# Molecular signaling pathways in GABAergic and glutamatergic synapses of developing neuronal networks

Ph.D. thesis

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Budapest, 2013.
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## 1. INTRODUCTION

The development of the brain consists of a series of events, that follow each other in time, and determine each other. During this process a simple knob of tissue – consisting of only a few cells – evolves into the most complex and highest level structure, the material substrate of human perception, consciousness, soul and spirit.

This astonishingly complex process follows a general scheme that arches over species, and during which several consecutive, partly overlapping subtask has to be completed by the developing system. First, sufficient number of cells have to born, these cells have to migrate to their final destination, and finally, they have to reach their complex three-dimensional structure and build up their connections with other cells. The basic characteristics of all these processes is their activity-dependence, which makes the presence of an intrinsic, spontaneous neuronal activity necessary.

Any cell in a developing neuronal network will first recieve GABAergic synaptic inputs, followed by glutamatergic connections later. These glutamatergic synapses possess only NMDA-receptors in the beginning, and will express AMPA-receptors later in development. The question arises: How can a cell recieve the activity necessary for its own development and maturation, if it has only inhibitory synapses in the beginning? The answer is that during development GABAergic transmission is not inhibitory, but depolarizing on the contrary; and will exert hyperpolarizing effect only later in development, after the network has reached maturity. This process – called the „GABA-switch" – is caused by the high intracellular chloride concentration of developing neurons, which is caused by the expression of the chloride-importer NKCC1. The expression of this transporter declines during development, and is gradually replaced by the chloride-exporter KCC2. During development, the higher intracellular chloride-concentration is sufficient to reverse the electrochemical gradient of chloride, and although the ion-channel GABA-receptor works the same way – allows the flow of chloride ions – due to the reversed electrochemical gradient, chloride will flow out of the neurons, causing depolarization.

It is well known that GABAergic depolarization is indispensable for the presence of spontaneous synchronous activity (SSA) – characteristics of the early postnatal developmental period. This activity pattern disappears in paralell with the GABA-switch, and is gradually replaced by adult activity-patterns. The depolarizing GABAergic synaptic transmission, as well as the SSA – that depends on the former – are playing fundamental roles in the proper development of neuronal networks.

NMDA-receptors are voltage- and ligand-gated ion-channel glutamate-receptors, they need coincident depolarization and ligand binding to take up the open conformation. Wide range of experiments has proven clearly that NMDAR-activation plays very important roles in the postnatal development of the brain, since their pharmacological blockade, or genetic inactivation caused radical damage to protein-transcription, the development of dendritic and axonal arborization, synaptogenezis and memory functions. The activation of these receptors contibutes largely to the maintanence of SSA. The question emerges: without a large number of AMPA-receptor containing glutamatergic synapses what provides the depolarization, indispensable for NMDAR-activation and its downstream developmental effects? The

answer is that during development the GABAergic depolarization is sufficient to extrude the $Mg^{2+}$-block from NMDA-receptors, leading to their activation and subsequent postsynaptic calcium-influx. This synergistic cooperation between $GABA_A$- and NMDA-receptors plays fundamental roles in the proper development of neuronal networks and synaptogenezis, yet, until now the precise cell-surface distribution of NMDA-receptors was unknown during this critical period of postnatal development.

Throughout the early postnatal period the depolarizing GABAergic and the likewise depolarizing glutamatergic synaptic transmission dominates. Thus, the presence of an effective, activity-dependent negative feedback system is inevitable. The retrograde nitric-oxide system could be able to fulfill these roles. In general, the postsynaptically located neuronal nitric oxide synthase (nNOS) will be activated upon the binding of calcium-calmodulin complex. This leads to the production of the signalling molecule nitric oxide (NO), that can freely cross cell membranes, and bind to its receptor in the presynaptic terminal. The NO-receptor – NO sensitive guanylate cyclase (NOsGC) – produces the second messenger cGMP when activated by NO. cGMP will then modulate transmitter release from the presynaptic terminal via several, secondary messenger pathways.

The experiments directed to explore the developmental effects of the NO-signalling pathway proved that during postnatal development, blocking this pathway either at the level of synthesis, or the level of the receptor lead to severe morphological and synaptic damage in a wide variety of brain areas. These results confirm that the nNOS-NO-NOsGC-cGMP signalling pathway plays an important role in the postnatal development of neuronal networks, and its uniterrupted operation is critical to the proper morphological and synaptic development of the network. Nevertheless, the subcellular distribution of the molecular machinery underlying the NO-signalling pathway during development is unknown.

## 2. AIMS

1. NMDA-receptors an the cooperation of the $GABA_A$-NMDA signaling systems play fundamental roles in the development of neuronal networks. Yet, until now the precise subcellular deitribution of NMDA-receptors is unknown in this critical developmental period, thus, their location relative to GABAergic synapses – the main sources of depolarization at this age – is also unknown. Our previous results – confirming the presence of NMDA-receptors in the GABAergic synapses of adult mice – made this question even more important. Because of these reasons, in our first series of experiments we aimed to explore the exact subcellular distribution of NMDA-receptors at an age through postnatal development, when the first, synapse-driven synchronous network activity is the most prominent. To achieve this, we wanted to investigate the following questions:

- Which synapses and in what proportion do express NMDA-receptors during postnatal development?
- Is the expression of these NMDA-receptors pre- or postsynaptic in these synapses, or both?
- Quantitative comparison of receptor-density between different synapse-types and extrasynaptic areas.

2. The spontaneous synchronous activity that emerges during development is brought about mainly by the depolarizing GABAergic, and the likewise depolarizing glutamatergic transmission. Although the GABAergic system is able to exert some inhibition through shunting, it can be assumed that the network possessed some kind of an effective, activity-dependent negative feedback regulatory system, in order to avoid over-excitation. Altough the retrograde nitric-oxide signalling system could be an ideal candidate, the precise subcellular distribution of the molecular elements of this pathway has not yet been described during development. Therefore, in the second series of experiments we aimed to examine whether the retrograde nitric-oxide signalling system could regulate synaptic transmission or spontaneous synchronous

activity during postnatal development. To achieve this, we asked the following questions:

- Is nNOS present in the GABAergic and glutamatergic synapses during development?
- Where is NO-receptor expressed relative to GABAergic and glutamatergic synapses?
- By sensing NO, could the NO-receptor produce cGMP in the vicinity of GABAergic and glutamatergic synapses of the developing brain?
- Can the NO-system influence the operation of GABAergic and glutamatergic synapses during development?
- Is the NO-system able to regulate spontaneous synchronous activity during postnatal development?

## 3. MATERIALS AND METHODS

### 3.1. Ethics Statement

All experiments were performed in accordance with the Institutional Ethical Codex and the Hungarian Act of Animal Care and Experimentation guidelines, which are in concert with the European Communities Council Directive of November 24, 1986 (86/609/EEC). The Animal Care and Experimentation Committee of the Institute of Experimental Medicine of Hungarian Academy of Sciences and the Animal Health and Food Control Station, Budapest, have specifically approved the experimental design under the number of 2302/003/FŐV/2006.

### 3.2 Selection of the developmental period for examination

We wanted to examine a developmental period during which the GABAergic depolarization is able to activate NMDA-receptors, thus we needed to choose a period before the GABA-switch. Another important criteria was that the examined period is needed to coincide with the maximum of the sponateous synchronous activity. In the case of mouse hippocampal pyramidal cells the GABA-switch happens between postnatal 12-16. days. The spontaneous synchronous activity is the most prominent between postnatal days 6-9. both in hippocampus and neocortex. Considering all this, we found the period between postnatal days 6-9. suitable for our experiments.

### 3.3. Animals and Tissue Preparation

Animals were perfused transcardially after general anaesthesia. The fixative contained 4% freshly depolymerized paraformaldehyde in the first series of experiments for the labeling of NMDA-receptors, except for the pre-embedding vGluT1-immunoperoxidase and GAD-immunoperoxidase single immunoreactions, where it was supplemented with 0.25% glutar-aldehyde. In the second series of experiments for the labeling of nNOS the fixative contained 1% paraformaldehyde, for the labeling of NOsGC subunits it contained 4% paraformaldehyde. Coronal sections were prepared from the removed brains at 40–60 μm thickness for immunofluorescent experiments, 70–80 μm thickness for pre-embedding electron microscopy reactions and 300 μm thickness for freeze-substitution and low-temperature embedding. In all preembedding experiments, where we labeled NMDARs, the tissue was treated with pepsin for epitope retrieval.

### 3.4. Preparation of acute slices

We used 5-8 days old animals for the praparation of acute slices. After general anaesthesia animals were decapitated, and brains were removed under ice-cold cutting solution, followed by the cutting of coronal or horizontal slices. For electophysiological and multineuron-calcium imaging experiments we placed the 450

μm thick slices into submerged type dual-superfusion chambers. For the cGMP-immunohistochemistry experiments the 300 μm thick slices were transferred to sterile, 12-chambered cell culture plates. Every chamber contained 1 mL ACSF, which was supplemented with blockers of phosphodiesterases (PDE). Chambers were individually bubbled with carbogan gas at equal rates. The slices were incubated for 10 minutes after the application of different drugs, then the solutions in chambers were quickly changed to ice-cold 4% paraformaldhyde solution. Slices were postfixed for 48h in the same fixative at 4 $^{o}$C before the immunohistochemical reactions.

### 3.5. Primary antibodies

In the first series of experiments we used two antibodies for the labeling of GABAergic terminals: Millipore MAB5406, clone-1G10.2 antitbody, and the number 1440 Oertel GAD65/67 antibody. To label glutamatergic terminals we used two antibodies against type-1 vesicular glutamate transporter (vGluT1): Millipore AB5905, and Synaptic Systems cat. nr.: 135 303, aa 456-560. To label synapses, we used a mouse monoclonal anti-Bassoon antibody: Abcam clone-SAP7F. The labeling of NMDA-receptors was performed using rabbit antibodies directed against the C-terminal of GluN1, GluN2A and GluN2B subunits. The specificity of these antibodies had been well characterized using immunoblot, antigen peptides and null mutant mice or conditioned knockout mice in both pre- and postembedding experiments. We also used a monoclonal mouse antibody directed against the extracellular loop of the NMDA-receptor, Millipore MAB363, clone-54.1. To label $GABA_A$-receptors, we used a guinea pig antibody raised against the beta3 subunit (produced and characterized in the laboratory of Ryuichi Shigemoto, Okazaki, Japan), or a rabbit polyclonal antibody raised against the gamma2 subunit (SynapticSystems, raised against amino acids 39–67, cat. nr.: 224 003).

In the second series of experiments to label nNOS we used a rabbit polyclonal antibody (Zymed Laboratories, cat. nr.: 61-7000), that gave absolutely no labeling on brain tissue from nNOS knockout animals. To label GABAergic terminals, we used a mouse monoclonal antibody: Millipore cat. nr.: MAB351. To label NOsGC subunits, we used rabbit polyclonal antibodies: anti-NOsGC α1 (Sigma-Aldrich cat. nr.: G4280) and anti-NOsGC β1 antibody (Cayman Chemical cat. nr.: 160897). The labeling of cyclic guanosine monophosphate (cGMP) was performed using a sheep anti-cGMP antibody, that has been produsedin the laboratory of Jan de Vente.

### 3.6. Immunofluorescent Labeling and Confocal Laser Scanning Microscopy

After washing, sections were incubated in mixtures of primary antibodies overnight at room temperature. After this, sections were washed, and incubated the mixture of secondary antibodies. This was followed by washes, and the sections were mounted onto glass slides and coverslipped. Immunofluorescence was analyzed using a Nikon Eclipse Ti-E inverted microscope and an A1R laser confocal system.

In the second series of experiments we used immunofluorescent labeling and confocal laser scanning microscopy to examine cGMP production in drug-treated acute slices. Fixed slices were washed, embedded in agar, and resliced at a thickness

of 50 μm. After further washes sections were blocked, and incubated in solutions of primary antibodies. Sections were washed again, and incubated in secondary antibody solutions. This was followed by further washes, and sections were put on slides and coverslipped. Immunfluorescent signal was analysed this time using an Olympus Optical FluoView300 confocal laser scanning miroscope.

### 3.7. Preembedding immunoelectron-microscopy

To determine the sizes of GABAergic and glutamatergic synapses, we performed immunoperoxidase reactions. After washing the samples, they were incubated in solution of primary antibodies. This was followed by washes, and incubation in solution of secondary antibodies. After further washes and ABC-treatment the reaction was made visible using 3,3-diaminobenzidine. Following further washes the sections were treated with osmium, and dehydrated.

For the labeling of the different NMDA-receptor subunits, we performed combined immunogold-immunoperoxidase reactions on 4% paraformaldehyde fixed tissue, that has been digested using pepsin. After primary and secondary antibody incubations sections were washed, and incubated in ABC solution. The reaction was developed using 3,3-diaminobenzidine. Immunogold labeling was intensified using a silver-enhancement solution (SE-EM) for 40-60 minutes at room temperature. percen keresztül, szobahőmérsékleten. Following further washes the sections were treated with osmium, dehydrated and embedded in epoxy-resin (Durcupan). After polimerisation 80-100 nm thick sections were cut, and analysed using a Hitachi H-7100 electron microscope, equipped with a Veleta CCD camera.

In the combined immunogold-immunoperoxidase reactions for localising nNOS we used 1% paraformaldehyde fixed tissue. In these experiments pepsine treatment was not used. Samples were washed, incubated in solutions of primary antibodies, washed again, and incubated in solutions of secondary antibodies. After this the development of the immunogold and immunoperoxidase labelings, the resin embedding and electron microscopic analysis of the samples was performed as described above.

### 3.8. Post-embedding Immunoelectron-microscopy

For Lowicryl embedding 300 μm thick sections from 4% paraformaldehyde fixed tissue samples were cryoprotected, slammed onto gold-plated copper blocks cooled in liquid nitrogen. This was follwed by low temperature dehydration, freeze-substitution, and embedding in Lowicryl HM20 resin. Postembedding immunohistochemistry was performed on 70 nm thick sections. The sections were incubated on drops of blocking solution, followed by incubation on drops of primary antibodies overnight. After this, the samples were washedand incubated on drops of secondary antibodies. This was followed by several washes, sections were rinsed in ultrapure water and contrasted with saturated aqueous uranyl acetate. The sections were examined using a Hitachi H-7100 electron microscope and a Veleta CCD camera.

### 3.9. In vitro electrophysiology (work of partner-workgroup)

Whole-cell recordings were performed at 31-32 °C under visual guidance using Zeiss Axioscope. ACSF, equilibrated with carbogen gas, was superfused with the flow rate of 2-3 mL/min. Patch electrodes had resistances of 3-6 MX when filled with the intracellular solution. In some experiments, the pipette solution contained biocytin, and post hoc morphological identification of recorded cells using an immunofluorescent method confirmed that these were all pyramidal neurons. PSCs were recorded at a holding potential of –70 mV. Slices were superfused with ACSF containing kynurenic acid or picrotoxin to block ionotropic glutamate receptors or $GABA_A$-receptors, respectively. Using a Supertech timer and isolator, stimulation of fibers was delivered in every 10 s (0.1 Hz) via a theta glass pipette filled with ACSF. To evoke glutamate receptor-mediated synaptic currents (GluR-PSCs) or $GABA_A$-receptor-mediated PSCs ($GABA_A$R-PSCs), the pipette was placed in the stratum radiatum (str. rad.) or in the str. pyramidale, respectively, in the CA1 region of the hippocampus. Access resistances were frequently monitored and remained constant (±20%) during the period of recording.

### 3.10. Multineuron calcium imaging (in collaboration with partner-workgroup)

The recordings were performed at a flow rate of 8-10 mL/min, at 32-33 °C. Areas from CA1 str. pyramidale were loaded with the cell-permeant calcium indicator Fura-2 AM. Ratiometric imaging was performed using alternating excitation at 340 and 380-nm wavelengths with 100-ms exposure time. Relative changes in Ca2+ levels were calculated from the ratio of the emitted fluorescent light in the 2 wavelengths (340 and 380 nm). Each active cell was marked manually as a region of interest (ROI), and the changes in the ratio during the measured periods were calculated in each ROI by the software. Events were considered synchronous, if Ca2+ signals increased simultaneously in more than 90% of the active cells. After recording the control period, different drugs were bath applied to the slices for 10-12 min, followed by the second recording to monitor the effects of the drugs on SEs. In every slice, we compared the number of SEs during the control period and after drug application. In some cases, the activities of single cells were recorded simultaneously with the optical imaging of their Ca2+ changes. In these experiments, unit activity was recorded in loose patch mode, using patch pipettes (3-6 MX) filled with ACSF. All data were recorded with a Multiclamp 700B amplifier, filtered at 2 kHz, digitized at 10 kHz, and analyzed off-line.

### 3.11. Drugs

L-NAME, SNP, and 8-Br-cGMP were dissolved in distilled water; IBMX, BAY-73 6691, and ODQ in dimethyl sulfoxide. Drugs were prepared as stock solutions and diluted to the required concentrations. Drugs were obtained from Tocris or from Sigma.

### 3.12. Analysis

When data populations in this work had a Gaussian distribution according to the Shapiro-Wilks W test, we reported parametric statistical features (mean+SD), otherwise we reported nonparametric statistical features (median, interquartile range). Two independent groups were compared using the nonparametric Mann--Whitney U test; 2 dependent groups were compared using the nonparametric WMP test; the null hypothesis was rejected when the P level was under 0.05. In such cases, the differences were considered significant.

In the first series of experiments glutamatergic as well as GABAergic synapses were selectively labeled. In the second series of experiments terminals were considered to be GABAergic if they were GAD65 immunopositive, while terminals that were GAD65 immunonegative and established asymmetric synapses were considered to be glutamatergic. All examined synapses in this work were sampled from the stratum radiatum of the hippocampal CA1 area. The sizes of glutamatergic and GABAergic synapses were measured from serial sections of fully reconstructed synapses. In the preembedding experiments for localising NMDA-receptors or nNOS, immunogold particles were counted within the defined GABAergic and glutamatergic synapses and along the extrasynaptic membrane. Immunogold particles were considered to be membrane or synapse-associated, if they were not further away from the membrane than 40 nm. In the post-embedding immunogold reactions, 40 nanometer-wide bands were chosen on the two sides of the synaptic membrane as an area representing membrane-associated gold particle labeling. For the measurements, we used the open-source image analyzing software ImageJ/Fiji.

## 4. RESULTS

### 4.1.1. Confocal Microscopy Reveals an Association of NMDARs with Both Glutamatergic and GABAergic Synapses during Development

Synapses were labeled with an antibody against Bassoon, a presynaptic active zone protein. Glutamatergic and GABAergic terminals were labeled with anti-type 1 vesicular glutamate transporter (vGluT1) and an anti-glutamate decarboxylase 65/67 (GAD65/67) antibody, respectively. During development, numerous glutamatergic and GABAergic synapses are present in stratum radiatum of the hippocampal CA1 region, therefore we investigated this area. In this region the vast majority of dendrites belong to pyramidal cells, thus it is very likely that the postsynaptic targets of the examined synapses were pyramidal-cell dendrites. vGluT1 and GAD65/67 staining appeared as a non-overlapping punctate labeling pattern, showing the distribution of glutamatergic and GABAergic synapses, respectively. In the quadruple-labeling experiments, we found that Bassoon-positive patches were associated to either vGluT1 or GAD65/67 positive terminals. In several cases, GluN1subunit labeling was found to be associated to the Bassoon-labeled synapses of both vGluT1-positive (glutamatergic) and GAD65/67-positive (GABAergic) terminals at P6-7 (n=4 animals). Furthermore, we used confocal laser-scanning microscopy to show direct colocalization of $GABA_A$- and NMDA-receptors within the same synapses. First, we used a triple labeling protocol for Bassoon, GluN1, and $GABA_AR$ β3 subunit (n=2 animals), and in some cases we observed that labeling for GluN1 and $GABA_AR$ β3 subunits overlapped, and were juxtaposed to Bassoon-positive patches. Second, we used double-labeling against the extracellular loop of the GluN1 subunit, combined with $GABA_AR$ γ2 subunit (n=2 animals) and we also found a frequent association of the GluN1 subunit with $GABA_AR$ γ2 labeling. Although the specificity of this method is high, due to its moderate sensitivity, quantification was not performed. Our results obtained using these fluorescent staining experiments suggested that GluN1 labeling associated with $GABA_AR$-positive puncta, which showed the synaptic co-expression of NMDARs and $GABA_ARs$ during postnatal development. Although these data suggested a close association of NMDARs to both types of synapses, these receptors could still be either pre- or postsynaptic, as well as peri- or intrasynaptic. Therefore, to obtain data that are more precise, we performed electron microscopic experiments as well.

### 4.1.2. Preembedding Immunogold Electron Microscopy Confirms the Presence of Three Types of NMDAR Subunits Postsynaptically in Glutamatergic and GABAergic Synapses during Development

We performed immunogold-immunoperoxidase reactions in several combinations. GluN1, GluN2B or GluN2A subunits were labeled with immunogold particles, while glutamatergic and GABAergic terminals were labeled for vGluT1 or GAD67 with the peroxidase method, respectively. Randomly sampled synapses from the stratum radiatum of the CA1 area were examined in each reaction. We found that

all three tested subunits of NMDARs were present postsynaptically in both glutamatergic and GABAergic synapses at P6-7. Synapses were fully reconstructed from serial electron-microscopic sections from two mice, and at least 51±16% of all glutamatergic (n=41) and 53±13% of all GABAergic synapses (n= 40) were found to be positive for the GluN1 subunit. The linear density of immunolabeling was 1.93±0.03 gold particles/µm in glutamatergic and 0.87±0.29 gold particles/µm in GABAergic synapses, and only 0.09±0.01 gold particles/µm extrasynaptically, on 100 nm thick sections. Our data showed that at least 83±3% of all glutamatergic (n=40) and 63±13% of all GABAergic synapses (n=40) were positive for the GluN2B subunit. The linear density of labeling was 3.16±1.01 gold particles/µm in glutamatergic and 1.38±0.34 gold particles/µm in GABAergic synapses, and only 0.03±0.01 gold particles/µm were found extrasynaptically. At least 88±8% of all glutamatergic (n=40) and 49±1% of all GABAergic synapses (n=43) contained GluN2A subunits. The linear density of the labeling was 3.40±0.81 gold particles/µm in glutamatergic and 0.59±0.28 gold particles/µm in GABAergic synapses, and only 0.02±0.003 gold particles/µm were extrasynaptic.

### 4.1.3. Quantitative Postembedding Immunogold Method Reveals Only Small Differences in the Number of NMDARs in Glutamatergic and GABAergic Synapses during Development

Because of technical limitations, the results of pre-embedding experiments are only semi-quantitative; therefore, we performed post-embedding immunogold labeling to obtain more precise quantitative data on the subcellular distribution of NMDARs. GluN1 staining represents well the distribution of NMDARs, because GluN2 subunits are not transported to the cell-membrane without GluN1 subunits, therefore GluN1 is present in all NMDARs. We employed the so-called mirror technique to measure the density of immunogold labeling for the GluN1 subunit. This method enabled us to count these immunogold particles over synapses on the very same sections from two mice and to identify GABAergic and glutamatergic synapses on the mirror sections. Using this method we could quantitatively compare receptor expression of synaptic and extrasynaptic areas in the P6-7 mice. We randomly collected identified glutamatergic and GABAergic synapses from the stratum radiatum of the CA1 area. NMDAR labeling was detected in both glutamatergic and GABAergic synapses. Our measurements showed that the labeling density was 2.46±0.71 gold particles/µm in glutamatergic (n=32) and 0.81±0.16 gold particles/µm in GABAergic synapses (n=28), and only 0.03±0.01 gold particles/µm extrasynaptically (along 103 mm extrasynaptic membrane segment). In order to establish the ratio of the absolute number of NMDARs in glutamatergic versus GABAergic synapses, we measured the synaptic areas and multiplied them by the labeling densities measured above in the two types of synapses. For the reliable estimation of synaptic areas, we performed single preembedding immunoperoxidase reactions (on strongly fixed tissue) with antisera against either vGluT1 or GAD67 in two animals, and samples were embedded in Durcupan resin. All synapses were sampled from stratum radiatum. The glutamatergic synaptic area (n=29) was found to

be 0.097±0.013 μm2, and GABAergic synapses (n=32) were 0.186±0.026 μm2, i.e. the latter being 1.91 times larger than the former. These data led to the unexpected conclusion that glutamatergic synapses contain only 1.6 times more NMDARs than GABAergic synapses. Preembedding results clearly showed a purely postsynaptic localization of NMDARs in these synapses, nevertheless, we confirmed this also by measuring the distance of NMDAR immunogold labeling from the postsynaptic membrane. The distance distribution of gold particles measured perpendicularly from the postsynaptic membrane was very similar in GABAergic (median: 3.63 nm, interquartile range: -8.12–13.78 nm, n=28) and glutamatergic synapses (median 7.26 nm, interquartile range -7.87–13.49 nm, n=32, positive values refer to intracellular positions), confirming the postsynaptic origin of the immunogold labeling.

### 4.1.4. nNOS Is Associated with the Postsynaptic Active Zones of GABAergic and Glutamatergic Synapses in the Developing Hippocampus

Since synchronous neuronal activity is a characteristic phenomenon of developing cortical networks that is most prominent around postnatal day 6 in mice, when GABAergic transmission is still depolarizing, we focused most of our experiments to investigate this developmental stage. We localized nNOS during these early postnatal days and performed single and double immunogold-immunoperoxidase stainings for nNOS and GAD65 on wild type (WT) and nNOS–/– mice. In the single immunoreactions for nNOS in the P6 WT mice, the pyramidal cells were stained weakly, and some strongly immunopositive interneurons were detected in the CA1 and CA3 areas. In nNOS–/– mice, no labeling was found. Mostly postsynaptic densities were labeled in the hippocampal CA1 and CA3 areas, with some labeling also associated with extrasynaptic cell membranes, and a few gold particles localized on intracellular membrane organelles close to synapses. During development, numerous glutamatergic and GABAergic synapses are present in stratum radiatum of the hippocampal CA1 region, therefore we investigated this area. In this region the vast majority of dendrites belong to pyramidal cells, thus it is very likely that the postsynaptic targets of the examined synapses were pyramidal-cell dendrites. The linear density of the nNOS labeling was 1.36 gold particles/μm in the synapses (median, 0.7-1.37 min-max, 3 mice) and 0.06 gold particles/μm extrasynaptically (median, 0.06-0.07 min-max, 3 mice), while in the nNOS–/– mice, no such labeling was detected. A remarkable 46% of the fully reconstructed randomly sampled synapses from str. rad. of CA1 of P6 mice were labeled for nNOS (median, 41-50% min-max; n=66 synapses in 3 mice). Using GAD65 labeling and other morphological criteria, we identified GABAergic and glutamatergic terminals and found that postsynaptic nNOS immunolabeling was present in both types of synapses in the CA1 and CA3 regions of P4, P6, P10, and P14 mice. We found that 40% of GABAergic (median, 27-47% min-max; n=45 synapses in 3 P6 mice) and 33% of glutamatergic (median, 29-40% min-max; n=44 synapses in 3 P6 mice) fully reconstructed synapses expressed nNOS postsynaptically.

### 4.1.5. Both α1 and β1 Subunits of the NO receptor Are Present in GABAergic Axon Terminals during Development

In order to examine the subcellular distribution of α1 and β1 subunits of NOsGC, we performed single and double stainings for these subunits alone or in combination with GAD65. The α1 subunit labeling of P4-P14 mice was similar to the developmental labeling pattern described for GAD, suggesting that it labels mostly GABAergic terminals. Unlike pyramidal cells in the CA1 and CA3 areas, interneuron somata were α1 subunit immunopositive, along with a dense meshwork of axonal fibers and terminals localized predominantly in the str. rad. at P4-P10. At P14 the labeling was weaker but similar to the adult pattern, that is, the cell bodies of interneurons were labeled in all layers and their axon terminals were also α1 subunit positive, especially in the str. pyramidale. In the adult hippocampus, both in situ hybridization and immunohistochemical experiments showed that the α1β1 subunit composition of the NO receptor is interneuron specific, and indeed, unlike pyramidal cells in the CA1 and CA3 areas, interneuron somata were α1 subunit immunopositive at P4-P10. In addition, we found that α1 subunit-positive terminals always made symmetric synapses, and never asymmetric ones nor did they contact spines or spine-like structures at any ages examined. These data suggests that most if not all α1+ terminals are GABAergic already during development, and indeed, in the double-labeling experiments, we frequently found synaptic terminals positive for both GAD65 and α1 subunits in the CA1 and CA3 regions at P4, P6, P10, and P14 ages. Fifty-four percent (median, 53-57% min-max; n=88 synapses in 3 P6 mice) of the reconstructed, randomly sampled GAD65-positive synaptic terminals in CA1 were positive for the α1 subunit. The β1 subunit labeling was similar to the adult pattern. Pyramidal cells showed weak cytoplasmic staining, and the neuropil showed a diffuse, punctate labeling. Interneuron somata and immunopositive axon terminals were stained for β1 subunit as early as P4-P6. The β1 subunit-containing axon terminals formed symmetric synapses. In the double-immunostained preparations, GAD65 and β1 subunit double-positive axon terminals formed synapses in the str. rad. of CA1 and CA3 regions from P4, P6, P10, and P14 mice. These results show that the NOsGC α1β1 subunit composition is present in GABAergic terminals during postnatal development, and it is in an ideal position to receive retrograde NO signal synthesized in the postsynaptic compartment by nNOS.

### 4.1.6. Activation of the NO Receptor Induces cGMP Production in GABAergic Axon Terminals of the Developing Hippocampus

To test whether the NO receptor is functional during the early postnatal days, we prepared acute hippocampal slices from P6 mice, incubated them in oxygenated ACSF containing PDE inhibitors, and treated them either with no drugs, with the NO-donor SNP (200 μM), or with a selective inhibitor of the NO receptor (ODQ, 10 μM), followed by the addition of the NO donor. These incubations were followed by fixation and double immunofluorescent labeling for GAD65 and cGMP. After incubation with no drugs (control conditions), we did not find labeled terminals in the

randomly collected samples, but some glia-like cGMP immunoreactivity could be detected in these control hippocampal slices. After NO-donor (SNP) treatment, strongly cGMP-immunopositive fibers and terminals could be observed, along with a few labeled neuronal somata and some glia-like staining. After pretreatment with ODQ, SNP could not induce any detectable cGMP signal. In the SNP-treated slices, we found that 28% (median, 23-34% min-max; n=508 terminals in 3 mice) of the randomly sampled GAD65-positive terminals of the str. rad. showed cGMP immunoreactivity, and at least 41% (median, 39-41% min-max; n=335 terminals in 3 P6 mice) of the cGMP-positive terminals were positive for GAD65. Therefore, NOsGC is able to produce cGMP in these terminals already during the first postnatal days.

### 4.1.7. NO Decreases both GABAergic and Glutamatergic Postsynaptic Currents in a NOsGC-Dependent Manner

Using a whole-cell patch-clamp method, we recorded evoked PSCs in CA1 pyramidal cells in slice preparations from P5-P8 mice. First, we examined the effect of the NO donor, SNP (200 μM) on the amplitude of pharmacologically isolated $GABA_A$R-PSCs, evoked by electrical stimulation in the str. pyramidale. Application of SNP significantly decreased the peak amplitude of $GABA_A$R-PSCs to 67% of control values (control median amplitude: 186.5 pA and interquartile range: 149.2-240.8 pA; median amplitude in SNP: 125.6 pA and interquartile range: 92.8-184.8 pA, n=12 slices, P=0.00097, WMP test). The sensitivity of $GABA_A$R-PSCs to SNP was not homogenous. In the majority of cases, SNP reduced the amplitude of events significantly to 52% of control (control median amplitude: 231.7 pA and interquartile range: 161.6-443.9 pA; median amplitude in SNP: 120.3 pA and interquartile range: 56.1-329.6 pA, n=7 slices, P=0.015, WMP test). In the other cases, SNP caused no significant change in the peak amplitude (control median amplitude: 151.3 pA and interquartile range: 142.7-180.9 pA; median amplitude in SNP: 137.6 pA and interquartile range: 126.8-77.2 pA, n=5 slices, P=0.12, WMP test). We then tested whether the suppressing effect was mediated by NOsGC. We pretreated slices with ODQ, the blocker of NOsGC, followed by coapplication of ODQ and SNP. After the control period, we bath-applied 10 lM ODQ, which caused a small reduction in the peak amplitudes of $GABA_A$RPSCs (control median amplitude: 130.8 pA and interquartile range. 92.8-226.2 pA; median amplitude in ODQ: 99.5 pA and interquartile range: 50.2-171.8 pA, n=9 slices, P=0.004, WMP test). Then, we treated the slices with SNP in the presence of ODQ. In all but one instance, SNP could not reduce the amplitude (median amplitude in ODQ: 132.3 pA and and interquartile range: 63.3-194.2 pA; median amplitude in SNP + ODQ: 131.8 pA and interquartile range: 56.7-181.4 pA, n=8 slices, P=0.25, WMP test). The comparison of the control peak amplitudes of $GABA_A$R-PSCs before application of drugs in the 2 sets of experiments (SNP experiments [control: 186.5 pA] or ODQ + SNP experiments [control: 130.8 pA]) revealed no significant difference (P=0.18, Mann-Whitney U test), suggesting that similar sets of GABAergic fibers were stimulated in both

experiments. These results showed that NO can control the efficacy of GABAergic synaptic transmission via activation of NOsGC.

Next, we investigated the impact of NO on glutamatergic transmission as well. First, we tested the effect of SNP on the peak amplitude of pharmacologically isolated ionotropic GluR-PSCs evoked by focal stimulation in the str. rad. Application of SNP invariably reduced the GluR-PSC amplitude by half (control median amplitude: 63.2 pA and interquartile range: 26.1-104.9 pA; median amplitude in SNP: 30.58 pA and interquartile range: 9.1-60.5 pA, n=7 slices, P=0.015, WMP test). Next, we pretreated the slices with ODQ, which in itself did not alter significantly the peak amplitude (control median amplitude: 42.8 pA and interquartile range: 24.3-120.6 pA; median amplitude in ODQ: 37.9 pA and interquartile range: 30.5-69.6 pA, n=7 slices, P=0.57, WMP test) but again occluded the effect of SNP (median amplitude in SNP + ODQ: 31.1 pA and interquartile range: 25.2-70.1 pA, n=7 slices, P=0.22, WMP test). The control amplitudes of GluR-PSCs were similar in both sets of experiments (P=0.79, Mann-Whitney U test). These data show that the glutamatergic synaptic communication is also depressed by NO signaling via NOsGC in postnatal hippocampal slices.

### 4.1.8. Modulation of NO Signaling Alters Synchronous Network Activity in Developing Hippocampal Slices

To test whether NO signaling can influence synapse-driven synchronous network activity, we used multineuron calcium imaging in acute hippocampal slices. Areas from CA1 str. pyramidale were loaded with the calcium indicator Fura-2 AM (20 μM). In control conditions, simultaneous calcium imaging and recording of the unit activity of the same cell in loose patch mode revealed that the calcium elevations corresponded to burst firings of the same cells. The number of SEs during recordings (155 s) was 8.6 ± 4.7 SD (n=26 slices). Then, we examined the effect of pharmacological blockade of the NO signaling on the occurrence of SEs. The NOS inhibitor L-NAME (100 μM) significantly increased the number of SEs to 157% of control in WT (median, 145-700% min-max; P = 0.0117, WMP test; n=8 slices, in 4 mice) but not in nNOS–/– mice (n=3, in 3 mice). Similarly, the NO-receptor inhibitor ODQ (10 μM) also increased the number of SEs significantly to 356.6% of control (median, 178-583% min-max; P=0.0277, WMP test; n=6, in 3 mice). These results indicated that there was a basal NO signaling in the network that restrained the synapse-driven SEs. Stimulation of the NO-signaling pathway was also tested. We bath applied either the NO-donor SNP (200 μM) or the membrane-permeable cGMP-analogue Br-cGMP (50 μM). These drugs significantly decreased the number of SEs to 15.5% of the control (median, 0-31% min-max; P=0.0277, WMP test; n=6, in 4 mice) or to 41% of control (median, 17-60% min-max; P=0.0277, WMP test; n=6, in 3 mice), respectively. The effect of Br-cGMP could be completely washed out in 4 of 6 slices (2 slices were not tested). Thus, modulation of NO signaling can have a strong influence on hippocampal synchronous activity in early stages of development.

## 5. CONCLUSIONS

GABA – the main inhibitory transmitter in the adult brain – exerts depolarizing effects during development. This GABAergic depolarization cooperates with NMDAR-s to produce spontaneous synchronous activity. All these processes are indispensable for the proper formation of neuronal networks.

In the first series of experiments we wanted to determine the exact subcellular distribution of NMDAR-s during this period of postnatal development. Using electron-microscopy, we found that GluN1, GluN2A and GluN2B NMDAR subunits are present both in glutamatergic and GABAergic synapses, exclusively postsynaptically. Quantitative post-embedding immunogold reactions confirmed that the density of NMDARs is 3x higher in glutamatergic than in GABAergic synapses. Since GABAergic synapses are about 2x larger, the two types of synapses contained similar amounts of NMDARs. Using immunfluorscent imaging, we also showed direct colocalization of synaptic GABAARs and NMDARs. Based on our results we suggest that the developmentally important GABA-NMDA cooperation takes place primarily in GABAergic synapses.

Since GABAergic transmission is mainly depolarizing during early postnatal development, the existence of a powerful negative feedback mechanism is necessary. In our second series of experiments we wanted to answer wether the retrograde NO-system could fulfill this role. Using whole-cell recording we showed that NO signaling modulates not only glutamatergic but also GABAergic synaptic transmission during the early postnatal period. We identified the precise subcellular localization of key elements of the underlying molecular cascade using immunohistochemistry at the light—and electron microscopic levels. As predicted by these morpho-functional data, multineuron calcium imaging in acute slices revealed that this NO-signaling machinery is involved also in the control of synchronous network activity patterns. We suggest that the retrograde NO-signaling system is ideally suited to fulfill a general presynaptic regulatory role and may effectively fine-tune network activity during early postnatal development, while GABAergic transmission is still depolarizing.

## 6. LIST OF OWN PUBLICATIONS

### 6.1. 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

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.
J Neurosci. 2007 Jul 25;27(30):8101-11.
* Equal contribution

### 6.2. Non-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

### 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.