Quantitative analysis of synaptic connections between hippocampal principal cells and interneurons

PhD thesis

Virág Tresóné Takács

János Szentágothai School of Ph.D. Studies
Semmelweis University

Supervisor:
Attila Gulyás, PhD, DSc

Official reviewers:
Zita Puskár, PhD
Lucia Wittner, PhD

Head of the Final Examination Committee:
András Csillag, MD, PhD, DSc

Members of the Final Examination Committee:
Katalin Halasy, PhD, DSc
József Kiss, MD, PhD, DSc

Institute of Experimental Medicine, Hungarian Academy of Sciences
Budapest, 2012

INTRODUCTION

The hippocampal formation is an archicortical part of the cerebral cortex that plays a pivotal role in the formation of episodic memory traces and in spatial navigation. During my doctoral studies I investigated the connectivity between hippocampal principal cells and interneurons using quantitative neuroanatomical techniques. In particular, I analyzed the postsynaptic target selectivity of the main excitatory inputs to CA1 area and investigated the GABAergic hippocampo-septal (HS) cells that project to the medial septum-diagonal band of Broca complex (MS-DB) in order to describe the main quantitative properties of their synaptic connections.

Major glutamatergic pathways to CA1 area

The vast majority of hippocampal neurons are glutamatergic principal cells (~90%) and a smaller proportion of them are GABAergic interneurons (~10%). The parallelly arranged dendritic trees of hippocampal principal cells and the layer-specific termination of incoming pathways result in a laminar organization of the hippocampus. The majority of glutamatergic inputs to CA1 area derive from CA3 pyramidal cells that project to CA1 stratum (str.) radiatum and str. oriens via their ipsilateral Schaffer collaterals and contralateral commissural fibers. Layer III pyramidal cells of the entorhinal cortex innervate CA1 str. lacunosum-moleculare through the temporo-ammonic pathway. The later pathway can be subdivided into two group of fibers that follow different paths to reach CA1 str. lacunosum-moleculare: most of the axons perforate the subiculum before they enter str. lacunosum-moleculare. The other, smaller group of fibers, forming the alvear path travels through the alveus and then turns, radially traverses str. oriens, pyramidale, and radiatum and join the former group of fibers in str. lacunosum-moleculare. In addition to the glutamatergic inputs arriving

from extrinsic sources, the CA1 area has sparse intrinsic inputs from CA1 pyramidal cell local collaterals which travel at the border of str. oriens and alveus. All of these glutamatergic pathways were shown to innervate both pyramidal cells and interneurons, but quantitative data on the relative proportions of their glutamatergic and GABAergic cell targets were not previously available.

Characteristic input properties of different interneuron types

The hippocampal neuronal network contains a morphologically and physiologically heterogeneous population of interneurons which can be subdivided into at least 21 different types in the CA1 area. Our research group demonstrated that dendrites of different interneurons receive excitatory and inhibitory inputs in characteristically different densities and ratios. These differences can affect the integrative properties of distinct interneuron types, thus the quantitative analysis of synaptic inputs is essential for developing realistic computational models of the CA1 network.

The medial septum-diagonal band of Broca complex (MS-DB)

The MS-DB is a brain area located in the anteromedial part of the telencephalon which sends GABAergic, cholinergic and glutamatergic fibers to the hippocampus. The hippocampal theta rhythm is a field potential oscillation that is closely related to memory processes. According to a widely accepted hypothesis, pacemaker cells of the MS-DB might be involved in the generation of hippocampal theta rhythm. GABAergic fibers of the pacemaker parvalbumin positive cells selectively innervate interneurons in the hippocampus, whereas, hippocampal axons of the cholinergic cells have mostly non-synaptic boutons.

Hippocampo-septal (HS) cells

Besides the sensu stricto interneurons innervating only local neurons, there are long-range projecting GABAergic cells as well in the hippocampus, these cells target extrahippocampal areas. HS cells are long-range projecting GABAergic cells that send collaterals to MS-DB and subiculum. HS cells form a heterogeneous population according to their morphological and neurochemical properties. The heterogeneity might also be manifested in the target selectivity of their local axons. Interneuron-selective HS cells were reported in acute slices of young rats, whereas HS cells juxtacellularly labeled in adult rats predominantly innervated pyramidal neurons. HS cells project back to the MSDB where they innervate parvalbumin-positive and cholinergic MSDB cells, including neurons that project to the hippocampus. Due to this neuronal network, HS cells may have an important role in the synchronization of the two brain areas and in the regulation of theta rhythm. Thus the exploration of synaptic connections of HS cells can help us in understanding the generation and regulation of theta rhythm.

AIMS

In our experiments, using quantitative neuroanatomical techniques we aimed to answer to the following questions:

1. It is well known that the major glutamatergic pathways to the CA1 area (i.e. the entorhinal input, the Schaffer collaterals and the local axons of CA1 pyramidal cells) target both pyramidal cells and interneurons. Do these pathways innervate different cell classes in their proportion of occurrence or rather show a preference for a certain cell type? What is the proportion of excitatory and inhibitory cells among the postsynaptic targets of different glutamatergic pathways?
2. Distinct interneuron types show characteristic input properties. The number and distribution of excitatory and inhibitory inputs on somata and dendrites have been described for several hippocampal interneuron types, but no quantitative data were available on the input properties of hippocampo-septal (HS) cells. These cells establish part of the reciprocal loop connecting the hippocampus and medial septum-diagonal band of Broca complex (MS-DB), thus they can play a critical role in the synchronization of the two brain areas and in the regulation of hippocampal activity patterns. How many glutamatergic and GABAergic inputs innervate these cells?
3. The MS-DB sends cholinergic, glutamatergic and GABAergic projections to the hippocampus. The GABAergic septal fibers selectively innervate all of the hippocampal interneurons examined so far. Do the septal fibers form synapses also with HS cells? If so, what is the transmitter type of these axons?

4. Which neurons are the local postsynaptic targets of HS cells? What compartments of the target cells are innervated by the HS cells?

METHODS

All experiments were carried out according to the guidelines of the Institutional Ethical Code, and the Hungarian Act of Animal Care and Experimentation (1998, XXVIII, Section 243/1998).

Contributions

Examination of glutamatergic inputs to CA1 area were carried out in collaboration with the group of Prof. Peter Somogyi (MRC Anatomical Neuropharmacology Unit, Dept. of Pharmacology, Univ. of Oxford). The labeling of pathways was performed by the group of Prof. Peter Somogyi. The electron microscopic analysis of *in vitro* labeled cells and alvear pathway was carried out by Dr. Txema Sanz and formed part of his DPhil Thesis (The Univ. of Oxford, 1997). The electron microscopic analysis of *in vivo* labeled cells and entorhinal axons in str. lacunosum-moleculare was my work. For the examination of synaptic connections of HS cells, Dr. Attila Gulyás performed some of the MS-DB injections. All other surgeries, stainings, light- and electron microscopic analysis in this project was my work.

Anterograde-, and retrograde tracing

For visualization of HS cells we injected biotinylated dextran amine (BDA; 3 kDa; Molecular Probes) into MS-DB (n=8 male Wistar rats). To label both septally projecting HS cells and septo-hippocampal axons with different markers, a combination of an anterograde tracer [*Phaseolus vulgaris* leucoagglutinin (PHAL, Vector Labs)] and retrograde tracer fluorescent microbeads (FluoSpheresR, 0,04 µm, Invitrogen)] was delivered into MS-DB at different injection sites (n=1 male Wistar rat). For labeling of perforant pathway PHAL was injected into the medial entorhinal cortex of female Wistar rats (n=3).

In vitro, intracellular-, and *in vivo*, juxtacellular labeling

Hippocampal slice preparation (n=5 Wistar rats) and neuronal labeling was carried out by Dr. Txema Santz and the late Dr. Eberhard Buhl. CA1/3 pyramidal cells were intracellularly recorded and labeled with biocytin using sharp microelectrodes in an interface chamber. *In vivo* activity of CA1 pyramidal cells was recorded by Dr. Thomas Klausberger and collegues in male Sprague-Dawley rats (n=4) anaesthetized with urethane. Cells were extracellularly recorded with glass electrodes filled with neurobiotin and labeled by applying positive current steps.

Visualization of labeled cells

Operated animals were perfused under deep anaesthesia with a fixative containing paraformaldehyde and glutaraldehyde. Slices were immersion-fixed in the fixative. Labeled cells were visualized using the avidin-biotinylated horeseradish peroxidase system with 3,3'-diaminobenzidine (DAB) - 4HCL or nickel intensified DAB. For fluorescent visualization of PHAL we used an antiserum raised in rabbit (Vector). For DAB-staining of PHAL a biotinylated goat antibody (Vector) was used.

Analysis of input properties of HS cells

For measuring the total dendritic length of BDA-labeled HS cells (n=4), their complete dendritic arbors were drawn with a drawing tube and reconstructed in 3-dimensions using the ARBOR software. Total soma-surfaces were determined from serial thick sections made from entire cell bodies. Dendrites and somata of BDA-labeled HS cells were re-embedded and serially sectioned using an ultramicrotome. On the ultrathin sections postembedding immunostaining was performed against GABA using a rabbit antibody kindly donated by Prof. Peter Somogyi. Labeled dendritic segments with known length (n=36) and parts of labeled somata with known surfaces (n=22) were traced in serial electron microscopic sections.

The contacting GABA positive/negative or BDA-labeled terminals were counted to calculate the density of synaptic inputs. The density values were multiplied by the total lengths of dendrites / total soma surface to estimate the total number of synaptic inputs per cell.

Analysis of postsynaptic target cell-selectivity of different glutamatergic pathways and HS cells

Labeled axons of HS cells, hippocampal pyramidal cells and the alvear pathway were analyzed with correlated light and electron microscopy. Axons were followed in serial electron microscopic sections and all synapses found were included in the sample. Synapses of labeled entorhinal axons in str. lacunosum-moleculare were sampled using a systematic random sampling technique, the disector method. Postsynaptic targets of labeled boutons were identified as pyramidal cells or interneurons according to the characteristic of their synaptic inputs.

RESULTS

SYNAPTIC CONNECTIONS OF GLUTAMATERGIC PATHWAYS TO THE CA1 AREA

The main postsynaptic targets of local CA1 pyramidal cell collaterals are interneurons

The majority of *in vitro* labeled CA1 pyramidal cell boutons (n=41 synapses, n=2 animals and cells) innervated interneurons (pooled total, 65.9% of synapses). Within this category, most of the postsynaptic profiles were interneuronal dendritic shafts (63.4%) and some of them were spines of interneurons (2.4%). A smaller percentage (29.3%) of synapses was formed on pyramidal cell dendritic spines. The analysis has been repeated on axon collaterals of *in vivo* labeled cells (n=130 synapses, n=4 animals, n=1,1,2,4 cell/animal): like in slices, most of the terminals established synapses with interneurons (pooled, 53.8%; 46.2% of the targets were interneuronal dendritic shafts and 7.7% were interneuronal spines). A smaller proportion, 39.2% of the postsynaptic targets was pyramidal cell dendritic spines.

Schaffer collaterals predominantly innervate pyramidal cells

Postsynaptic targets of in vitro labeled Schaffer collaterals (n=70 synapses, n=46 from str. oriens and n=24 from str. radiatum, n=4 animals and cells) were mostly pyramidal cell dendritic spines (92.9%). Only 7.1% of the synapses were formed on dendritic shafts of interneurons.

Entorhinal axons show different target selectivity in str. lacunosum-moleculare and outside str. lacunosum-moleculare

The vast majority (90.8%) of entorhinal boutons in str. lacunosum-moleculare (n=130, n=3 animal) targeted pyramidal cells, whereas 8.5% of them innervated interneurons. In the case of innervated pyramidal cells,

most of the targets were spines (88.5%); whereas some of them were pyramidal cell dendritic shafts (2.3%). Labeled boutons of alvear pathway (n=127 synapses, n=3 animals) were examined in the alveus/str. oriens, str. pyramidale and str. radiatum (n=52, 30 and 41 synapses, respectively). Most of the innervated targets of alvear pathway were pyramidal cell dendritic spines (78.7%) too, but the alvear axons innervated a significantly higher proportion of interneurons (21.3%) than did the entorhinal axons in str. lacunosum-moleculare.

Statistical comparison of glutamatergic inputs to CA1 area

Statistical analysis revealed that local axons of CA1 pyramidal cells innervated a significantly higher proportion of interneurons (56.7% of all targets), than the other three examined pathways which innervated a much higher proportion of pyramidal cell targets (78.7% - 92.9% of all targets).

SYNAPTIC CONNECTIVITY OF HS CELLS

Types of HS cells

Based on the strongly labeled HS cells we could distinguish two different morphological types: the sparsely-spiny HS cells and the densely-spiny HS cells, which had characteristic location within the hippocampus. Sparsely-spiny cells were found in CA1-3 str. oriens and CA3 str. radiatum, whereas densely-spiny cells were exclusively located in CA3 str. lucidum and hilus. In the hilus, both types and cells with intermediate morphologies could also be found. Sparsely-spiny cells carried sparsely-distributed short spines only on their distal dendrites, whereas all of the dendrites as well as the somata of densely-spiny cells were profusely covered with long spines, which received many synaptic inputs. Both the HS cells in CA1-3 str. oriens and the HS cells in CA3 str. lucidum had horizontal dendritic trees: all of their dendrites were restricted to the same

layer (str. oriens and str. lucidum, respectively), while HS cells in CA3 str. radiatum showed multipolar morphology with dendritic trees spanning several hippocampal layers.

The HS cells are reciprocally connected with GABAergic septo-hippocampal cells

Using BDA injection into MS-DB (a tracer know to be transported both retrogradely and anterogradely) besides HS cells we could also label the septo-hippocampal projecting axons of MS-DB cells. These fibers massively innervated the BDA-labeled HS cells. To identify putative connections of BDA-labeled septal fibers and HS cells (n=74) we combined correlated light and electron microscopic examination with postembedding GABA-immunogold staining. All examined boutons formed synapses with the labeled HS cells (n=36 with dendrites, n=32 with somata and n=6 with axon initial segments), and all of them proved to be GABAergic, with the exception of five boutons. After double tracer injections into MS-DB, we found, that hippocampal and hilar HS cells retrogradely labeled with fluorescent microbeads were surrounded by septo-hippocampal terminals, containing the anterograde tracer, PHAL. The findings of these experiments provide evidence that GABAergic cells of hippocampus and MS-DB are in reciprocal connection at cell-population level.

Sparsely-spiny and densely-spiny HS cells receive excitatory and inhibitory inputs in different numbers and ratios

Our electron microscopic observations revealed that both types of HS cells were densely covered by synaptic terminals, thus the number of inputs per 100 μm dendrite was extremely high (n=248-437 in case of sparsely-spiny cells; n=523-1688 in case of densely-spiny cells). Somata of HS cells also received inputs in a very high density (n=34-90 and n=80-162

inputs per 100 μm^2 in case of sparsely-spiny cells and densely-spiny cells, respectively). In case of sparsely-spiny HS cells ~13.7% of dendritic inputs and ~21.5% of somatic inputs were GABAergic. In contrast, only ~2.3% of dendritic inputs and ~1.2% of somatic inputs of densely-spiny HS cells originated from GABAergic inhibitory neurons. A considerable proportion of inputs on HS cells might have had septal origin: up to 54.5% of the GABAergic inputs on soma and up to 27.6% of the GABAergic inputs on dendrites were BDA-labeled in case of sparsely-spiny cells. Densely-spiny HS cells were innervated less frequently by septal inputs. Axon initial segments of sparsely-spiny cells were innervated by GABA-negative and positive boutons, in a similar ratio to their somatic innervation with septal inputs contributing to the GABAergic innervation.

According to our calculations, the total number of inputs innervating a typical sparsely-spiny HS cell in CA1 str. oriens is ~22 000, of which 14% is GABAergic. Due to their heavily innervated long spines, densely-spiny HS cells in CA3 str. lucidum receive an even larger number of inputs (~37 000), of which only 2.3% is inhibitory. The ultrastructural properties of the excitatory inputs of densely-spiny HS cells suggested that they derived from small-diameter *en passant* boutons or filopodial extensions of granule cell-mossy fibers.

Local postsynaptic targets of HS cells

We have partially reconstructed the local axonal tree of BDA-labeled CA1 HS cells (n=3) using a drawing tube and identified their postsynaptic targets in the electron microscope. The local axons of HS cells arborized in str. oriens and radiatum and spanned a very large area in rostro-caudal directions (bouton-bearing collaterals were found at ~900 μm from the soma in CA1 area). Postsynaptic targets (n=180) of local axons were predominantly dendrites of pyramidal cells (80.6%) or less frequently

pyramidal cell dendritic spines (11.7%). HS boutons targeted interneuron dendrites in case of 3.3% of the examined synapses. We also found two collaterals of a labeled HS cell that climbed along two ramifying dendrites of another labeled HS cell and formed nine synapses with them.

CONCLUSIONS

Our electron microscopic investigations revealed that extrinsic and local glutamatergic inputs to the CA1 area exhibited remarkable differences in the relative proportion of innervated pyramidal cells and interneurons. Schaffer collaterals in str. oriens and radiatum as well as entorhinal axons in str. lacunosum-moleculare predominantly innervated pyramidal cell dendritic spines. More than 90% of their synapses targeted pyramidal cells and less than 10% of their synapses innervated interneurons, thus they appear to select their postsynaptic targets in a ratio that is similar to the ratio for the two cell types in the CA1 area. In contrast, local axons of CA1 pyramidal cells at the alveus/str. oriens border preferentially targeted interneurons (>50%), and innervated pyramidal cells in a lesser extent. These data indicate that unlike in CA3 area, the network architecture of CA1 area does not include extensive recurrent connections between local pyramidal cells. We have also found that the entorhinal fibers showed layer-specific target selection: alvear entorhinal path fibers passing through str. oriens, pyramidale, and radiatum appeared to be biased towards targeting interneurons (20% of all targets) as compared to the fibers in str. lacunosum-moleculare. As a result, the powerful feed-forward inhibition activated by the entorhinal input can be strongly mediated also by interneurons with dendrites in layers other than str. lacunosum-moleculare.

The location of sparsely-spiny HS cells corresponds to those layers where local collaterals of principal cells in the given area arborize; therefore large proportion of their numerous excitatory inputs might have local principal cell origin. Densely-spiny HS cells are specifically located in str. lucidum and hilus, where they receive a very large number of glutamatergic inputs from small-caliber terminals of granule cells.

Therefore the two types relay the activity of large populations of local pyramidal cells (sparsely-spiny cells) and granule cells (densely-spiny cells) to the MS-DB neurons. Both types of HS cells are innervated by GABAergic septo-hippocampal axons, thus GABAergic cells of the MS-DB and the hippocampus establish reciprocal connections with each other. This reciprocal loop might play an important role in synchronization of network oscillations between the two brain areas. Densely spiny cells were innervated in an extremely low ratio of GABAergic inputs (2.3%) compared to sparsely-spiny cells (14%), which might explain the different vulnerability of the two cell types in pathological conditions of the brain (ischemia, epilepsy). Local axons of CA1 HS cells examined in this study predominantly targeted pyramidal cells. As earlier studies showed interneuron-selective HS cells in young animals, HS cells can be heterogeneous according to their postsynaptic targets or they might show a developmental change in target selection.

LIST OF PUBLICATIONS

Publications related to thesis

***Takács VT**, Freund TF, Gulyás AI.* 2008. Types and synaptic connections of hippocampal inhibitory neurons reciprocally connected with the medial septum. European Journal of Neuroscience 28(1):148-64.

***Takács VT**, Klausberger T, Somogyi P, Freund TF, Gulyás AI*. 2012. Extrinsic and local glutamatergic inputs of the rat hippocampal CA1 area differentially innervate pyramidal cells and interneurons. Hippocampus, in press. DOI 10.1002/hipo.20974.

Other publications

*Marchionni I, **Takács VT**, Nunzi MG, Mugnaini E, Miller RJ, Maccaferri G*. 2010. Distinctive properties of CXC chemokine receptor 4-expressing Cajal-Retzius cells versus GABAergic interneurons of the postnatal hippocampus. Journal of Physiology 588(Pt 15):2859-78.

*Dobó E, **Takács VT,** Gulyás AI, Nyiri G, Mihály A, Freund TF. 2011*. New silver-gold intensification method of diaminobenzidine for double-labeling immunoelectron microscopy. Journal of Histochemistry and Cytochemistry 59(3):258-69.