NMDA-receptor dependent nitric-oxide signaling in GABAergic synapses of the hippocampus

Ph.D. thesis

Dr. Cserépné Dr. Szabadits Eszter

Semmelweis University
Szentágothai János Neuroscience Doctoral School

Supervisor: Dr. Nyiri Gábor,Ph.D., senior research fellow

Official opponents: Dr. Halasy Katalin, D.Sc., professor
Dr. Altdorfer Károly, Ph.D., assistant professor

Chairman of final examination committee:
Dr. Csillag András, D.Sc., professor
Members of final examination committee:
Dr. Dobolyi Árpád, Ph.D., senior research fellow
Dr. Rácz Bence , Ph.D., assistant professor

Budapest, 2013
Institute of Experimental Medicine, Hungarian Academy of Sciences

1. Introduction

Nitric-oxide (NO) participates in various forms of synaptic plasticity, including long-term potentiation (LTP) and depression (LTD), this way it may be able to influence learning and memory processes. These processes are essentially determined by pyramidal-cell networks, regulated by different populations of interneurons. NO effect is mainly thought of as retrograde signal in glutamatergic synapses, where the NO is released from the postsynaptic neuron and regulates neurotransmitter release at the presynaptic site.

NO is produced by the postsynaptic neuronal NO synthase (nNOS) in pyramidal cells. Under physiological conditions the released NO reaches concentrations at nanomolar concentrations, and its range extends only to a few microns. Its receptor – the NO-sensitive guanylyl cyclase (NOsGC), located presynaptically – has a very high affinity, and even a few NO molecules can activate it. This activation leads to the synthesis of cGMP and to the ignition of several secondary signaling pathways, which in turn could modulate transmitter release. The quick tissue absorption of NO enables synapse-specific signaling.

nNOS is a Ca^{2+}-calmodulin dependent enzyme, and is anchored to the postsynaptic density of glutamatergic synapses via PDZ-domain proteins, in a close apposition to NMDA receptors. Local calcium influx via NMDA receptors leads to the synthesis of NO and consequent cGMP production.

NMDA receptors are voltage- and ligand gated cathion (primarily calcium) channels. They form functional heterotetramers, consisting of two obligatory GluN1 (formerly NR1) subunits, and two from GluN2 (formerly NR2) subunits (GluN2A-D). In the pyramidal cells of adult hippocampus, only GluN1, GluN2A and GluN2B subunits are expressed.

2. Aims

Inhibitory inputs on pyramidal cells are essential for network level synchronous activity. The activity of pyramidal cells can retrogradely influence this inhibitory input. One of these retrograde signalling pathways is the well known endocannabinoid system, that is present only in the terminals of the cholecystokinin (CCK) positive perisomatic interneurons. Another retrograde signaling system is the retrograde nitric oxide pathway, which plays important role in the plasticity of glutamatergic synapses. While this pathway is well known in glutamatergic synapses, its possible presence has not yet been investigated in GABAergic synapses. Our main question was whether this signaling machinery was present in GABAergic synapses on pyramidal cells, and if yes, does it show a cell-type-specific distribution like endocannabinoid system, or it is a general feature of these synapses?

Our questions were:

- Is nNOS present in GABAergic synapses?
- If yes, which perisomatic synapses (CCK/VIP) expresses nNOS?
- Is the NO-receptor present in GABAergic terminals?
- If yes, which perisomatic synapses (CCK/VIP) expresses the NO-receptor?
- If yes, what is the subunit-composition of these receptors?

The activation of the nitric-oxide pathway is a function of postsynaptic calcium level. In the second series of experiments we investigated whether NMDA-receptors could be responsible for this postsynaptic calcium-elevation in GABAergic synapses.

Our questions were:

- Can NMDA administration activate the NO-system in vitro?
- Are NMDAR subunits present in GABAergic synapses?
- If yes, which subunits?
- What proportion of perisomatic synapses express NMDARs?
- Which perisomatic synapses express NMDARs?

To answer these questions, we employed pre-embedding immunogold, combined immunoperoxidase-immunogold, post-embedding immunogold, SDS-digested freeze-fracture replica labeling, mRNA in situ hybridization techniques, and cGMP immunochemistry after drug application on acute slices in vitro.

3. Materials and methods

3.1. Transcardial perfusions

Animals were anesthetized with an intraperitoneal injection and perfused through the heart. For the pre-embedding nNOS immunogold reaction, the solution contained 1% paraformaldehyde, and 4% paraformaldehyde for the mirror-experiments, immunofluorescence, pre- and post-embedding NMDA-R immunogold reactions and in situ hybridization. Samples for replica experiments were fixed with a solution containing 2% paraformaldehyde and 15% picric acid. Subsequently, brains were removed from the skull, and this was followed by sectioning without further fixation.

3.2. Testing the specificity of the antibodies

Primary antibodies were tested by the laboratories of origin, and we also found the expected labeling pattern in cortex and hippocampus. We found no specific staining pattern with the nNOS antibodies in nNOS-KO mice. NOsGC antibodies were tested further in this work. Secondary antibodies were extensively tested for possible cross-reactivity with the other secondary or primary antibodies, and possible tissue labeling without primary antibodies was also tested to exclude autofluorescence or specific background labeling by the secondary antibodies. No specific staining was observed under these control conditions. The specificity of the cGMP and the GAD65 antibodies were tested extensively and were described previously. $GABA_A$ receptor ($GABA_AR$) β3 subunit antibodies also specifically labeled GABAergic synapses on pyramidal cell somata. nNOS and vesicular glutamate transporter 3 (vGluT3) antibodies were proved to be also specific in experiments with wild-type and nNOS-/- and vGluT3-/- null mutant mice. The specificity of the rabbit antibodies against the C terminus of the NMDAR subunits GluN1, GluN2A, and GluN2B had been well characterized by using immunoblot, antigen peptides, and null mutant mice or

conditioned knock-out mice in both preembedding and postembedding experiments. We used the same preembedding digestion protocol, as was used in the experiments for testing the specificity of the antibodies. We also performed additional control experiments to validate the specificity of the antibodies used in our experiments. We labeled freeze–fracture replicas of hippocampal slices of pyramidal cell-restricted GluN1 knock-out mice and wild-type mice to test specificity of the GluN1 antibody even further on replicas. We found no synaptic labeling and only negligible background labeling on pyramidal cell somata. In addition, GluN1, GluN2A, and GluN2B immunogold labeling displayed the same distribution in the tissue, using three different methods. Furthermore, in postembedding immunolabeling experiments, we performed mirror experiments as well: adjacent sections of the same synapses were incubated for GluN1, GluN2A, or GluN2B, respectively. If a synapse was labeled for any of the subunits, the very same synapse was also tested for other subunit labeling on adjacent sections. Although it is highly unlikely that the same channel could be tested in adjacent sections, using this method, the different subunits of theNMDARscould be colocalized in the same synapse. Indeed, we found that in the majority of the GluN2-positive synapses GluN1 was also detected in one of the adjacent sections (12 of 23 synapses in two mice), which demonstrates that these antibodies label the very same cell membrane domains, proving additional evidence for their specificity. We also tested the cross-reactivity of the fluorescent secondary and gold-conjugated secondary antibodies used in double-labeling experiments. No cross-reactivity was found in either case. Selective labeling, resembling that obtained with the specific antibodies, could not be detected if primary antibodies were omitted.

3.3. Drugs

3-Isobutyl-1-methylxanthine (IBMX), 1-(2-chlorophenyl)-6-[(2*R*)- 3,3,3-trifluoro-2-methylpropyl]-1,5-dihydro-4*H*-pyrazolo[3,4-*d*]pyrimidine- 4-one (BAY 73-6691), L-arginine, L-*N*G-nitroarginine methyl ester hydrochloride (L-NAME), and (5*R*,10*S*)-(_)-5-methyl-10,11-dihydro-5*H*dibenzo[*a*,*d*]cyclohepten-5,10-imine-hydrogen-maleate (MK-801) were produced by Sigma-Aldrich. NMDA, D-(_)-2-amino-5-phosphonopentanoic acid (D-AP5), 1*H*-[1,2,4]oxadiazolo[4,3-*a*]quinoxalin-1-one (ODQ),nifedipine, and SNX-482 were obtained from Tocris Bioscience.We obtained tetrodotoxin (TTX) from Alomone Labs and sodiumnitroprusside (SNP) from Fluka.

3.4. In situ hybridization experiments

Primarily nonoverlapping segments of the rat NOsGC α1 and NOsGC α2 coding sequences were amplified by reverse transcription. PCR products were cloned into the *Sma*I site of pBluescript II SK. The integrity and orientation of clones were verified by sequencing. NOsGC α1 probe was linearized by *Xba*I and *Pst*I digestion for the antisense and sense probe, respectively. NOsGC α2 was linearized by *Not*I and *Hin*dIII digestion for the antisense and sense probe, respectively. The linearized template was stored at -20°C after gel extraction. After *in vitro* transcription, riboprobes were labeled with digoxigenin, treated with DNase and purified using the

RNeasy MinElute Cleanup kit. Finally, the integrity and quantity of the riboprobes were determined using gel electrophoresis.

Incubation of the 50 μm-thick rat or mouse brain slices was performed in a free-floating manner in RNase-free sterile culture wells for all steps. First the sections were washed, hybridization was then performed overnight in hybridization buffer. After washing in wash solution 1 and wash solution 2, they were incubated in a solution containing anti-digoxigenin Fab fragment conjugated to alkaline phosphatase. Finally, the sections were washed and mounted in Vectashield onto glass slides.

3.5. Double immunofluorescent labeling experiments

Incubation of sections in 1% human serum albumin was followed by a 2 d incubation in mixtures of primary antibodies for either NOsGC α1 subunit (1:1000) together with CCK, PV, nNOS, or somatostatin (SOM) or for nNOS together with cannabinoid CB1 receptor or substance P receptor (SPR). The primary antibodies were diluted in TBS. After repeated washes in TBS, the sections were incubated in mixtures of fluorescent-labeled secondary antibodies for 5 h. We did not use any detergent during the immunostaining. The sections were treated with 0.025% $CuSO_4$ solution for 30 min to reduce autofluorescence and then were washed in TBS, transferred onto microscope slides, and covered with Vectashield. The sections were evaluated using a Zeiss Axioplan2 microscope.

3.6. Preembedding immunoperoxidase staining.

Incubation of sections in blocking solution was followed by a 48 h incubation in solutions of primary antibodies raised against NOsGC α1 subunit and NOsGC β1 subunit. After subsequent extensive washing in TBS, the sections were incubated with biotinylated anti-rabbit antibody solution, followed by avidin-biotinylated horseradish peroxidase complex. The immunoperoxidase reaction was developed using 3,3-diaminobenzidine (DAB) as a chromogen. The sections were treated with osmium tetroxide in PB on ice. After that, sections were dehydrated in ascending alcohol series and propylene oxide and embedded in Durcupan. During dehydration, the sections were treated with 1% uranyl acetate in 70% ethanol for 30 min.

3.7. Preembedding immunogold and combined immunogold–immunoperoxidase staining.

To localize nNOS, first we incubated sections in the solution of primary antibody for nNOS or in the combined staining experiments in mixtures of primary antibodies for either nNOS and cholecystokinin (CCK) or nNOS and parvalbumin (PV). After repeated washes sections were treated with blocking solution. This was followed by incubation with gold-conjugated antibodies. After this, the sections were washed, intensified, osmificated and dehydrated. For the immunogold-immunoperoxidase double labeling experiments, after intensification we incubated the sections in a solution containing biotinylated secondary antibodies, and treated them with avidin-biotinylated horseradish peroxidase complex. The reaction was developed using 3,3-diaminobenzidine (DAB).

For synaptic detection of NMDARs, pretreatment with pepsin was essential. Sections were incubated in HCl solution containing pepsin. Sections were then blocked, and incubated in either only one primary antibody or in mixtures of primary antibodies for NMDAR subunits and vGluT3 or NMDAR subunits and parvalbumin (PV). After repeated washes, sections were incubated in 1.4 nm gold-conjugated anti-rabbit antibody solution. After this, the sections were washed, intensified, osmificated and dehydrated. For the immunogold-immunoperoxidase double labeling experiments, after intensification we incubated the sections in a solution containing biotinylated secondary antibodies, and treated them with avidin-biotinylated horseradish peroxidase complex. The reaction was developed using 3,3-diaminobenzidine (DAB).

The sections were treated with 0.5% osmium tetroxide in 0.1 M PB on ice and were dehydrated in ascending alcohol series and in acetonitrile and embedded in Durcupan (ACM; Fluka). During dehydration, the sections were treated with 1% uranyl acetate in 70% ethanol for 20 min. For the electron-microscopic investigations, tissue samples were embedded into small Durcupan molds. After this, 60-nm-thick sections were prepared using ultramicrotome and picked up on single-slot copper grids. The sections were examined using a Hitachi H-7100 electron microscope. For the semiquantitative analyses of immunogold particles for NMDAR subunits, we counted gold particles within the anatomically defined GABAergic synapses and along the somatic membrane. A band of 50 nm was chosen along the membrane as an area representing membrane-associated receptor labeling in the digested hippocampal samples.

3.8. Lowicryl resin embedding and postembedding immunohistochemistry

The same embedding procedure was used as described previously. Briefly, after washing in PB, the sections from fixed hippocampi were transferred into sucrose solutions in 0.1 M PB for cryoprotection. After slamming onto gold-plated copper blocks cooled in liquid nitrogen, low temperature dehydration, and freeze-substitution, the sections were embedded in Lowicryl HM20 resin. Postembedding immunohistochemistry was performed on 50- or 70- nm-thick sections. Grids were incubated on drops of blocking solution for, followed by incubation on drops of primary antibodies overnight. After incubation in primary antibodies, sections were washed and incubated on drops of secondary antibodies coupled to gold particles. In double-sided reactions, immunoparticles for NMDAR subunits were counted within the anatomically defined GABAergic or glutamatergic synaptic junctions and along the somatic membrane. 45 nanometer- wide bands were chosen on the two sides of the synaptic membrane as an area representing membrane-associated gold particle labeling.

3.9. SDS-digested freeze–fracture replica immunolabeling

Mice were deeply anesthetized with sodium pentobarbital, followed by transcardial perfusion first with 25 mM PBS for 1 min, and then with 2% paraformaldehyde with 15% saturated picric acid in 0.1 M PB for 12 min. Coronal sections from the hippocampus were cut on a Dosaka microslicer at a thickness of 130 μm. The slices were cryoprotected in 30% glycerol in 0.1 M PB overnight at 4°C and

were frozen by a high-pressure freezing machine. Frozen samples were inserted into a double replica table and then fractured into two pieces at -140°C. Fractured faces were replicated by deposition of carbon (8 nm thickness), platinum (2 nm), and carbon (15 nm) in a freeze– fracture replica machine. Samples were treated with 15mM Tris buffer, pH 8.3, containing 2.5% SDS and 20% sucrose at 80°C for 20 h. The replicas were washed in 25mM TBS containing 0.05% bovine serum albumin (BSA) and then incubated in blocking solution (5% BSA in 25mM TBS) for 1 h. The replicas were then incubated first in one of the primary antibody solutions recognizing NMDAR subunits (rabbit; GluN1 6 μg/ml, GluN2A 10 μg/ml, GluN2B 8 μg/ml, in blocking solution), overnight at room temperature. After washing in TBS, replicas were incubated in the mixture of the GABAAR β3 subunit primary antibody (1:25; serum, guinea pig, raised and characterized in the laboratory of R. Shigemoto) and gold-conjugated anti-rabbit secondary antibodies for NMDARs (1:25; BBI; 5 nm goat anti-rabbit) overnight at room temperature. This was followed by the gold-conjugated secondary antibodies to label $GABA_A$Rs (1:25; BBI; 10 nm goat anti-guinea pig). After washing in TBS and distilled water, replicas were picked up onto piloloform-coated parallel copper grids and were examined using Philips Tecnai 10 or Hitachi H-7100 transmission electron microscopes.

3.10. Acute slice preparation and cGMP immunolabeling.

For acute slice preparation, mice were deeply anesthetized and decapitated, the brains were removed, and 300 μm-thick coronal hippocampal slices were cut. Slices were incubated for 1 h in artificial CSF (ACSF) equilibrated with carbogen gas, at room temperature in interface conditions before the experiments. Slices were then transferred to sterile 12-well cell culture plates and were individually bubbled with carbogen gas at equal rates. Each well was filled with 1 ml of modified ACSF (mACSF) containing phosphodiesterase inhibitors. After preincubation in mACSF for 20 min, no drugs were applied in the control wells, whereas 200 μM SNP was added for 10 min to "SNP wells" and 5 μM NMDA was applied for 3 min to the "NMDA wells." In experiments in which nNOS, NOsGC, voltage-dependent Ca^{2+}channels (VDCCs), or NMDARs were blocked, slices were preincubated for 20 min with mACSF containing the given blocker, and then 5 μM NMDA was applied for 3 min. After incubation, the solutions were quickly changed to ice-cold 4% paraformaldehyde fixative, and then the slices were postfixed in the same fixative for 48 h at 4°C. For the immunofluorescent staining, the slices were washed, embedded in 2% agar, and resectioned into 50 μm-thick sections. After washes, sections were incubated in primary antibody solutions against cGMP and GAD65. This was followed by washes in PBS and incubation in solutions of fluorochrome-labeled secondary antibodies. This was followed by washes, and sections were mounted onto glass slides and coverslipped. Immunofluorescence was analyzed using an Olympus Optical FluoView300 confocal laser-scanning or Zeiss Axioplan 2 epifluorescent microscope.

3.11. Statistical analysis

In the fluorescent experiments to localize the NO-receptor, coronal sections were tested per colocalization in a systematic random manner from the rostral, medial, and caudal parts of the rostrocaudal extent of the dorsal hippocampus. Colocalization of antigens was investigated only when the nucleus of a cell could be detected to ensure that a large part of the cell was tested. We measured the cross-sectional area of all examined cells. Cells were sampled with the help of a grid placed over the photos of each section in a systematic random manner. Once the cells were selected, they were scrutinized individually. We used NIH ImageJ image analyzer software for measuring the cells and StatSoft Statistica software for data analysis. We did not intend to define the absolute number of the examined cells in the hippocampus but performed a quantitative measurement of the ratios using Abercrombie's correction. In the immunogold experiments, after extensive testing and adjusting of reaction conditions, final measurements of gold particles were performed from serial sections from each animal, in which background labeling was minimal. Immunoparticles for nNOS were counted within the anatomically defined synaptic junctions and under other cell membrane segments adjacent to synapses, without synaptic junctions. Gold particles were considered to be associated with the cell membrane only when they were not farther away from the membrane than 40 nm. Because most data populations in this work did not have a Gaussian distribution according to the Shapiro–Wilk's *W* test, we used nonparametric statistics. Two groups were compared using the nonparametric Mann–Whitney *U* test; multiple groups of data were compared using the nonparametric Kruskal–Wallis test. The null hypothesis was rejected when the *p* level was under 0.05, and, in such cases the differences were considered significant throughout this paper.

4. Results

4.1. neuronal nitric-oxide synthase is associated to postsynaptic densities of GABAergic synapses on pyramidal cells

In previous studies in the hippocampal CA1 region, the NO synthesizing enzyme nNOS was found postsynaptically in pyramidal cells in their excitatory synapses. However, by using a preembedding immunogold technique on mildly fixed brains, here we show for the first time that nNOS can be found not only in dendritic spines in mice and rats, but it is also localized to symmetrical GABAergic synapses on pyramidal cells. We found no labeling in nNOS KO mice, whereas, for example, somatic synapses were strongly labeled in wild-type mice in the very same experiments. In addition, the labeling is not randomly associated with the pyramidal cell membrane, but it is specifically enriched in symmetrical synapses as well as in asymmetrical synapses on spines. Our measurements of the relative linear density of labeling show that, whereas labeling density was ~1,35 +/- 0,31 gold particles per micrometer membrane (mean +/- SD) in the synaptic active zone of somatic symmetrical synapses, it was only 0.03+/-0.02 gold particles per micrometer at extrasynaptic membrane domains in the vicinity of these synapses. Some labeling also occurs in the cytoplasm of pyramidal cells around cisternae of the endoplasmic reticulum, and strong labeling is detectable in the somata and dendrites of nNOSpositive interneurons. Two types of interneurons innervate the somata of

pyramidal cells: those immunoreactive for PV establish approximately two-thirds of terminals on pyramidal cell somata, whereas CCK-positive interneurons comprise approximately one-third of somatic terminals. In mice, we tested fully reconstructed symmetrical synapses, and we found that at least 76% of these fully reconstructed somatic synapses (median of the individual percentages of 16 of 19, 15 of 20, and 16 of 21 in three mice) and at least 32% of the AIS synapses (median percentage of 7 of 22, 10 of 26, and 6 of 20 in three mice) were identified as nNOS positive on the basis of immunogold particles associated with the postsynaptic density. Supposedly, these data underestimate the number of nNOS-labeled synapses, because high concentrations of scaffolding and other proteins in the postsynaptic density make detection of its constituting proteins difficult. Because perisomatic GABAergic inhibition is brought about by three types of interneurons (CCK- and Pvpositive basket cells and PV-positive axo-axonic cells), these data have strong predictive values. However, dendrite targeting interneurons are much more diverse; therefore, quantification of such synapses was not performed at the electron microscopic level. Nevertheless, many of the dendritic symmetrical synapses were also intensely positive in all three mice investigated. Similar to mice, somatic, dendritic, and AIS synapses are labeled in rats as well. In rats, we also performed a direct colocalization of nNOS in reconstructed synapses of both PV- and CCK-positive terminals.We found (in all three rats) that nNOS labeling was present in postsynaptic active zone of synapses established by CCK-positive and PV-positive terminals on somata, in synapses established by PV-positive terminals on AISs, as well as in synapses established by both CCK-positive and -negative terminals on pyramidal cell dendrites. Because different interneurons target different pyramidal cell surface domains, this means that at least five different types of interneurons establish synapses on pyramidal cells with postsynaptically located nNOS. The distribution of the NO receptors provides additional support to this conclusion.

4.2. Cell type-specific expression of the mRNA of NOsGC α_1 és α_2 subunits in the hippocampus

NOsGC is a heterodimeric enzyme composed of two different subunits: α (α_{1-2}) and β (β_{1-2}). There are only two kinds of functionally important subunit compositions of NOsGC in the hippocampus that detects NO signal: α1β1 and α2β1 complexes. Because binding of these subunit compositions to synaptic scaffolding protein and possible differences in their regulation may differentially support signaling in neurons, here we tested which neurons express these isoforms of NOsGC. Using digoxigenin-labeled riboprobes for *in situ* hybridization (developed with 5-bromo-4-chloro-3-indolyl-phosphate and nitro blue tetrazolium chloride dissolved in chromogen buffer solution) in rats, we found that α1 subunit mRNA was only present in interneurons, and these labeled neurons were found in all layers of the CA1 area. On the contrary, α2 subunit mRNA was only present in pyramidal cells of the CA1 area. We obtained identical results in mice.

4.3. The α1 subunit of NOsGC is present in interneurons and their terminals, but it is not detectable in pyramidal cells

Because the mRNA of NOsGC α1 subunit is selectively present in interneurons, we performed preembedding immunoperoxidase staining to test whether the axon terminals of interneurons express the α1 subunit protein. In addition, we also tested whether its complementary β1 subunit is also expressed in the very same cells. Mice and rats showed an identical staining pattern. We found that many of the hippocampal interneurons showed strong immunostaining for the α1 subunit, and these cells could be found in all layers of the hippocampus from the alveus to the stratum (str.) lacunosum-moleculare, similar to its mRNA distribution. In the light microscope, basket-like axon terminal labeling appeared in the str. pyramidale around cell bodies in both species. Using electron microscopy of immunoperoxidase-stained tissue and random samples from reconstructed terminals in mice, we found that at least 79% of the reconstructed somatic terminals (median percentage of 20 of 23, 18 of 24, and 23 of 29 in three mice) and at least 42% of the AIS terminals (median percentage of 10 of 21, 4 of 21, and 8 of 19 in three mice) were positive for the α1 subunit of NOsGC, and many of the terminals establishing dendritic symmetrical synapses were also strongly positive in all three mice. In rats, we also found that many of the reconstructed synaptic terminals on somata, dendrites, and AISs were also positive for the α1 subunit. Furthermore, we also tested β1 subunit labeling at the electron microscopic level in the rat and found that, as expected, many of the GABAergic terminals are also positive for the β1 subunit. The α1 subunits always heterodimerize with β1 subunits. To test whether other interneurons have abundant β1 subunit containing NOsGC without the α1 subunit, we investigated the colocalization between α1 and β1 subunits in rat hippocampal interneurons.

Because both antibodies were raised in rabbit, we used the so-called mirror technique, in which cells that were cut in half on the section surfaces can be examined in adjacent sections, one incubated with α1 and the other with β1 subunit antibody. As expected, β1 subunits were found in both pyramidal cells and interneurons, whereas the α1 subunit antibody labeled only interneurons. We found that α1 subunit-positive interneurons were practically always positive for the β1 subunit (in four rats, 27 of 27, 41 of 42, 34 of 37, and 18 of 19), and, also, β1 subunit-positive interneurons were positive for the α1 subunit (in four rats, 28 of 29, 41 of 43, 39 of 40, and 16 of 17; some false-negative cells may occur in the sections because of the soluble nature of the α1-containing NOsGC or because of the loss of some soma tissue during sectioning). Besides confirming the specificity of the α1 subunit antibody, these results also suggest that, because no β1 subunitpositive interneurons were detected without the α1 subunits, there are no interneurons that express α2β1 subunit composition, which is in line with our finding that α2 subunit mRNA expression was absent in interneurons.

4.4. Both perisomatic and dendrite targeting GABAergic neurons contain the α1 subunit of the NOsGC

As we have shown, many interneurons expressed the mRNA of NOsGC α1 subunit, and many of them contained NOsGC α1 subunit protein together with the β1 subunit, but not all interneurons were positive. Therefore, we performed a quantitative immunofluorescence study to test whether major interneuron classes, including CCK-,

PV-, nNOS-, or SOM positive perisomatic or dendrite targeting interneurons, express this NO receptor. Although direct colocalization data are not available for all of these markers in the rat hippocampal CA1 region, indirect observations suggest that these four markers label different, primarily nonoverlapping populations of interneurons. The general staining patterns with these markers were similar to those reported previously. NOsGC α1 subunit staining was identical to that found with immunoperoxidase (DAB) staining. No labeling could be detected in the cell nuclei. With immunofluorescent staining, we found a variable intensity of immunolabeling signal; however, we have not observed a correlation between the signal intensity and the type of interneuron markers. Thin axon-like processes could be identified in the surrounding neuropil with immunofluorescent staining as well. After investigating random samples of labeled interneurons in the CA1 area of the rat dorsal hippocampi, we found that the median proportions are the following (in parentheses are the number of examined cells in the given colocalization from the three rats and the percentages in the animals, respectively): from all NOsGC α1 subunit-positive cells, 21% (*n*=1383; 23, 15, and 21%) are CCK positive, 40% (*n* =1527; 38, 41, and 40%) are PV positive, 11% (*n*=1322; 11, 10, and 20%) are nNOS positive, and 7% (*n*=1599; 4, 7, and 7%) are SOM-positive. Measuring it from the side of the interneuron markers, we found that the proportion of NOsGC α1-positive cells are 68% (*n*=432; 68, 45, and 80%) of all CCK-positive cells, 74% (*n*=771; 74, 74, and 90%) of all PV-positive cells, 20% (*n*=756; 18, 20, and 34%) of all nNOS-positive cells, and 32% (*n*=264; 37, 32, and 31%) of all SOM-positive cells. Although direct and indirect data suggest that these four interneuron markers show only negligible colocalizations, little information was available about possible colocalization between nNOS- and CCK-positive cells. Because practically all CCKpositive cells are CB1 receptor-positive, we tested whether CB1 receptor labeling and nNOS labeling can be found in the same cells, but we observed no colocalization between these two markers in the random samples (*n*=206 CB1-positive cells in three rats).

4.5. NMDA induces cGMP production in GABAergic terminals in the CA1 subfield of acute hippocampal slices

GABAergic terminals in the hippocampus express functional NO receptors and are able to produce cGMP. We prepared acute slices from dorsal hippocampi and incubated them in mACSF containing PDE-inhibitors (to avoid the hydrolysis of cGMP) and L-arginine (which is the substrate of nNOS). After fixation, cGMP was visualized by immunofluorescent labeling using specific antibodies raised against cGMP bound to a carrier protein. In control slices (*n*=20, 10mice), detectable levels of neuronal cGMP immunoreactivity occurred only in a few interneuron (IN) somata and dendrites. Basket cell terminal labeling was absent in the CA1 region, whereas weak and sparse basket-like terminal staining was present in the pyramidal cell layer of CA3ab. Using double immunofluorescent labeling, we confirmed that they were indeed GABAergic terminals (96,5±2,7% of them were GAD65 positive; *n*=118, 4 slices, 2 mice). However, 38.9±2.7% of the GAD65-positive basket cell terminals were cGMP positive in the CA3ab (*n*=330, 4 slices, 2 mice). No basket-like terminal staining was detected in the dentate gyrus (DG). Although we focused on the CA1

region, the labeling pattern was identical in the CA3c in all experiments. Some glial cells were also stained in each subfield, probably as a result of the well known expression of both NO-sensitive and -insensitive guanylyl cyclases by astrocytes. Application of anNOdonor, SNP (200 μM for 10 min) caused a large increase in cGMP labeling in most neuronal elements. It was the strongest in basket terminals both in CA1 and CA3, but basket terminals remained unlabeled in the DG (5 slices, 2 mice). Then, we applied 5 μM NMDA to control slices for 3 min, which resulted in a remarkable region-specific enhancement of cGMP accumulation (27 slices, 10 mice). In addition to a strong homogenous neuropil staining in strata radiatum and oriens, a profound increase was detected in basket cell terminals of CA1. After application of 5 μM NMDA for only 30 s, terminals were still labeled in the superficial 50 μm of the slices. These were indeed GABAergic terminals in stratum (str.) pyramidale; 96.3±0.6% of the cGMP terminals were GAD65 positive (n=375, 9 slices, 3 mice), which corresponded to 57.3±0.6% of all GAD65-positive terminals in CA1 (n=513, 9 slices, 3 mice). No staining was observed in the str. lacunosum-moleculare. In the str. radiatum, some cGMP-labeled terminals, which appeared less intensely labeled than those around pyramidal cell somata, were also GAD65 positive. Furthermore, in the str. radiatum and oriens, most of the weaker cGMP-immunoreactive profiles did not coexpress GAD65 (likely representing either excitatory contacts or glial processes). In the CA3ab subfield, cGMP levels remained unchanged after NMDA application and were limited to GABAergic terminals (95.6±2.5% were GAD65 positive; n=201, 9 slices, 3 mice). This corresponds to 39.8±2.8% of all GAD65-positive basket cell terminals in CA3 (n=290, 4 slices, 2 mice). In summary, basket cell terminals of the DG showed no immunoreactivity for cGMP either before or after NMDA treatment. The CA3ab region had some weakly cGMP labeled GABAergic terminals in control slices, but NMDA treatment had no effect on their staining intensity or density (it changed from 38.9 to 39.8%). However, in CA1 and CA3c, NMDA had a massive effect on cGMP labeling in GABAergic axon terminals (from the original 0 to 57.3%). Furthermore, the positive terminals were stained much stronger than in CA3ab. Preincubation with competitive and noncompetitive NMDAR antagonists, D-AP5 and MK-801 (50–50 μM), prevented the effect ofNMDAadministration in CA1 but did not affect the basket terminal labeling in CA3ab, suggesting that only CA1 but not CA3ab basket synapses are regulated via NMDA-induced NO production (n=4 slices, 2 mice). To test the possible contribution of other voltage-dependent Ca^{2+} currents, we blocked postsynaptic L- and R-type Ca^{2+} channels (20_M nifedipine plus 100 nM SNX-482). The effect of 5 μM NMDA did not change (the results from double-immunofluorescent labeling were identical), suggesting that NMDARs are necessary and sufficient for triggering the NO–cGMP cascade (n=10 slices, 2 mice). The effect of NMDA could be completely blocked by the preincubation of slices with a NOS blocker, L-NAME (100 μM; n=5 slices, 3 mice), or the NOsGC blocker, ODQ (20 μM; n=14, 5 mice). To test the contribution of the potentially large number of spontaneous action potentials of pyramidal cells and interneurons, these studies were repeated using Mg^{2+}-free mACSF, containing 1 μM TTX, but the results were identical. To prove the exclusive role of nNOS, we performed experiments using nNOS-/- mice as well. All neuronal cGMP labeling was absent both from control

(*n*=8, 4 mice) and from NMDA-treated slices (*n*=9, 4 mice) in all hippocampal regions. cGMP staining in blood vessels was still detectable in nNOS-/- slices because of residual endothelial NOS activity. These results suggest that the most likely mechanism is that local Ca^{2+}-influx via NMDARs induces nNOS-dependent cGMP production in GABAergic terminals in the CA1 and CA3c regions of the hippocampus. Although the present work was focused on the GABAergic synapses, it is important to note that NMDA induced cGMP production was also detectable in the neuropil of the str. radiatum, whereas it was absent from the str. lacunosum-moleculare of the CA1 region. Interestingly, the induction requirements for long-term potentiation and long-term depression in these regions are characteristically different, which may be partly explained by the above-mentioned differences. On a technical note, interpretations of previous electrophysiological or pharmacological studies that involved applications of NMDAreceptor agonists or antagonists should take into account the indirect effects on GABAergic synaptic currents as well as on network patterns mediated by the retrograde NMDA–NO– cGMP pathway.

4.6. GluN1, GluN2A, and GluN2B subunits are expressed in perisomatic GABAergic synapses on CA1 pyramidal neurons

The experiments above suggested that NMDARs should be very close to GABAergic synapses. Therefore, we performed quantitative postembedding immunogold labeling for GluN1, 2A, and 2B with antibodies that were confirmed to be specific in subunit-specific knock-out mice. Pyramidal cell spines are known to express these subunits that we could also confirm.

In addition, GluN1, 2A, as well as 2B subunits were also clearly detectable in the postsynaptic active zones of GABAergic synapses on pyramidal cell somata. The average distance of immunogold labeling from the postsynaptic membrane, perpendicularly to the plane of the synapses, was 3.63 nm intracellularly (median; 0-12.8 interquartile range) (72.8% of gold particles were postsynaptic and 27.2% were in the synaptic cleft; *n*=104 gold particles), showing that NMDARs are postsynaptic in GABAergic synapses as well. No labeling was detected presynaptically. Next, using this quantitatively reliable method, we estimated the NMDAR labeling density in excitatory and perisomatic inhibitory synapses. We measured the labeling density of the GluN1 subunits, for it is present in all NMDARs uniformly. Random samples were collected from perisomatic GABAergic (*n*=54 synapses from 2 mice), and dendritic spine synapses (from the str. radiatum; *n*= 98, 2 mice). In GABAergic synapses, the density of NMDARlabeling was 9.73±1.34 times weaker than in spine synapses (GABAergic, 2.55±0.13; spine, 24.84±4.71 gold/ μm). Since somatic GABAergic synapses are 1.8 times larger than spine synapses, the former have 5.4±0.75 times less NMDARs than the latter. Some NMDARs were also found extrasynaptically on pyramidal cell bodies (0.198±0.03 gold/μm). To directly prove that different NMDAR subunits coexist in the very same synapses, we localized different subunits in consecutive sections of the same synapse. We found that GluN1–2A, GluN1–2B, and GluN2A-2B subunits frequently colocalized. Moreover, the three subunits could also be detected in the very same synapse. These results show that the

same GABAergic synapse may use both GluN2A and GluN2B subunit-containing NMDARs, postsynaptically.

4.7. The majority of somatic GABAergic synapses of CA1 pyramidal cells possess all three types of NMDAR subunits: quantitative data

To examine the proportion of GABAergic synapses containing NMDARs, a large number of reconstructed synapses are needed; therefore, we performed SDS-digested freeze– fracture replica immunolabeling. Complete synaptic active zones are revealed on the surface of pyramidal cell bodies, when the lipid bilayer of the plasma membrane fractures into two pieces: the exoplasmic (E)- face underlain by extracellular surface and the protoplasmic (P)-face underlain by cytoplasmic surface. An antibody against the C terminus of the $GABA_AR$ β3 subunit labeled dense clusters of IMPs on the P-face. Putative GABAergic synapses on the soma membrane, having a high local density of $GABA_AR$ subunit immunogold labeling, were identified by an unbiased delineation protocol. Then, using double immunogold labeling for $GABA_AR$ and NMDAR subunits, we estimated the minimum proportion of GABAergic synapses that express NMDAR subunits. All NMDAR subunit antibodies labeled the spines of pyramidal cells intensively but were also associated with $GABA_AR$-containing somatic synapses. The background labeling was measured on somatic E-faces, and it was found negligible (0.31±0.08 gold/μm^2 for GluN1, 0.79±0.16 gold/μm^2 for GluN2A, and 0.55±0.18 gold/μm^2 for GluN2B subunits). However, the density of NMDAR labeling in $GABA_AR$ clustering areas (putative GABAergic synapses) was as high as 29.80±6.85 gold/μm^2 (n=82 synapses, 3 mice) for GluN1, 47.77±6.77 gold/μm^2 (n=48 synapses, 2 mice) for GluN2A, and 45.83±11.58 gold/μm^2 (n=44 synapses, 2 mice) for GluN2B. Thus, synaptic NMDAR density was 99.06, 62.78, and 94.79 times higher than the background for GluN1, 2A, and 2B antibodies, respectively. Accordingly, we found that 66.2±6.8% (n=82), 65.5±11.1% (n=48), and 70.5±9.6% (n=44) of the GABAergic synapses were GluN1, 2A, and 2B positive, respectively, and in each of these synapses, the subunit labeling density was at least 30 times higher than background. The average number of gold particles for NMDAR subunits was 3.02±0.51, 5.21±0.95, and 4.41±0.54 gold/synapse for GluN1, 2A, and 2B subunits in NMDAR-positive synapses, respectively. These measurements may slightly underestimate synaptic NMDAR density compared with other membranes, because the higher density of $GABA_AR$ label labeling may hamper the access of NMDAR-associated immunogold particles to synapses. The number of gold particles and synaptic areas showed a significant positive correlation for each subunit (Spearman's R-correlation; r=0.3652, p=0.00074 for GluN1; r= 0.5002, p=0.00029 for GluN2A; r=0.4018, p=0.0069 for GluN2B); that is, the larger the synapse was, the more subunits were detected in it. The density of labeling did not change with the synaptic area (Spearman's R correlation; r=0.0031, p= 0.9781 for GluN1; r=0.1357, p=0.3576 for GluN2A; r=0.2923, p=0.0542 for GluN2B). We could not detect any synaptic gold particles for GluN1 on pyramidal cells of pyramidal cell-restricted GluN1 knock-out mice (n=34, 2 mice). However, glutamatergic synapses on the dendritic shafts of INs were densely labeled in these mice (data not shown), indicating that the lack of labeling was not attributable to technical reasons in the knock-out

mice. Because electrophysiological experiments have already suggested that NMDARs may be expressed extrasynaptically, we also investigated the labeling of these membranes on the soma. The extrasynapticNMDARdensity was 6.76±1.72, 9.10±1.51, and 2.65±0.66 gold/μm^2 for GluN1, 2A, and 2B subunits, respectively. This was 4.49, 5.41, and 17.62 times lower than synaptic labeling, but still 22.05, 12.02, and 5.49 times higher than background for GluN1, 2A, and 2B subunits, respectively. These extrasynaptic NMDARs may be mobile receptors that may later be targeted to GABAergic synapses, or they may be associated with lipid-raft proteins to promote NMDAR internalization. Together, at least approximately two-thirds of the somatic GABAergic synapses of CA1 pyramidal cells possess NMDAR subunits in the adult hippocampus.

4.8. NMDARs are present postsynaptically in synapses formed by both basket cell types

To identify the source of NMDAR-positive GABAergic synapses and to further confirm that NMDARs are exclusively postsynaptic, we performed preembedding immunogold labeling experiments for GluN1, 2A, and 2B subunits in the CA1 area. All NMDAR-subunit labelings were postsynaptic in somatic GABAergic synapses. GABAergic terminals were not labeled. The linear density of gold particles for GluN1 labeling in somatic GABAergic synapses was 0.520 gold/µm, whereas extrasynaptic labeling was much lower (0.058 gold/µm). This finding further confirms – now using the preembedding technique – the specific association of NMDARs with GABAergic synapses. Then, we performed double immunogold-immunoperoxidase experiments for NMDAR-PV and NMDAR-vGluT3. The latter is known to be expressed only in cholecystokinin (CCK)-containing basket cells in the hippocampus and labels ~90% of the CCK-positive basket terminals in the CA1 area. There is no overlap between the CCK- and the PV-containing subsets of interneurons in the hippocampus. First, we serially reconstructed synapses of PVand vGluT3-containing terminals. We found that, in the case of the synapses established by PV-positive terminals, 42.6% (n=47 synapses from 2 mice) were labeled for GluN1, 36.4% (n=33, 2 mice) for GluN2A, and 30.6% (n=36, 2 mice) were labeled for GluN2B. In the case of the synapses of vGluT3-positive terminals, 61.0% (n=41, 2 mice) were positive for GluN1, 42.9% (n=28, 2 mice) for GluN2A, and 37.9% (n=29, 2 mice) for GluN2B. Although we focused on somatic synapses in this study, some dendritic inhibitory synapses were also found to be positive for NMDAR subunits. In our previous work, we demonstrated that nNOS is present in somatic GABAergic synapses established by PV- and CCKpositive terminals. Using colocalization experiments, here we demonstrate directly that nNOS is also present in synapses established by vGluT3-positive terminals. Because the antibodies for nNOS and NMDARs were both raised in rabbits, there is no reliable method to directly colocalize these antibodies. Nevertheless, the fact that nNOS and NMDARs were found in basket cell synapses (in a very similar proportion) also strongly suggests that they are colocalized in the postsynaptic compartment of somatic GABAergic synapses.

5. Conclusions

The main conclusions of the thesis are:

(1) nNOS is associated to the postsynaptic active zones of GABAergic synapses made either by PV, or CCK/vGluT3 positive interneurons

(2) NOsGC is present in the somato-dendritic and axon terminal compartments of these interneurons

(3) NOsGC in interneurons is expressed as α1β1-heterodimers, and in pyramidal cells as α2β1-heterodimers

(4) administration of NMDA caused robust, NMDAR-, nNOS- and NOsGC-dependent cGMP production in GABAergic basket-terminals in the CA1 and CA3c regions of hippocampus

(5) GluN1, GluN2A and GluN2B subunits of NMDAR were enriched in GABAergic synapses, and were present exclusively postsynaptically

(6) at least two-thirds of perisomatic GABAergic synapses contained all three tested NMDAR subunit – with a density ten times smaller than in glutamatergic synapses

(7) synapses of both PV, and CCK/vGluT3 positive perisomatic terminals contained NMDARs

Our results suggest that the local activation of NMDARs in perisomatic GABAergic synapses is capable of providing a sufficient postsynaptic calcium influx for the activation of nNOS. Consequently, this signaling pathway can be an effective, activity-dependent modulator of neurotransmission at these synapses.

Because of the coincidence-detector characteristic of NMDARs, activation of the NO pathway can be achieved only by the coincident discharge of the pre- and postsynaptic cell, or the coincident activation of the postsynaptic cell and glial cells – responding to network activity. This makes the precise regulation of the communication of the pre- and postsynaptic cell possible.

6. List of own publications

6.1. First-author publications in the topic of the thesis:

NMDA receptors in hippocampal GABAergic synapses and their role in nitric oxide signaling
Szabadits E., Cserép C., Szőnyi A., Fukazawa Y., Shigemoto R., Watanabe M., Itohara S., Freund TF and Nyiri G.
The Journal of Neuroscience, 2011; 31(16):5893-5904

Hippocampal GABAergic synapses possess the molecular machinery for retrograde nitric oxide signaling.
Szabadits E, Cserép C, Ludányi A, Katona I, Gracia-Llanes J, Freund TF, Nyíri G.
The Journal of Neuroscience, 2007 Jul 25;27(30):8101-11.

6.2. Non-first-author publications in the topic of the thesis:

NMDA Receptors in GABAergic Synapses during Postnatal Development.
Cserép C, **Szabadits E**, Szőnyi A, Watanabe M, Freund TF, Nyiri G.
PLoS ONE 2012; 7(5):e37753. Epub 2012 May 25

Nitric oxide signaling modulates synaptic transmission during early postnatal development.
Cserép C, Szőnyi A, Veres JM, Németh B, **Szabadits E**, de Vente J, Hájos N, Freund TF and Nyiri G.
Cerebral Cortex, 2011;21:2065-2074

6.3. Other publications – not in the topic of the thesis:

CB1 cannabinoid receptors are enriched in the perisynaptic annulus and on preterminal segments of hippocampal GABAergic axons
Nyiri G, Cserep C, **Szabadits E**, Mackie K, Freund TF
Neuroscience, 2005, Quantitative Neuroanatomy Special Issue, 2005., 136(3):811-22;

GABA(B) and CB1 cannabinoid receptor expression identifies two types of septal cholinergic neurons
Nyiri G, **Szabadits E**, Cserep C, Mackie K, Shigemoto R, Freund TF
Eur J Neurosci. 2005; 21:3034-3042.