hippocampus paralleled the neuronal cell loss observed in TLE and AD. In TLE cases with mild overall neuronal loss in the hippocampus and in AD the number of PSA-NCAM-positive infragranule cells was increased, whereas in severe hippocampal damage in TLE no PSA-NCAM-positive infragranule cells were observed. The granule cell layer is preserved in AD but disappears in severe TLE cases. Whether the loss of granule cells is related with the reduced capacity of granule cell differentiation remains to be explored. Together, however, the findings indicate that reorganization processes are somewhat different in these diseases. These studies provide evidence that the embryonic form of NCAM is upregulated in hippocampal areas where neurons are thought suffer from a lack of inputs, or are targets of mossy fiber sprouting, or where neurofibrillary tangles, amyloid plaques, and neuronal loss occur.

In conclusion, the present studies provide new information about the entorhinal cortex which is involved in memory processing, highly vulnerable in AD and is damaged in some patients with TLE. These studies extend the knowledge of the normal organization of human entorhinal cortex and confirm that the human entorhinal cortex is a neurochemically heterogeneous structure in which information flow is presumably differentially modulated by local interneurons. Furthermore, the subfields and laminae of the human entorhinal cortex have differences in their vulnerability to AD and TLE. Moreover, these studies provide evidence that reorganization of neuronal circuitries occurs in the entorhinal cortex and its hippocampal target areas in TLE and AD.

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