Biogenesis, intracellular trafficking, and biophysical and pharmacological properties of ion channels are studied using molecular biology, biochemistry, fluorescence microscopy, electrophysiology, and molecular modeling. We routinely use the following experimental models and methods to study ligand-gated ion channels.

Cultured hippocampal neurons and cerebellar granule cells

To study glutamate receptors we use primary hippocampal or cerebellar granule cell cultures (Fig. 1). Neurons in these cultures express AMPA and NMDA glutamate receptors and form functional synapses.

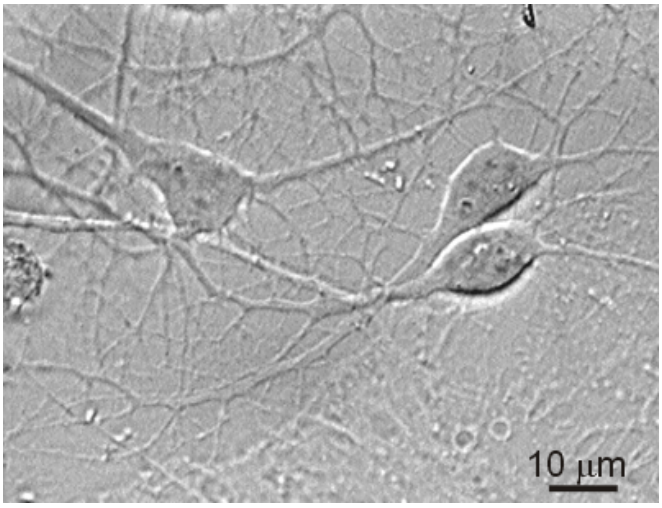


Fig. 1: Primary cell culture prepared from rat hippocampus. Three neurons with a number of dendrites are growing on a glial feeder layer.

Cultured primary sensory neurons of dorsal root ganglia

To study molecular mechanisms of nociception we use Dorsal Root Ganglion (DRG) culture (Fig. 2). Sensory neurons in this culture express transient receptor potential (TRP) channels involved in pain sensation.

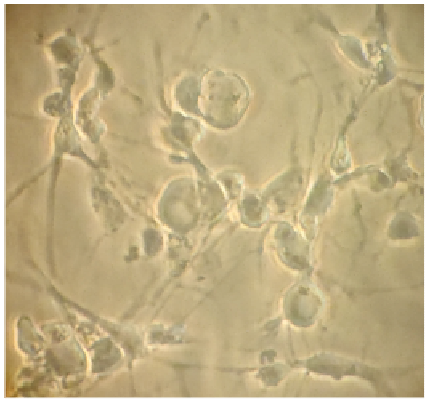


Fig. 2: Primary tissue culture prepared from rat DRG.

Expression of recombinant genes in HEK293 and COS cells

To study the properties of membrane ion channels, we use heterologous expression systems of HEK293 or COS cells transfected with recombinant plasmid DNAs (Fig. 3). We can also express recombinant ion channels in cultured neurons. To investigate relationships between the structure and the function of ion channels, we create targeted mutations of genes in two ways: (a) by generating a point mutation leading to the exchange of a single amino acid or the artificial formation of a stop codon, or (b) by constructing chimeric ion channels (hybrid ion channels made by combining two homologous channels). To identify successfully transfected cells, cDNA coding for green fluorescent protein (GFP) is used (Fig. 4).

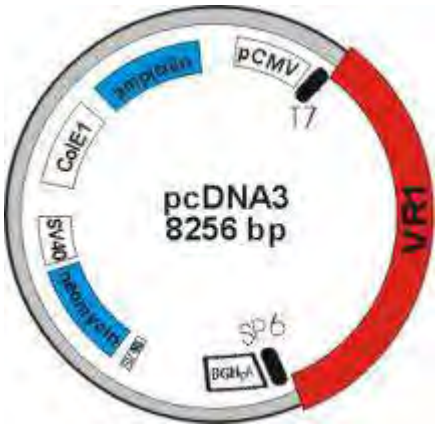


Fig. 3: A diagram of plasmid DNA carrying a gene of an ion channel subunit (red).

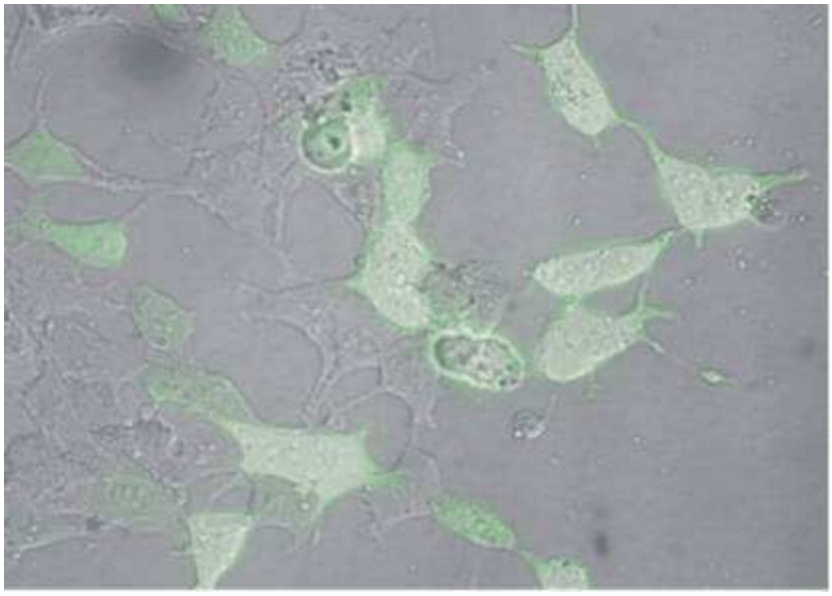


Fig. 4: HEK293 cells transfected with green fluorescent protein.

Patch-Clamp Electrophysiology

The patch-clamp technique makes it possible to measure small electric currents (10^{-13} A) induced by activation of ion channels. A glass microelectrode is brought close to the surface of the cell membrane and, using suction, a connection with high electrical resistance („gigaseal“) is formed. Several possible configurations of microelectrode tip and the membrane are shown in the picture (Fig. 5). Cell attached, inside-out and outside-out configurations enable us to record currents through one or several ion channels isolated in the membrane patch. In the whole-cell configuration it is possible to measure currents through all the channels present in the cell membrane, including synaptic currents.

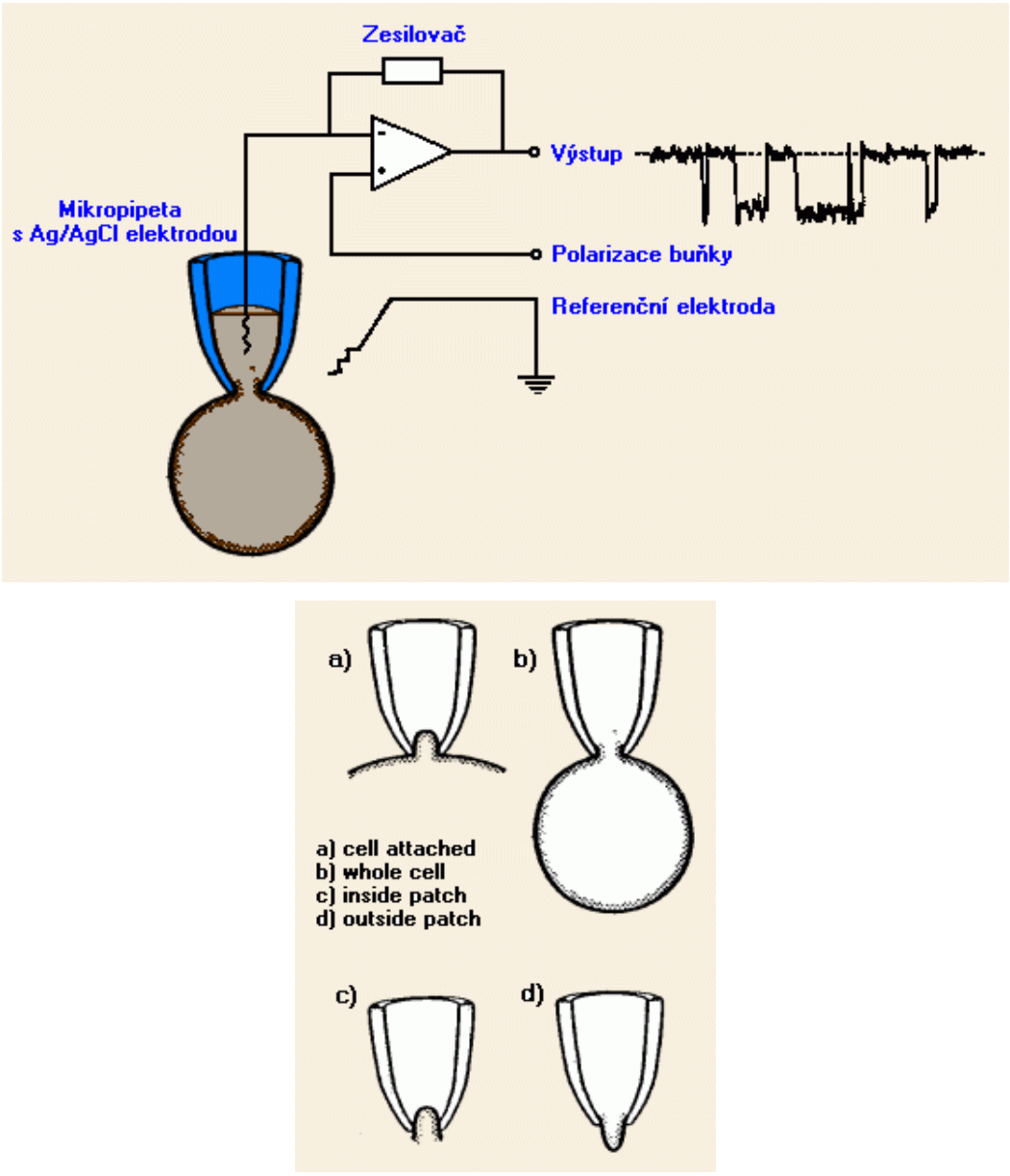


Fig. 5: A diagram illustrating the patch-clamp technique ***(top)*** and the different recording configurations ***(bottom).***

Fast application of solutions

Fast application of substances at known concentrations enables us to investigate the quantitative aspects of ligand-gated ion channel activation. In our department, we have developed an application system that makes it possible to exchange solutions in the local environment of cells in <5 ms, and in isolated patches in less than 1 ms (Fig. 6). For the study of thermally activated channels, equipment was supplemented with the control of the temperature of the applied solutions.

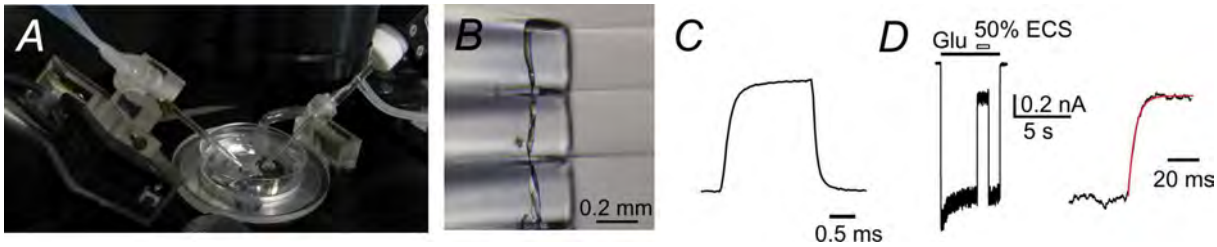


Fig. 6: Fast application of solutions. **(*A*)** Experimental setup: application tubes attached to a piezo actuator (left) and the patch-clamp recording pipette (right). **(*B*)** Borosilicate glass multibore square tube opening. **(*C*)** Open-tip current measurement. Pulse duration 2 ms, 10-90% rise time 380 µs, 10-90% decay time 220 µs. The pulse was delivered using a triple-stream method, where the outer tubes are filled with ECS (extracellular solution) and the middle tube is filled with ECS diluted by water. The step is made from the first tube to the third tube thus crossing the middle tube at a high speed and producing a short pulse of the diluted solution. **(*D*)** Glutamate-induced current in a lifted HEK293 cell transfected with GluN1/GluN2B subunits. Standard ECS was switched to 50% diluted ECS during glutamate application. A change in the glutamate-induced current elicited by the solution switch is shown on the expanded time-scale. The fit (τ = 4.5 ms, red line) reflects the rate of solution exchange around the cell.

Recording synaptic currents in hippocampal microisland cultures

Hippocampal microisland cultures are a very practical tool to study the pharmacology of synaptic transmission (Fig. 7). These cultures are prepared in several steps. First, the coverslip is coated with non-permissive substrate which does not support cell attachment. Second, small (0.3 mm) microislands of permissive substrate are stamped onto the coverslips. Next, rat glial cells are allowed to attach to and grow on the microislands. Finally, rat hippocampal neurons are plated at a very low density, such that many islands contain a solitary neuron forming synapses only with itself (autapses). Autaptic currents can readily be evoked and recorded using whole-cell patch-clamp techniques.

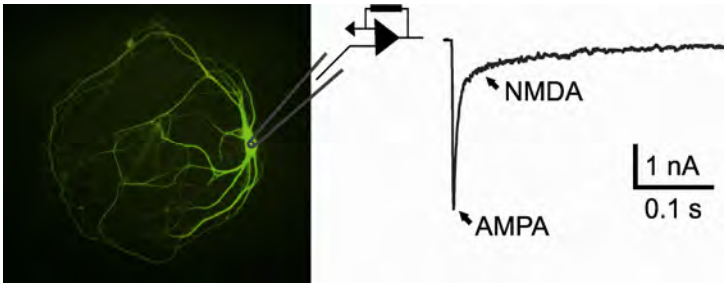


Fig. 7: A solitary autaptic hippocampal neuron expressing green fluorescent protein grown on a microisland ***(left)***. Example trace of a dual excitatory postsynaptic current mediated by AMPA and NMDA receptors ***(right)***.

Ca^{2+} imaging

Local and global changes of cytoplasmic levels of free calcium ions are important signals connected with activation of particular types of receptors or with cell excitation. The most frequently used method for measuring the intracellular calcium concentration is fluorescence microscopy combined with calcium-sensitive fluorescent probes – „calcium imaging". The principle of this method is based on changes of the spectral properties of the probe molecule after binding a calcium ion. The excited probe emits light at 510 nm. The probe saturated with calcium is excited at 340 nm while the free probe is excited at 380 nm (Fig. 8). Local free calcium concentration is estimated by comparing the emission of the free and the occupied fractions of probe molecules in the sample. A frequently used calcium-sensitive probe is FURA-2. The apparent dissociation constant of this probe is 100-300 nM (depending on the ionic strength and the composition of intracellular space). This implies that FURA-2 can measure calcium ions in range from 10 nM to several mM.

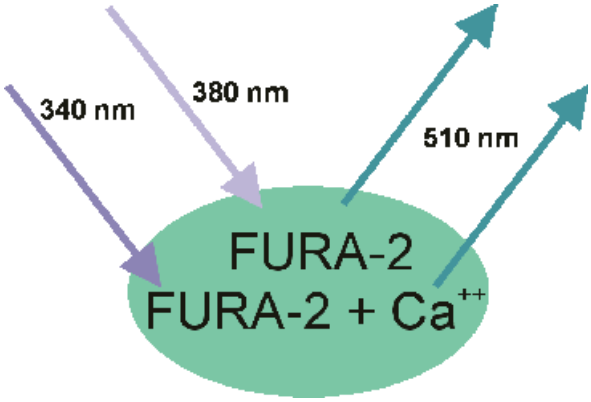


Fig. 8: A diagram illustrating the shift of the spectral properties of FURA-2 caused by calcium binding.

Polymerase chain reaction (PCR)-based analysis

We use various modifications of standard PCR to amplify and subsequently analyse DNA/RNA of glutamate receptors in our experimental models. PCR techniques allow us to determine changes in the level of expression of various iGluR subunits (Q-PCR) and to monitor the presence of subunits and alterations in mRNA processing (RT-PCR). Amplified PCR product from genomic DNA samples, when subjected to Sanger sequencing, can provide information about genetic alterations that may contribute to the emergence and development of neurological disorders (Fig. 9).

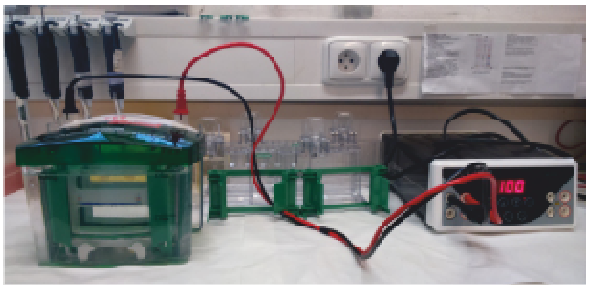
C

GluN3A	rs2900317			rs12338602		
(n=10)	A/G (%)	A/A	G/G	C/T	C/C	T/T
SCH	15	85	0	13	87	0
CTRL	67	33	0	67	33	0

Fig. 9: PCR amplification and direct sequencing performed on control (CTRL) and schizophrenic (SCH) patient genomic DNA samples to screen for single nucleotide polymorphism (SNP) locations and frequency. **(*A*)** EtBr gel shows PCR amplicons for NR1/3A promoter regions. **(*B*)** Sequencing chromatographs (reverse strands) document the positions of two SNPs (rs2900317, rs12338602) in the NR3A promoter region. (***C***) Table presents the observed allele distribution for the two NR3A SNPs in CTRL/SCH samples (n=10).

Biochemistry

Identification, expression and post-translational modification of proteins are studied using sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (Fig. 10).



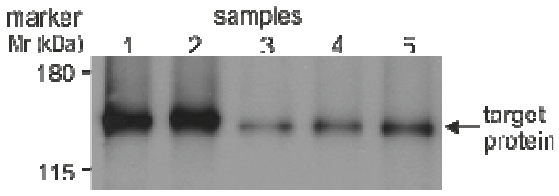


Fig. 10: SDS-PAGE system for the separation of proteins ***(left)***. Example of an immunoblot – detection of a target protein using specific antibodies visualized with a chemiluminescence detection system ***(right)***.

Immunofluorescence

Immunofluorescence methods are used for localizing proteins in cells. Secondary antibodies conjugated with a fluorescent dye visualize primary antibodies, targeted against selected proteins (Fig. 11). We use immunohistochemical staining to localize e.g. different subunits of NMDA receptors (NR2A-D) and vanilloid receptors in neuronal populations in spinal cord slices and primary dissociated cultures.

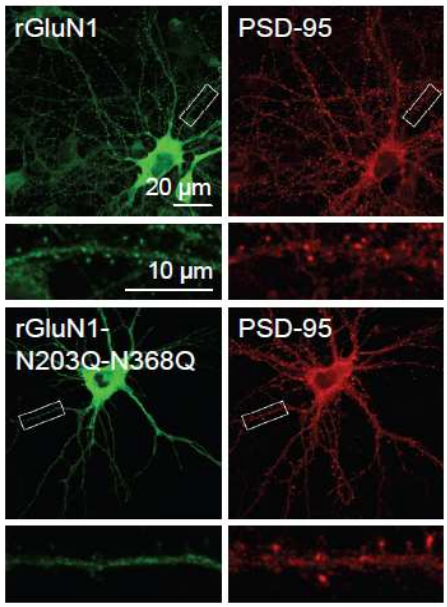


Fig. 11: Hippocampal neurons infected with lentiviruses were immunostained for GluN1 and PSD-95 (a marker of excitatory synapses). Note that the rGluN1-N203Q-N368Q subunit (which lacks two specific glycosylation sites) does not reach excitatory synapses, in contrast to the wild type GluN1 subunit.

In-vitro models of excitotoxicity

When primary hippocampal cultures are challenged with an excitotoxic insult, such as NMDA application, or oxygen-glucose deprivation, a proportion of neurons succumb to necrotic cell death. Necrotic cells can be visualized and counted using a fluorescent dye propidium iodide. We use this method to test the ability of different substances to protect neurons from excitotoxic cell death (Fig. 12).

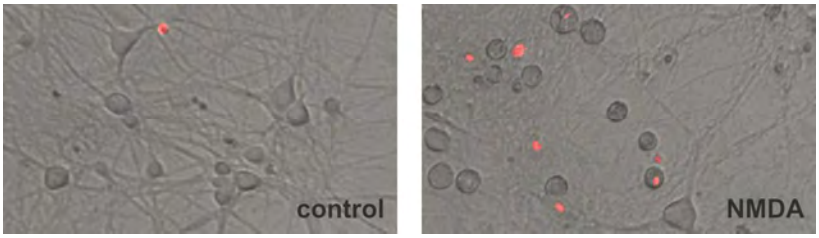


Fig. 12: Phase-contrast images overlayed with images of propidium iodide staining (red) from a control culture ***(left)*** and a culture treated with 20 µM NMDA for 2 h ***(right)***.

Molecular modeling

A combination of tools of molecular modeling, sequence analysis, and structural bioinformatics is employed for understanding the molecular mechanism of NMDAR opening and modulation of function by interaction with neurosteroids and membrane components. The tools we are using cover the sequence analysis and alignment (Ugene, muscle, clustalw), homology modeling (Modeller), visualization and analysis of structures and models (pymol, VMD, chimera, FoldX), docking of proteins and neurosteroid ligands (ClusPro, AutoDock, DOCK), molecular dynamics simulation (Amber, Gromacs) as well as accurate quantum chemical calculations (TurboMole, openmopac).

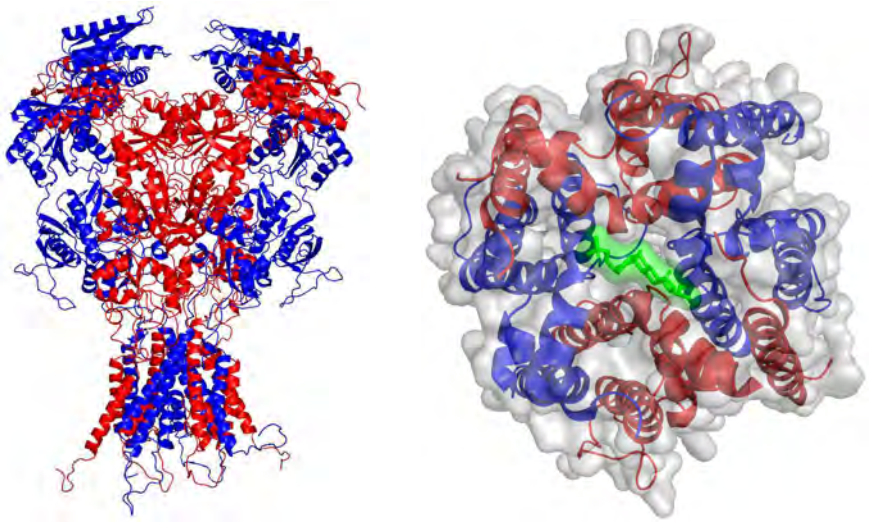


Fig. 13: Visualization of the NMDAR structure based on the pdb id 4pe5 ***(left)*** Model of blocking of open channel entrance by neurosteroid ***(right)***.