Doctoral Thesis

Analysis of the diversity of the postsynaptic membrane proteins neuroligins
identification of at least one new isoform

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ANALYSIS OF THE DIVERSITY OF THE POSTSYNAPTIC MEMBRANE PROTEINS NEUROLIGINS: IDENTIFICATION OF AT LEAST ONE NEW ISOFORM

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY
ZÜRICH

for the degree of

Doctor of Natural Sciences

presented by

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Prof. Dr. Yves Barral, co-examiner
PD Dr. Sergio M. Gloor, co-examiner

2001
This work is dedicated to my family.

I owe thousand thanks to all superiors and colleagues for countless fruitful discussions, technical assistance, and a pleasant atmosphere in the lab.
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Neuroligins 1, 2 and 3, first discovered in rat, constitute a family of brain-specific proteins whose structural and biochemical characteristics are indicative of a role in cell adhesion. These integral plasma membrane proteins have structural similarity in the extracellular portion to acetylcholinesterases, without having, however, catalytic activity. Among each other, neuroligins have a related structural organization with an N-terminal signal peptide, the esterase-like domain containing two sites of alternative splicing, a small linker region of low sequence identity in front of the transmembrane domain, and a short cytosolic part. Immunoelectron microscopy analysis of rat brain sections has established a restriction of neuroligin 1 to the postsynaptic membrane of excitatory synapses, where the protein could form an intercellular complex with β-neurexins, another class of synaptic plasma membrane proteins. The neuroligin/β-neurexin junction represents the only known heterophilic cell adhesion system of the synapse. In addition, rat neuroligins bind via their intracellular tails to PDZ domains of several synaptic scaffolding proteins like PSD-95/SAP90; therefore neuroligins are suggested to participate in the assembly of protein complexes involved in signal transduction. Co-cultures of primary neurons and non-neuronal cells overexpressing mouse neuroligins have illustrated that neuroligin proteins can induce presynaptic differentiation.

Based on the hypothesis that neuroligins might represent a family of molecules for selective intercellular recognition of neurons, the aim of this study was to investigate whether further neuroligin isoforms can be identified in mouse and human. For this purpose extensive reverse transcription-polymerase chain reactions and database analyses were performed. A DNA database search on mouse sequences resulted in entries encoding three proteins that were homologous to the rat neuroligins 1-3. From a mouse brain cDNA library a clone was isolated coding for neuroligin 1. Overexpression of this cDNA in COS-7 cells revealed that most protein was not delivered to the plasma membrane as expected, but accumulated within the cell, probably in the endoplasmic reticulum and/or the Golgi apparatus. Western blot analysis showed that the deglycosylated protein runs at a lower mass than calculated.

Using reverse transcription-polymerase chain reaction on human polyadenylated RNA and database searches, also the human homologs of the three rat neuroligins could be identified. In addition, sequences were obtained encoding a further member of the family which was named neuroligin 4. This protein has 63-73% identity with the other human neuroligins, and the same predicted domain structure. DNA database analyses, furthermore, indicated that a possible fifth neuroligin gene may be present in the human genome. Northern blot analysis revealed expression
of neuroligin 4 in heart, liver, skeletal muscle and pancreas, but hardly in brain. Transfection of COS-7 cells with a neuroligin 4 cDNA led to the production of a 110 kDa protein which was glycosylated. Immunofluorescence microscopy analysis demonstrated that the protein was integrated into the plasma membrane. Overexpressing cDNAs encoding neuroligin 4 and PSD-95/SAP90 in COS-7 cells resulted in the formation of detergent-resistant complexes. Neuroligin 4 did not bind to ZO-1, another PDZ domain protein, confirming that the interaction with PSD-95/SAP90 is specific. Together these data show that the human neuroligin family is composed of at least one additional member, and suggest that these proteins may also be produced outside of the central nervous system.
ZUSAMMENFASSUNG


Mittels reverse Transkription-Polymerase Kettenreaktion auf humaner polyadenylierter RNS und Datenbanksuchen konnten auch die humanen Homologe der drei Ratten-Neuroligine identifiziert werden. Zusätzlich wurden Sequenzen gefunden, die für ein weiteres Mitglied der Familie kodieren: Das abgeleitete Protein wurde Neuroligin 4 genannt. Neuroligin 4 hat 63-73%
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>ABP</td>
<td>AMPA receptor-binding protein</td>
</tr>
<tr>
<td>AMPA</td>
<td>α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate</td>
</tr>
<tr>
<td>ANK</td>
<td>ankyrin</td>
</tr>
<tr>
<td>AP</td>
<td>alkaline phosphatase</td>
</tr>
<tr>
<td>APS</td>
<td>ammonium peroxodisulfate</td>
</tr>
<tr>
<td>BBS</td>
<td>Bes-buffered saline</td>
</tr>
<tr>
<td>Bes</td>
<td>N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid</td>
</tr>
<tr>
<td>bis-acrylamide</td>
<td>N,N'-methylene-bis-acrylamide</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C1 (2)</td>
<td>protein kinase C, conserved domain 1 (2)</td>
</tr>
<tr>
<td>CAM</td>
<td>cell-adhesion molecule</td>
</tr>
<tr>
<td>CaMK</td>
<td>Ca$^{2+}$/calmodulin-dependent protein kinase</td>
</tr>
<tr>
<td>CASK</td>
<td>CaMK/SH3/GuK domain protein</td>
</tr>
<tr>
<td>CAZ</td>
<td>cytomatrix assembled at the active zone of neurotransmitter release</td>
</tr>
<tr>
<td>Cdh</td>
<td>cadherin</td>
</tr>
<tr>
<td>chapsyn</td>
<td>channel-associated protein of synapses</td>
</tr>
<tr>
<td>CNR</td>
<td>cadherin-related neuronal receptor</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>CortBP</td>
<td>cortactin-binding protein</td>
</tr>
<tr>
<td>CRIP1</td>
<td>cysteine-rich interactor of PDZ three</td>
</tr>
<tr>
<td>CSPD</td>
<td>disodium 3-(4-methoxyxpyrlio {1,2-dioxetane-3,2'-(5'-chloro)tricyclo [3.3.1.1^{3,7}]decan}-4-yl)phenyl phosphate</td>
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<tr>
<td>DABC0</td>
<td>1,4-diazabicyclo[2.2.2]octane</td>
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<td>DAP</td>
<td>Dlg-associated protein</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>Dlg</td>
<td>Drosophila discs large</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle's medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>Doc2α</td>
<td>double C2α</td>
</tr>
<tr>
<td>DPBS</td>
<td>Dulbecco's PBS</td>
</tr>
<tr>
<td>DTT</td>
<td>DL-dithiothreitol</td>
</tr>
<tr>
<td>EC</td>
<td>extracellular</td>
</tr>
<tr>
<td>ECL</td>
<td>enhanced chemiluminescence</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>EM</td>
<td>electron microscope (microscopy)</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>EST</td>
<td>expressed sequence tag</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FNIII</td>
<td>fibronectin type III</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
</tr>
<tr>
<td>GBM</td>
<td>glioblastoma multiforme</td>
</tr>
<tr>
<td>GEF</td>
<td>GDP-GTP exchange factor</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>GKAP</td>
<td>GuK-associated protein</td>
</tr>
<tr>
<td>GluR</td>
<td>glutamate receptor</td>
</tr>
<tr>
<td>GPI</td>
<td>glycosylphosphatidylinositol</td>
</tr>
<tr>
<td>G-protein</td>
<td>GTP-binding protein</td>
</tr>
<tr>
<td>GRASP</td>
<td>GRIP-associated protein</td>
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<tr>
<td>GRIP</td>
<td>GluR-interacting protein</td>
</tr>
<tr>
<td>GTC</td>
<td>guanidine thiocyanate</td>
</tr>
<tr>
<td>GuK</td>
<td>guanylate kinase</td>
</tr>
<tr>
<td>htds</td>
<td>unfinished high throughput genomic sequence</td>
</tr>
<tr>
<td>Ig</td>
<td>immunoglobulin</td>
</tr>
<tr>
<td>iGluR</td>
<td>ionotropic GluR</td>
</tr>
<tr>
<td>LB</td>
<td>Luria-Bertani</td>
</tr>
<tr>
<td>LNS</td>
<td>laminin, neurexin, sex hormone-binding globulin</td>
</tr>
<tr>
<td>LTP</td>
<td>long-term potentiation</td>
</tr>
<tr>
<td>MAG</td>
<td>myelin-associated glycoprotein</td>
</tr>
<tr>
<td>MAGuK</td>
<td>membrane-associated GuK</td>
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<td>MAP</td>
<td>microtubule-associated protein</td>
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<td>mGluR</td>
<td>metabotropic GluR</td>
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<td>Mint</td>
<td>Munc18-interacting protein</td>
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<tr>
<td>MOPS</td>
<td>3-morpholinopropanesulfonic acid</td>
</tr>
<tr>
<td>Munc</td>
<td>mammalian uncoordination mutant</td>
</tr>
<tr>
<td>NCAM</td>
<td>neural CAM</td>
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<tr>
<td>NEAA</td>
<td>non-essential amino acids</td>
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<tr>
<td>NL</td>
<td>neuroligin</td>
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<tr>
<td>NMDA</td>
<td>N-methyl-D-aspartate</td>
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<tr>
<td>nNOS</td>
<td>neuronal nitric oxide synthase</td>
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<tr>
<td>NP-40</td>
<td>Nonidet P-40</td>
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<tr>
<td>Osp</td>
<td>oligodendrocyte-specific protein</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
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<tr>
<td>PBGG</td>
<td>PBS-BSA-gelatin-glycine</td>
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<tr>
<td>PBH</td>
<td>piccolo bassoon homology</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>Pcdh</td>
<td>protocadherin</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
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<tr>
<td>PDZ</td>
<td>PSD-95/SAP90, Dlg, ZO-1</td>
</tr>
<tr>
<td>PICK</td>
<td>protein interacting with C kinase</td>
</tr>
<tr>
<td>PRA</td>
<td>prenylated Rab acceptor protein</td>
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<tr>
<td>proSAP</td>
<td>proline-rich SAP</td>
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<tr>
<td>PSA</td>
<td>polysialic acid</td>
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<tr>
<td>PSD</td>
<td>postsynaptic density</td>
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<tr>
<td>RC-PCR</td>
<td>rapid cloning of cDNAs by screening pools of cDNA by PCR</td>
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<tr>
<td>RHM</td>
<td>radiation hybrid mapping</td>
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<td>RIM(-BP)</td>
<td>Rab3-interacting molecule (-binding protein)</td>
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<tr>
<td>RT</td>
<td>room temperature</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcription-PCR</td>
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<tr>
<td>SAM</td>
<td>sterile alpha motif</td>
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<tr>
<td>SAP</td>
<td>synapse-associated protein</td>
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<td>SDS</td>
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<td>SNARE</td>
<td>soluble N-ethylmaleimide-sensitive factor attachment protein receptor</td>
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<tr>
<td>SSC</td>
<td>standard saline citrate</td>
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<tr>
<td>S-SCAM</td>
<td>synaptic scaffolding molecule</td>
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<td>somatostatin receptor-interacting protein</td>
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<td>SynGAP</td>
<td>synaptic GTPase-activating protein</td>
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<td>Tris-boric acid-EDTA</td>
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<td>Tris-buffered saline(-Tween)</td>
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<td>Tris-EDTA</td>
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<td>tris[hydroxymethyl]aminomethane</td>
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<tr>
<td>Veli</td>
<td>vertebrate LIN-7 homolog</td>
</tr>
<tr>
<td>ZO-1</td>
<td>zonula occludens</td>
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1. INTRODUCTION

1.1. Discovery of the Synapse

At the end of the 19th century Santiago Ramón y Cajal proposed that the brain consisted of enormous numbers of individual cells (neuron theory; figure 1.1). Camillo Golgi, on the other hand, was convinced that the brain was a continuous syncytium of tissue (reticular theory; figure 1.1). Ramón y Cajal made elegant use of a technique for staining tissue, which had been discovered accidentally by Golgi. For reasons that are not understood to the present day, this technique stains only a small number of the nerve cells present in a brain section. As a result, individual neurons show up clearly in tissue sections that actually contain a large number of neurons. Using other methods that stain all the neurons, these same sections would have appeared as tangled thickets. Although both Golgi and Ramón y Cajal used the same staining method, they came to opposite conclusions. Ramón y Cajal supposed that the nervous system is made up of discrete nerve cells, whereas Golgi never accepted this interpretation and continued to put forward his reticular theory of a continuous meshwork. The debate was finally won by the supporters of the neuron theory, and it is now universally accepted that the brain, like other organs, is cellular.

While Ramón y Cajal provided the anatomical basis for modern neuroscience, the studies of Charles Scott Sherrington were laying the basis for the physiological principles. Sherrington supplied the experimental findings which showed that the region of contact between two neurons might allow action potential transmission in one direction only and realized that the separation between nerve cells would allow for a new form of intercellular communication (i.e. chemical transmission). He introduced the term synapse which is derived from greek words meaning 'to fasten together'. The word actually appears for the first time in the 1897 edition of Michael Foster's *A Text Book of Physiology* (Sherrington, 1897). This proposal brought together the neuroanatomical and the physiological evidence into one term. Sherrington postulated that the connection of one nerve cell with another at the synapse is merely a contact without continuity of substance, but only the introduction of EM (electron microscopy) proved him correct when a small gap was directly sighted between the presynaptic and postsynaptic membranes.

Sherrington's pioneering work established the idea that understanding synaptic structure and function is essential for understanding how the brain works. A synapse is a highly dynamic structure. During early brain development a large number of newly generated synapses is eliminated while others are strengthened. Similar dynamic changes in synaptic strength occur in the adult brain, where they are thought to underlie learning and memory processes.
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Figure 1.1: Reticular versus neuron theory. Over a century ago, the nervous system was thought to be a syncytium of cells that were joined together by their processes (left). This arrangement would permit electrical activity to travel through the syncytium in either direction upon stimulation (thin and thick arrows). As evidence mounted that neurons were separate cells (right), it was recognized that a chemical synapse (inset) would permit electrical activity to travel in only one direction (thin arrows). From Sanes et al., 2000.

1.2. Structure of the Synapse

The neurons are the cells of the brain that are responsible for intracellular and intercellular information transfer. They are asymmetric cells with morphologically and functionally distinct regions that specialize them for signaling. A neuron is bounded by a continuous plasma membrane and consists of a cell body (soma) which is the metabolic center of the cell and gives rise to two types of processes: The dendrites and the axon (figure 1.2). Dendrites usually branch extensively in the vicinity of the cell body, spread out for several millimeters and have the appearance of a tree. Small budlike projections (dendritic spines) of a variety of shapes are frequently seen on the more distal branches of the dendritic tree. These spines are the synaptic input sites at which the neuron receives information from another cell. In contrast to the dendrites only one single axon, usually of small diameter, arises from the cell body and can extend for long distances (from a few micrometers to more than a meter). Axons in the CNS (central nervous system) often end in fine branches known as terminal arbors. In most neurons, each axon terminal is capped with small terminal boutons which correspond to functional points of contact between nerve cells (synapses). Information flows unidirectionally from the dendrites to the cell body to the axon (and its terminals), and then to the next cell through the synapse. Each neuron makes pre- and postsynaptic contacts with many other nerve cells; one single cell can receive input from more than $10^5$ presynaptic cells and makes contact with another $10^5$ postsynaptic neurons. The total number of synapses formed by the $10^{10}$-$10^{11}$ neurons of the human brain can be estimated to reach about $10^{15}$. Thus synapses are abundant in the brain, and the pattern of synaptic connectivity is complex.
Synapses are key structures for communication between nerve cells and their target cells, i.e. other neurons, muscle cells or gland cells. Although the synapse is actually a physiologic entity, it has traditionally been defined by its morphologic characteristics. In general, there are two types of synapses: Electrical synapses and chemical synapses. Electrical synapses are rarely found in the mammalian CNS, but are common both in fishes and invertebrates. The most outstanding morphological feature of the electrical synapse is the gap junction, a cell-to-cell pore that allows ions and small molecules to pass freely from the cytoplasm of one cell to the next. Thus, the gap junction allows current to pass directly between adjacent cells, resulting in very fast synaptic transmission. At chemical synapses electrical activity is translated by the presynaptic neuron into a chemical signal, the neurotransmitter, which is detected and processed by the postsynaptic cell. The release and diffusion of transmitter takes time, so there is a synaptic delay between the arrival of the presynaptic action potential and the onset of synaptic current in the postsynaptic neuron. Associated with the morphological asymmetry of chemical synapses is a fundamental functional asymmetry: In contrast to electrical synapses, the transfer of information in the chemical synapse occurs unidirectional.

Chemical synapses can be typified because synapses require structural specializations for different forms of synaptic transmission (Burns and Augustine, 1995). The best-studied example of a chemical synapse is the one formed between motor neurons and muscle cells in the peripheral nervous system (Sanes and Lichtman, 1999). This synapse provides excitation of the postsynaptic muscle cell, ensuring muscle contraction whenever the motor neuron is active. The neuromuscular synapse is optimized for very rapidly releasing large quantities of acetylcholine, an excitatory neurotransmitter. A second type of synapses is found in the mammalian CNS (tom Dieck and Gundelfinger, 2000). This kind of synapse seems to be specialized for rapidly releasing small quantities of neurotransmitter from a presynaptic terminal and for generating small postsynaptic electrical responses. Thus, to excite the postsynaptic cell, many such inputs must sum together. In the following paragraphs the physiology and molecular organization of this type of synapse will be described in more detail. Another kind of chemical synapses are the ribbon synapses that are found in certain primary sensory cells, such as photoreceptors (Rao-Mirotznik et al., 1995) and mechanoreceptors.
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(Roberts et al., 1990). The ribbon, a proteinaceous structure within the presynaptic terminal, is believed to allow constant Ca\(^{2+}\)-regulated secretion of transmitters. While all of the above-mentioned types of synapses are specialized to transmit rapidly, other types act over slower time scales.

1.3. Physiology and Morphology of the Synapse

Neurons, like all other cells, exhibit a voltage difference (membrane potential) across their plasma membrane. However, nerve cells are unique in their ability to manipulate the flow of charge across the cell membrane of other neurons or effector cells. The membrane of the neuron controls ion transport so that Na\(^+\) and Cl\(^-\) ions are concentrated outside the cell, whereas K\(^+\) ions are concentrated inside. The submembrane region ends up with a relative excess of negative charge, so a voltage exists across the cell membrane which is called resting potential and has typically a value of about -70 mV. Signals that make the cytoplasm more positive are said to depolarize the membrane, and those making it more negative are said to hyperpolarize it. Changes in membrane potential are produced by the opening or closing of populations of ion channels, whereas the resting potential is restored and maintained by the activity of the Na\(^+\)/K\(^-\)-ATPase. An action potential, the conducting signal of neurons, is a brief, spike-like depolarization (to +50 mV) that propagates as an electrical wave without decrement and at a high velocity (up to 100 m/sec) from one end of the axon to the other. It is normally elicited when the cell membrane is depolarized by some type of stimulus to beyond a threshold level (-60 to -50 mV); it is said to be produced in an all-or-nothing manner because a subthreshold stimulus gives no propagated response, whereas every suprathreshold stimulus elicits the stereotyped propagating wave. Underlying the propagated action potential is a regenerative wave of opening and closing of voltage-gated Na\(^+\) and K\(^+\) channels that sweeps along the axon.

Almost all parts of the neuron can receive synaptic input, the dendrites, the cell body, the axon hillock where axon potentials are initiated, and even the nerve terminal itself. A neuron in the brain is covered with thousands of synaptic inputs, and a single action potential usually produces only a small depolarization of the postsynaptic cell, too small to reach threshold for triggering an action potential. Therefore, an individual presynapse has to fire a rapid series, or several neurons have to fire simultaneously. Reaching threshold is determined by the sum of all existing excitatory and inhibitory synaptic potentials, a process called neuronal integration which takes place in the soma of the neuron. In contrast, each muscle cell receives input from only a single synapse, and a single action potential is sufficient to trigger the postsynaptic action potential.

A typical chemical synapse consists of a presynaptic element (axon terminal), a postsynaptic element (dendritic spine) and the synaptic cleft which is about 20-30 nm in width (figure 1.3). The synapse is surrounded by glial cells. They not just fill in the gaps between synapses, but promote the formation of synapses, take up released neurotransmitter and provide energy substrates and neurotransmitter precursors to synapses, thus helping to maintain their proper function (Pfrieger and Barres, 1996). The axon terminal contains mitochondria and several hundred SVs (synaptic vesicle) which contain the neurotransmitter (Burns and Augustine, 1995; Peters et al., 1991). These vesicles aggregate near sites on the presynaptic membrane called active zones, which
are the places of neurotransmitter release. Depolarization of the presynaptic membrane by an arriving action potential causes Ca^{2+} channels to open. The resulting influx of Ca^{2+} ions into the cell mediates fusion of SVs with the presynaptic plasma membrane, resulting in release of neurotransmitter into the cleft (Bajjalieh, 1999; Brunger, 2000; Südhof, 1995). Thereafter SVs are rapidly retrieved by dynamin/clathrin-dependent endocytosis, recycled and refilled with neurotransmitter (Brodin et al., 2000; Südhof, 1995). The transmitterdiffuses rapidly across the synaptic space and binds to specific receptor molecules in the postsynaptic membrane, where a highly electron-dense structure, the PSD (postsynaptic density), appears on electron micrographs. As a consequence of transmitter-receptor binding, a modification of postsynaptic ion channel permeability occurs (Seeburg, 1993).

Depending on the neurotransmitter type and the corresponding postsynaptic receptor apparatus, chemical synapses may be excitatory or inhibitory, causing either depolarization or hyperpolarization of the postsynaptic membrane, respectively. The major excitatory neurotransmitter in the mammalian brain is glutamate which activates Na^{+} or Ca^{2+} channels. Abundant inhibitory neurotransmitters of the CNS are GABA (γ-aminobutyric acid) and glycine which regulate Cl⁻ channels. Two different classes of receptors can be distinguished: Ionotropic receptors that harbor an intrinsic ion channel (fast transmission), and metabotropic receptors that activate G-proteins (GTP-binding protein) and an effector protein which alters the concentrations of intracellular second messengers (slow transmission). The transmitter-receptor complex is shortlived, and free transmitter molecules are removed from the synaptic gap either by enzymatic degradation or by removal using transporter proteins in the presynaptic membrane or in glial cells positioned near the synaptic cleft (Schloss et al., 1994).
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1.4. Molecular Organization of the Excitatory Chemical Synapse in the CNS

At the ultra-structural level both sides of the synapse are characterized by electron-dense projections that provide correct alignment of pre- and postsynaptic specializations (Gundelfinger and tom Dieck, 2000). The CAZ (cytomatrix assembled at the active zone of neurotransmitter release) defines and organizes the site of neurotransmitter release from presynaptic nerve terminals (Dresbach et al., 2001; Garner et al., 2000a; Hirokawa et al., 1989). The PSD tethers neurotransmitter receptors and the postsynaptic signal transduction machinery (Garner et al., 2000b; Kennedy, 1997; Ziff, 1997). Both sides of the synapse are kept in register by CAMs (cell-adhesion molecule), which are anchored to the subsynaptic cytoskeleton on both sides of the synapse and which interact to bridge the synaptic cleft (Benson et al., 2000; Brose, 1999). In the next sections, the molecular structure of excitatory synapses of the CNS using the neurotransmitter glutamate will be described.

1.4.1. Protein-Protein Interaction Domains

Several modular protein domains specialized for the specific recognition and binding of other proteins have been discovered. The PDZ (PSD-95/SAP90, Dlg, ZO-1) domain consists of about 90 amino acids and is viewed as a protein interaction module that recognizes a short C-terminal consensus sequence of target proteins (Kornau et al., 1997). The motif consists of six β-strands and two α-helices, and the three or four C-terminal residues of the interacting protein bind to a hydrophobic groove in the domain (Doyle et al., 1996). Three different C-terminal consensus motifs for PDZ domain ligands have been defined: Class I (-S/T-X-Φ), class II (-Φ-X-Φ), and class III (-E/D-X-W-C/S), whereas Φ stands for a hydrophobic residue (Maximov et al., 1999; Songyang et al., 1997). In addition to their interaction with the C termini of binding partners, PDZ domains also can associate with other PDZ domains (Brenman et al., 1996a). The SH3 (Src homology type 3) domain was defined as a signaling module in Src tyrosine kinase and was later identified in many other proteins. The domain consists of approximately 60 amino acids and binds short proline-rich peptides with the consensus sequence -P-X-X-P-. The GuK (guanylate kinase) domain is homologous to yeast GuK and is present in various membrane-associated proteins. It is supposed that several short amino acid repeats in the interacting protein are essential for binding to GuK domains. The C1 (protein kinase C, conserved domain 1) domain binds phorbol esters and diacylglycerol, whereas the C2 (protein kinase C, conserved domain 2) domain binds Ca\(^{2+}\) ions and phospholipids. The SAM (sterile alpha motif) domain appears to form homotypic and heterotypic dimers. The WW domain is a very small module of 35 to 40 residues that binds proline-rich motifs, commonly with the consensus sequences -P-P-X-Y- or -P-P-L-P-. Thus, proline-rich regions can act as binding sites for SH3 and WW domains, but also for PDZ-like domains. Further sites of protein-protein interactions are the CaMKII (Ca\(^{2+}\)/calmodulin-dependent protein kinase) domain, the PBH (piccolo bassoon homology) domain, the Zn\(^{2+}\) finger domain, and the ANK (ankyrin) repeat.
1.4.2. The CAZ of Chemical Synapses

The terminals of presynaptic neurons need to synthesize and store neurotransmitters, and then secrete them in a regulated manner upon arrival of an action potential. The CAZ is thought to play an essential role in the organization of the SV cycle. Various structural and functional compartments can be distinguished in axon terminals of conventional CNS synapses (Dresbach et al., 2001; Garner et al., 2000a; Pieribone et al., 1995). These include the active zone, the SV domain, and the mitochondrial domain (figure 1.4).

The active zone is defined as the region of the presynaptic membrane where regulated neurotransmitter release takes place. Like the PSD, the active zone plasma membrane is characterized by an electron-dense projection at the ultra-structural level (Peters et al., 1991). Filamentous strands originate at the active zone membrane and extend into the nerve terminal (Landis et al., 1988). This cytomatrix is believed to represent the machinery which guides SVs to the plasma membrane and is necessary for regulated exocytosis and endocytosis of SVs. It is hypothesized that a class of proteins, the SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) complex, is the basic machine to control docking and fusion of SVs (Bajjalieh, 1999). The SV domain can be divided into three functionally different subpools (figure 1.4): The release-ready pool which contains the fusion-competent vesicles (about 50 nm in diameter) docked at the active zone plasma membrane; the proximal pool which includes two to four vesicle layers and consists of SVs that appear to be clustered near the active zone via filamentous structures of the CAZ; and the reserve pool which stores SVs more than 200 nm away from the active zones by binding to the cytoskeleton (mainly actin filaments) through members of the synapsin family (Dresbach et al., 2001; Garner et al., 2000a; Hirokawa et al., 1989; Pieribone et al., 1995). The mitochondrial domain is a common feature of presynaptic nerve terminals (Peters et al., 1991). In addition to ATP provision as energy supply and regulation of the SV cycle, mitochondria may play also a role in synaptic Ca\textsuperscript{2+} homeostasis and neurotransmitter synthesis (Melamed-Book and Ramimoff, 1998).

Figure 1.4: Schematic illustration of the structural organization of a presynaptic nerve terminal. The presynapse can be divided into mitochondrial domain, SV domain consisting of release-ready pool, proximal pool and reserve pool, and active zone. Upper inset: SVs in the large reserve pool (R) are tethered to microfilaments by short fine filaments composed of synapsin. Lower inset: A smaller proximal pool (P) of SVs is imbedded in a meshwork of fine filaments associated with the presynaptic plasma membrane at neurotransmitter release sites (CAZ). SVs of the release-ready pool are physically docked (D) with the active zone membrane. They are closely associated with voltage-gated Ca\textsuperscript{2+} channels. After fusion (F) and neurotransmitter release, SV proteins are recycled via clathrin (T)-mediated endocytosis (E). Modified from Dresbach et al., 2001, and Garner et al., 2000a.
Recent studies have led to the characterization of four proteins that are specifically localized at the active zone and thus are putative molecular components of the CAZ (figure 1.5): Munc13 (mammalian uncoordination mutant), RIM (Rab3-interacting molecule), bassoon and piccolo/aczonin. In addition, a number of cytoskeletal and cytoskeletal-associated proteins are present in the CAZ, the distribution of which is not restricted to the presynapse. All these proteins of the CAZ contain several domains and are thought to organize the exocytotic and endocytotic machinery precisely at active zones.

Figure 1.5: Molecular model of the structure and interactions of CAZ proteins. The membrane-associated cytoskeleton is thought to provide a scaffold for different classes of proteins involved in SV exo- and endocytosis, as well for ion channels and CAMs. For details see text. Modified from Garner et al., 2000a.

Munc13: Munc13-1 is highly enriched near active zones of rat brain synapses and is a 200 kDa protein that contains one C1 domain which binds phorbol esters and diacylglycerol, and three C2 domains which serve as Ca\(^{2+}\)/phospholipid-binding or protein interaction domains (Brose et al., 1995, 2000). In mammals, Munc13-1 has at least two additional isoforms, Munc13-2 and Munc13-3 (Brose et al., 1995). Experimental evidence suggests that Munc13-1 is involved in regulating SV exocytosis (Betz et al., 1998), as disruption of the Munc13-1 gene dramatically reduced the release of neurotransmitters (Augustin et al., 1999). Munc13-1 is thought to be involved in priming of SVs at the active zone through its interactions with the SV-associated protein Doc2α (double C2α), a Ca\(^{2+}\)/phospholipid binding protein (Orita et al., 1997), and syntaxin, a key component of the exocytotic fusion machinery (SNARE complex) (Betz et al., 1997). An association of Munc13-1 with a brain-specific isoform of β-spectrin may act to anchor this molecule to the CAZ (Sakaguchi et al., 1998). Thus, in interacting with proteins of the plasma membrane, SVs, the cytosol and the cytoskeleton as well with lipids, Munc13 proteins may occupy a central controlling position at the active zone.
RIM: A second CAZ-specific protein is RIM1, a 180 kDa protein containing an N-terminal double Zn\(^{2+}\) finger domain, a PDZ domain and two C-terminal C2 domains (Wang et al., 1997). The ability of RIM1 to bind Rab3 in a GTP-dependent manner, similar to rabphilin, suggests a function in the translocation of SVs from the proximal to the release-ready pool (Wang et al., 1997). Rab3 is an SV-associated protein that may regulate exocytosis by limiting the number of vesicles that can be fused as a function of Ca\(^{2+}\) (Geppert and Südhof, 1998). In addition to Rab3, RIM1 also binds to a new family of proteins, the RIM-BPs (RIM-binding protein) through contact of its proline-rich region situated between the two C2 domains with the SH3 domains of RIM-BPs (Wang et al., 2000). RIM2/oboe, a second member of the RIM family (Fenster et al., 2000), as well as shorter RIM isoforms containing only the C-terminal C2 domain (named NIM2 and NIM3) are also supposed to be involved in the regulation of neurotransmitter release (Wang et al., 2000). In general, RIMs could act as scaffolding proteins at the active zone plasma membrane.

Bassoon and piccolo/aczonin: These proteins are characterized by their enormous sizes of 420 kDa and 530 kDa, respectively (Cases-Langhoff et al., 1996; tom Dieck et al., 1998). They are restricted to the CAZ within the nerve terminals of both excitatory and inhibitory synapses throughout the CNS, but are absent in neuromuscular junctions (Fenster et al., 2000). Bassoon and piccolo are structurally related proteins and share ten regions of high homology called PBH domains including two double Zn\(^{2+}\) fingers at their N termini and three potential coiled coil-forming regions. At the C terminus the two molecules diverge. Whereas bassoon contains a region with limited homology to synapsin, piccolo contains a PDZ and two C2 domains. For piccolo, whose chicken and mouse homologs were named aczonin (Wang et al., 1999), two potential interacting partners have been identified. The Zn\(^{2+}\) fingers have been found to bind PRA1 (prenylated Rab acceptor protein), an SV-associated protein (Fenster et al., 2000; Martincic et al., 1997). This interaction might play a role in the maturation of SVs as they pass through the proximal pool to the release-ready pool. The second binding partner identified for piccolo is profilin, a small G-actin-binding protein involved in the regulation of the dynamics of the actin cytoskeleton (Wang et al., 1999). Bassoon and piccolo/aczonin are likely to be scaffolding proteins of the CAZ that may serve in assembling the machinery for neurotransmitter release. Due to their large sizes, bassoon as well as piccolo/aczonin reach out into the distal pool of SVs. This raises the possibility that the high-molecular weight proteins of the CAZ may coordinate events occurring both in the proximal SV pool and at the transition zone between reserve and proximal pools.

Other components: A number of additional proteins that are, however, not CAZ-specific have been identified as being involved in the organization of the active zone. These include classical cytoskeletal proteins like fodrin (or brain spectrin), actin and myosin (Hirokawa et al., 1989), as well as members of the MAGuK (membrane-associated GuK) superfamily. The MAGuKs are composed of one or more PDZ domains, an SH3 domain and a GuK domain (Fanning and Anderson, 1999; Fujita and Kurachi, 2000; Garner et al., 2000b). They are involved in the assembly and organization of a variety of cell junctions including synapses. Three MAGuKs, PSD-95/SAP90 (synapse-associated protein) (Cho et al., 1992; Kistner et al., 1993), SAP97/hDlg (human homolog of Dlg) (Lue et al., 1994; Müller et al., 1995) and CASK (CaMK/SH3/GuK domain protein) (Hata et al., 1996), are found in presynaptic boutons. A trimeric complex consisting of CASK and the proteins Veli (vertebrate LIN-7 homolog) and Mint1 (Munc18-interacting protein) may play an essential organizing role in the presynapse (Butz et al., 1998). Both voltage-gated Ca\(^{2+}\) channels
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and the CAM β-neurexin are in contact with this complex via CASK (Hata et al., 1996; Maximov et al., 1999). These interactions may serve several functions. On the one hand, β-neurexin binds the postsynaptic CAM neuroligin (Ichtchenko et al., 1995, 1996), which in turn is linked to the postsynaptic cytomatrix by members of the PSD-95 subfamily of MAGuKs (Irie et al., 1997) suggesting that this complex is involved in the alignment and/or physical attachment of pre- and postsynaptic membranes. On the other hand, the trimeric CASK/Veli/Mint1 complex may be involved in coupling voltage-gated Ca\(^{2+}\) channels to the SV fusion apparatus.

The current concept of CAZ function includes the idea that a core of matrix proteins recruits a set of otherwise broadly distributed proteins to synapses. The identification of Munc13, RIM, bassoon, piccolo/aczonin and CASK probably represents only the tip of the iceberg with respect to the complexity of the protein components of the CAZ. Summarized, it can be supposed that CAZ proteins act as adapters that connect CAMs, ion channels/receptors, the cytoskeleton and components of the neurotransmitter release machinery. These interactions may hold the active zone in register with the postsynaptic reception apparatus, may regulate the recruitment of SVs to the release-ready pool, and may restrict neurotransmitter release to active zones.

1.4.3. The PSD of Chemical Synapses

The postsynaptic cell has structural specializations that allow it to sense the presence of neurotransmitters secreted from the presynaptic cell, namely the neurotransmitter receptor proteins that are highly concentrated at the portion of the postsynaptic membrane directly opposite the presynaptic terminal. A characteristic submembraneous structure, called PSD, is responsible for the organization of the postsynaptic reception apparatus (Kennedy, 1997). Many components of the density remain to be characterized, but it is known that it contains cytoskeletal and regulatory proteins, some of which contact the cytoplasmic domains of ion channels in the postsynaptic membrane (Ziff, 1997). This location places the PSD directly in the path of the intracellular ionic fluxes and second messenger cascades generated by neurotransmitters.

The PSD has a diffuse cytoplasmic boundary which suggests a fibrillar structure, and filamentous components are visible on electron micrographs (Peters et al., 1991). The cytoskeleton is composed of proteins like actin, α-actinin-2, or fodrin. As shown in figure 1.6, two families of multi-domain proteins likely form two different levels of a framework within the PSD (Garner and Kindler, 1996; Garner et al., 2000b; Sheng, 2001). The PSD-95 subfamily of MAGuKs connect NMDA (N-methyl-D-aspartate)- and kainate-subtypes of GluRs (glutamate receptor), K\(^{+}\) channels, and CAMs, as well as components of signal transduction pathways. The shanks/proSAPs (SH3 domain and ANK repeat-containing protein/proline-rich SAP), in turn, are supposed to constitute the master scaffold of the PSD, holding together the different types of GluR complexes of the postsynapse and linking them to the cytoskeleton. The two levels are connected by SAPAPs/GKAPs (SAP90/PSD-95-associated protein/GuK-associated protein). Although the AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionat)-subtypes of GluRs may physically interact with the MAGuK-shank/proSAP meshwork through GRIP/ABP (GluR-interacting protein/AMPA receptor-binding protein), they appear to be less tightly linked to the PSD core than the NMDA receptors. In addition, at other synapses different sets of proteins appear to be involved
in orchestrating the synaptic clustering of neurotransmitter receptors. At the vertebrate neuromuscular junction, the 43 kDa protein rapsyn seems to cluster acetylcholine receptors (Apel and Merlie, 1995), and at inhibitory synapses in the CNS, the 93 kDa protein gephyrin directly interacts with glycine receptors (Kuhse et al., 1995).

Figure 1.6: Molecular model of the structure and interactions of PSD proteins. Frameworks of MAGuKs and shanks/proSAPs are linked by proteins of the SAPAP/GKAP family. Together they have the potential to tether all types of GluRs, CAMs, signaling components and other adapter proteins to the postsynaptic membrane and link the PSD complex to the cytoskeleton. For details see text. Modified from Garner et al., 2000b.

**GluRs:** A neurotransmitter reception apparatus is located in the postsynaptic membrane, exactly opposite the transmitter release site. Glutamate, the principal excitatory neurotransmitter in the mammalian brain, activates both mGluRs (metabotropic GluR), which mediate transmembrane signal transduction via G-proteins, and iGluRs (ionotropic GluR), which are coupled directly to an intrinsic neurotransmitter-gated cation channel (Ozawa et al., 1998). Eight mGluRs have been cloned termed mGluR1-8. The receptor contains seven membrane-spanning domains. The binding site for the neurotransmitter is just within the lipid bilayer on the extracellular surface of the cell, and the G-protein associates with intracellular loops of the receptor molecule. iGluRs can be subdivided into three categories on the basis of pharmacological and molecular criteria (Seeburg, 1993). The members of the first class, termed AMPA receptors, activate channels with fast kinetics and are primarily permeable for Na⁺ ions. AMPA receptors are probably tetrameric complexes of four homologous subunits termed GluR1-4 that assemble in varying combinations to form functional channels (Rosenmund et al., 1998). The second class of iGluRs, the NMDA receptors, are activated only after partial depolarization of the postsynaptic membrane (e.g. as a result of the previous activation of AMPA receptors) and conduct primarily Ca²⁺ ions. NMDA receptors are also multimeric complexes of homologous subunits termed NR1 and NR2A-D. The third class are named kainate receptors after their preferred ligand (Frerking and Nicoll, 2000). The five known subunits are encoded by two gene families, GluR5-7 and KAI-2, but their role in excitatory synaptic transmission is less clear to date. The structure of the iGluR subunits is conserved throughout
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the three classes, including a large extracellular N-terminal domain that binds the ligand, four transmembrane domains and an intracellular C-terminal regulatory domain. The four types of GluRs differ in their subsynaptic distribution, i.e. mGluRs are located at the periphery of the PSD, whereas NMDA and AMPA receptors occupy a central location. This suggests that distinct mechanisms underlie the subsynaptic distribution of each GluR. Recent studies demonstrated that the NMDA receptor can interact directly with the actin cytoskeleton through the protein α-actinin-2 (Wyszynski et al., 1997). Another possibility to tether ion channels to the PSD is mediated by PDZ domain-containing proteins (Bolton et al., 2000; O’Brien et al., 1998).

MAGuKs: In mammals, four members of the PSD-95 subfamily of MAGuKs have been detected in the postsynaptic terminal: PSD-95/SAP90 (Cho et al., 1992; Kistner et al., 1993), SAP97/hDlg (Lue et al., 1994; Müller et al., 1995), chapsyn110/PSD-93 (channel-associated protein of synapses) (Brenman et al., 1996b; Kim et al., 1996) and SAP102 (Müller et al., 1996). They all are multi-domain proteins consisting of three tandem PDZ domains, an SH3 domain and an inactive GuK domain (Fujita and Kurachi, 2000). Each of these domains is a site of protein-protein interaction and seems to play a role in the localization of ion channels and adhesion molecules, and in the assembly of synaptic junctions (Craven and Bredt, 1998; Garner et al., 2000b; Kirsch, 1999). First clues to the function of PSD-95 family members in the structural organization of the PSD came from the observation that their PDZ domains can interact with the C termini of NR2 and certain splice variants of the NR1 subunits of NMDA-type GluRs, and of voltage-gated K⁺ channels (Kim et al., 1995; Kornau et al., 1995; Müller et al., 1996). Another study showed that kainate receptor subunits GluR6 and KA2 can be bound and clustered by MAGuKs via their PDZ domains or their SH3 and GuK domains, respectively (Garcia et al., 1998). Moreover, SAP97/hDlg specifically interacts through a PDZ domain with GluR1 subunits of AMPA receptors (Leonard et al., 1998). In addition, PSD-95/SAP90 may function to appose postsynaptic NMDA receptors to presynaptic terminals by interacting with neuroligins (Irie et al., 1997). Many studies have revealed a number of additional interaction partners for PSD-95 family members, including cytoskeletal elements. For example, PSD-95 family members may be linked to microtubules via the microtubule-associated protein CRIPT (cysteine-rich interactor of PDZ three) (Niethammer et al., 1998) or via MAP1A (microtubule-associated protein) (Brenman et al., 1998) which bind to the PDZ3 and the GuK domains, respectively, or to the actin cytoskeleton via a PSD-95/SAPAP/shank/cortactin complex (Naisbitt et al., 1999). Various proteins that are involved in the intracellular signaling stimulated by GluR activation, including nNOS (neuronal nitric oxide synthase) (Brenman et al., 1996a) and SynGAP (synaptic GTPase-activating protein) (Kim et al., 1998), also interact with PSD-95 family members. In summary, MAGuKs are multi-domain adapter proteins that directly interact with synaptic membrane proteins and play an important role in organizing subcompartments within the PSD. Cluster formation is driven by the ability of some PSD-95 family members to homomultimerize by their unique N-terminal domains (Hsueh et al., 1997) or to heteromultimerize with each other through their PDZ domains (Kim et al., 1996), and to be anchored to the plasma membrane by the palmitoylation of a pair of N-terminal cysteine residues (Topinka and Bredt, 1998). Another member of the MAGuK superfamily is S-SCAM (synaptic scaffolding molecule). Compared with PSD-95 family members, the structure of S-SCAM is inverted, having the GuK domain at the N terminus, followed by two WW and five PDZ domains (Hirao et al., 1998). This protein binds with the PDZ5 domain to NR2 subunits of NMDA receptors and with the PDZ1 domain to neuroligins. It further interacts with SAPAP/GKAP.
through the GuK domain. S-SCAM may therefore assemble receptors and CAMs at synaptic junctions.

Shanks/proSAPs: A second group of proteins that are localized in the PSD were discovered by several groups and are hence known under many different names, such as shanks, proSAPs, CortBP (cortactin-binding protein), SSTRIP (somatostatin receptor-interacting protein) and synamon (Sheng and Kim, 2000). The family contains at least three members, and the proteins are composed of multiple structural domains (in N- to C-terminal order): Multiple ANK repeats, an SH3 domain, a PDZ domain, a long proline-rich region and a SAM domain. All three shanks/proSAPs have been located in the PSD of excitatory synapses, although the expression of at least shanks 2 and 3 is not restricted to the nervous system (Sheng and Kim, 2000). Via their PDZ domains, all three shanks/proSAPs can bind to the C termini of SAPAPs/GKAPs, which in turn bind to the GuK domain of MAGuKs (Naisbitt et al., 1999; Yao et al., 1999). There have been found two further binding partners for the PDZ domain, namely the somatostatin receptor 2 (Zitzer et al., 1999) and a receptor for the spider toxin α-latrotoxin (Kreienkamp et al., 2000; Tobaben et al., 2000). One of the proline-rich elements of shank 2 has been shown to bind to the SH3 domain of the actin-binding protein cortactin, thus linking shanks/proSAPs to the actin-based cytoskeleton (Du et al., 1998). Homer is a small protein that binds with its single, PDZ-like domain to proline-rich elements of shanks/proSAPs (Tu et al., 1999), but also to the C termini of GluRs (Brakeman et al., 1997). Through homo- and heteromultimerization through its C-terminal coiled coil structure, homer is thus able to connect mGluRs to the protein scaffold (Xiao et al., 1998). Further, the AMPA-type GluR-interacting protein GRIP/ABP has been suggested to bind the SH3 domain of shanks/proSAPs (Sheng and Kim, 2000). Finally, the SAM domain is thought to be responsible for multimerization of the proteins (Naisbitt et al., 1999). The fact that shanks/proSAPs interact with SAPAP/GKAP, homer and GRIP/ABP suggests that shanks/proSAPs are a master scaffold holding together the NMDA receptor, mGluR and AMPA receptor complexes in the postsynaptic specialization.

SAPAP/GKAP: The SAPAPs compromise a family of abundant PSD proteins also known as GKAPs or DAPs (Dlg-associated protein). These proteins bind directly to the GuK domains of the postsynaptic scaffolding proteins PSD-95/SAP90 (Kim et al., 1997; Takeuchi et al., 1997) and S-SCAM (Hirao et al., 1998). With their C termini, SAPAPs/GKAPs bind to the PDZ domains of shanks/proSAPs; this interaction is important in neurons for the synaptic localization of shanks (Naisbitt et al., 1999). Thus, SAPAPs/GKAPs may maintain the structure of the PSD by concentrating its components to the membrane area.

GRIP/ABP and PICK: Although largely colocalized with NMDA receptors at glutamatergic synapses, AMPA receptors interact with a different complement of intracellular proteins. The first such protein identified was a seven PDZ domain-containing protein termed GRIP1 (Dong et al., 1997). GRIP1, the closely related GRIP2 (Dong et al., 1999), and the GRIP2 splice variant lacking the seventh PDZ domain and referred to as ABP (Srivastava et al., 1998) together form a novel non-MAGuK family of neuronal adapter proteins. The fourth and fifth PDZ domains of GRIP/ABP are involved in binding to the C-terminal tails of AMPA receptor subunits GluR2 and GluR3. Furthermore, GRIP/ABP interacts with ephrin receptors through PDZ6 (Brückner et al., 1999; Torres et al., 1998), whereas PDZ7 binds to GRASP1 (GRIP-associated protein), a novel
neuronal rasGEF (GDP-GTP exchange factor) (Ye et al., 2000). This would bring the ephrin receptor into close proximity with a rasGEF and therefore potentiate the activation of ras. Thus GRIP/ABP is likely an adapter protein that physically and functionally couples AMPA receptors to a variety of cytoskeletal and signaling molecules. It is currently unclear how GRIP/ABP is tethered into the complex of postsynaptic scaffolding proteins, but it is supposed that it interacts with the SH3 domain of shank/proSAP proteins (Sheng and Kim, 2000). PICK1 (protein interacting with C kinase) is another non-MAGuK scaffolding protein that binds AMPA receptor subunits (Staudinger et al., 1995). It contains a single PDZ domain that interacts with the C-terminal tails of GluR2 and GluR3, followed by a central coiled coil domain thought to mediate dimerization. As PICK1 promotes the clustering of GluR2 subunits in the plasma membrane of transfected cells, a possible role in regulating the synaptic clustering of AMPA receptors can be suggested (Xia et al., 1999).

As a result of their modular organization, all these proteins are ideally suited for generating protein scaffolds that couple neurotransmitter receptors to their associated secondary signaling machinery and anchor CAMs at sites of synaptic contact. The cytoskeleton might play a fundamental role in the organization, structure and function of synaptic junctions.

1.4.4. Cell-Adhesion Molecules at the Synapse

The remarkable features of information-processing performed by the brain are determined to a large extent by the complicated network of connections between the neurons. There are two mechanisms by which cells communicate with one another: Signaling by secreted molecules or signaling by membrane-bound molecules. In addition to being a site of cell-to-cell communication, the synapse is also a site of cell-cell adhesion, having many of the properties associated with other cell-cell junctions. The principal difference is that most junctions are functionally symmetric, joining identical cell types across the same cellular domains, whereas synapses are polarized, most often joining functionally distinct cellular domains: Axon to dendrite or soma. The synaptic contact includes a narrow space between the presynaptic and postsynaptic cells, i.e. the synaptic cleft. The cleft is slightly wider than the gap between adjacent apposed membranes and is filled with electron-dense material. CAMs play many roles in the nervous system including neuronal cell migration, neurite outgrowth, axonal fasciculation, pathfinding, target recognition, and also synapse formation (figure 1.7). In this section, classes of CAMs will be described that were localized to the synapse and which presumably are relevant in target finding and synaptic stabilization/modulation.
CAMs are required both for building and maintaining synaptic structures during brain development (synaptogenesis), but also play important roles in modulating synaptic strength in maturity (synaptic plasticity), a key feature of the cellular changes thought to underlie learning and memory as well as recovery of function after brain injury (Benson et al., 2000; Fields, 1998; Fields and Itoh, 1996). How could changes in adhesive strength affect synaptic physiology? There are several possibilities: (i) The distance between pre- and postsynaptic membranes might be altered. This could affect the glutamate concentration in the cleft, which is increased at potentiated synapses. (ii) The size of the active zones in the pre- and postsynaptic membranes might be altered. This could affect the density, compartmentation or composition of postsynaptic GluRs. (iii) Adhesion molecules could directly modulate GluR channel properties. (iv) The extent to which astrocyte processes surround the edges of the synapse might be altered by changes in the strength of adhesion between neuronal and glial membranes. This could modify the rate of glutamate re-uptake from the synaptic cleft by affecting the density or proximity of glutamate transporters which are localized predominantly to the astrocytic processes. (v) Finally, altered outside-in signaling by adhesion proteins could produce rapid effects on other signaling pathways.

Formation of new synaptic sites as well as the loss of old ones occurs throughout life and represents another aspect of synaptic plasticity in which synaptic communication is modified for longterm periods (Purves and Lichtman, 1980; Vaughn, 1989). Migration of the neuron into the target area, genesis of the growth cone and directed axon extension together restrict the array of neurons that can be targets for an individual cell during synaptogenesis. In principle, the synapse-building process can be divided into two steps (Haydon and Drapeau, 1995; Verderio et al., 1999). First, the arriving axonal growth cone identifies its appropriate partner cell and creates an initial contact. This process is extremely specific as any given axon makes contacts to only a selected group of target cells, and it is most likely mediated by CAMs (Brose, 1999; Ruegg, 2001). In the second phase of synaptogenesis, recruitment of presynthesized axonal and dendritic protein components leads to the formation of a synapse at the site of initial contact.
Different types of CAMs must be present to mediate various levels of synaptic specificity: CAMs identify target cells, determine pre- and postsynaptic compartments, and influence synapse strength, size, and stability. Several groups of CAMs are specifically enriched at CNS synapses: Integrins, members of the Ig (immunoglobulin) superfamily, the cadherin superfamily including classic cadherins and CNRs (cadherin-related neuronal receptor), and β-neurexins/neuroligins (figure 1.8). Within each of these adhesion molecule families, membership has been defined largely by amino acid sequence similarity, which is reflected in common structural features. With the exception of integrins, which bind also to the ECM (extracellular matrix), CAMs bind through homophilic or heterophilic interactions to molecules on adjacent cell membranes. The intracellular domains of these CAMs interact with the cytoskeleton and with molecules of signal transduction pathways.

Figure 1.8: Schematic structures of CAMs at the synapse. Four groups of CAMs have been localized to CNS synapses, integrins, members of the Ig superfamily, members of the cadherin superfamily, and β-neurexins/neuroligins. Integrins composed of various α and β subunits bind to a variety of different ECM components and to cadherins or Ig superfamily CAMs. Some members of the Ig superfamily can bind homophilically, while others are heterophilic. Cadherins are Ca\(^{2+}\)-dependent homophilic adhesion molecules. Neuroligins and β-neurexins have been shown to interact with each other. From Benson et al., 2000.

1.4.4.1. Integrins

Integrins make up a large family of heterodimeric glycoproteins consisting of two non-covalently associated subunits, α and β (Hynes, 1992). Each subunit contains a large extracellular domain required for dimerization, a transmembrane segment, and a small, cytoplasmic tail (figure 1.9). The α subunits vary in size between 120 and 180 kDa, the β subunits have about 90-110 kDa. The α subunits contain three or four Ca\(^{2+}\)-binding regions in the extracellular domain, and in some, two disulfide-linked heavy and light chains constitute a single subunit. Characteristic of all β subunits is a four-fold repeat of a cysteine-rich segment believed to be internally disulfide-bonded. Analyses of chimeric integrin receptors or mutant α and β subunits have indicated that the β cyto-
plasmic domains are necessary and sufficient to target integrins to subcellular sites in a ligand-independent manner, whereas the $\alpha$ cytoplasmic domains regulate the specificity of the ligand-dependent interactions (Sastry and Horwitz, 1993).

Integrin receptors on cell surfaces mediate cell-ECM and cell-cell adhesion. The extracellular domains of both subunits together form the ligand-binding site, which, for many integrins, recognizes a sequence -R-G-D- found in many matrix proteins (Ruoslahti and Pierschbacher, 1987). However, not all integrins bind to ligands via -R-G-D-containing domains. Although the classic integrin interaction is to join cells with substrates such as fibronectin, laminin and tenascin, integrins can also function in cell-cell adhesion through cadherins and members of the Ig superfamily of adhesion molecules (Higgins et al., 1998; Staunton et al., 1988). The extracellular domain is followed by a single transmembrane segment and an intracellular C-terminal domain which is involved in interactions with the cytoskeleton through binding to actin via talin, $\alpha$-actinin, tensin or vinculin. These assemblages of structural proteins are believed to play important roles in stabilizing cell adhesion and regulating cell shape, morphology, and mobility. They may also serve as a framework for the association and activation of signaling proteins like FAK (focal adhesion kinase) that regulate signal transduction pathways leading to integrin-induced changes in cell behaviour (Clark and Brugge, 1995).

In mammals, at least eight $\beta$ integrin subunits have been described so far, and each of them associates with a certain subset of the 17 or more $\alpha$ subunits. Alternative splicing of the $\alpha$ and $\beta$ subunits adds additional complexity (Hynes, 1992). This suggests that the interactions
within the integrin family must be very complex because of the number of possibilities of linking a
given \( \alpha \) with any \( \beta \) subunit to make a functional cell-surface integrin (Jones, 1996). Each of the
five subunits \( \beta_1-\beta_8 \), for example, might bind only to a single \( \alpha \) subunit, while \( \beta_1 \) binds to at least
nine different \( \alpha \) subunits. All of these combinations of \( \alpha \) and \( \beta \) subunits form a functional integrin
that then binds fairly specifically to certain molecules of the ECM - such as laminin in the case of
\( \alpha_6\beta_4 \) - or to some other adhesion molecules. Some integrins, like \( \alpha_3\beta_1 \), are able to recognize sev-
eral ECM ligands, but most appear to bind preferentially to particular ECM molecules.

Integrin expression appears to be universal. At least one member of the integrin family has
been found on every cell or tissue studied (Hynes, 1992). There is an extensive literature on the
importance of integrins in neural development and cancer, but evidence for their existence in the
adult CNS has emerged only recently (Jones, 1996). Perhaps the most exciting contribution that
integrins make to the function of the adult CNS results from their possible involvement in synaptic
plasticity (Benson et al., 2000; Fields, 1998). In 1990 Staubli et al. published a pioneering paper
which demonstrated that infusion of -R-G-D-containing peptides into hippocampal slices disrupted
LTP (long-term potentiation), an activity-dependent, long-lasting increase in synaptic transmission
that is thought to be the cellular basis for certain types of learning and memory (Malenka and
Nicoll, 1999). At least some members of the large family of integrins are present in the adult brain
and are differentially localized (Paulus et al., 1993). EM has shown that \( \alpha_8 \) and \( \beta_8 \) are concentrated
at some postsynaptic densities, thus suggesting that integrins may play a role in the maintenance,
formation and/or plasticity of subsets of central synapses (Einheber et al., 1996; Nishimura et al.,
1998).

1.4.4.2. Immunoglobulin Superfamily

The largest class of Ca\(^{2+}\)-independent CAMs are members of the Ig superfamily. They are
either type I or GPI (glycosylphosphatidylinositol)-linked membrane proteins and are defined by
regions that have sequence similarity with Igs. Such Ig-like domains contain a unique barrel
structure composed of two opposing \( \beta \) sheets which are stabilized by a disulfide bond between
them. There are three subclasses of Ig-like domains which are defined by their sequence similarity
to variable (V) or constant (C) regions of Igs (Walsh and Doherty, 1997). For V-like domains,
there are 70-110 amino acids spanning the two cysteines that form the disulfide bond, allowing
formation of seven to nine \( \beta \) strands. C-like domains have about 50 amino acids spanning the sta-
bilizing cysteines and, consequently, carry seven \( \beta \) strands. The third class of Ig-like domains is
termed a C2 domain. This class has the \( \beta \) strand distribution of a C-like domain but bears more
sequence similarity to V-like domains. Members of the Ig superfamily of adhesion molecules may
have only one Ig-like domain, as is the case for the myelin protein P\(_0\), or, as for most of the family,
have many Ig domains (figure 1.10). Ig family members are subdivided into three groups (Uye-
mura et al., 1996): (1) Simple types that have only Ig-like domains, e.g. P\(_0\), Thy-1 or MAG (mye-
lin-associated glycoprotein); (2) complex types that have Ig domains and FNIII (fibronectin type
III) repeats, e.g. L1 or NCAM (neural CAM); and (3) mixed types that have Ig domains and motifs
other than FNIII repeats, e.g. the C2 protein that has a kinase domain. For many of these mole-
cules, multiple isoforms which differ in the length of the cytoplasmic domain or in their post-
translational modifications, or whether they are membrane-spanning or GPI-anchored, are known.
Furthermore, alternative splicing in the extracellular domain may distinguish additional isoforms of a particular molecule. All these isoforms can have distinct spatial or temporal expression patterns during development.

Figure 1.10: Schematic structures of some members of the Ig superfamily of adhesion molecules in the nervous system. Several varieties of Ig domain-containing molecules are contained within this gene family. Therefore, the proteins can be subdivided into three groups: Simple type, complex type and mixed type (see text). Most are type I membrane proteins, some are anchored to the membrane by GPI. From Colman and Filbin, 1999.

Most members have preferences for either homophilic or heterophilic interactions (e.g. with integrins or other Ig superfamily proteins), but many can engage in both, and the strength of adhesion varies widely. Studies investigating the structure/function relationships of these proteins have highlighted the importance of Ig-like domains near the N terminus to be both necessary and sufficient for binding (Brümmendorf and Rathjen, 1996). The cytoplasmic domains of some can interact with cytoskeletal proteins (Davis and Bennett, 1994) or activate signal transduction cascades (Walsh and Doherty, 1997). In this way adhesion is transduced into an intracellular response. With respect to homophilic adhesion of members of the Ig superfamily, the cytoplasmic domain does not seem to be essential.

CAMs of the Ig superfamily, including NCAM, L1 and Thy-1, are involved in both synaptogenesis and synaptic plasticity (Fields, 1998; Fields and Itoh, 1996). NCAM and L1 were shown to modulate the development or the stabilization of LTP in hippocampal slices (Lüthi et al., 1994). Interestingly, fasciclin II, a Drosophila homolog of NCAM, is present both pre- and postsynaptically (Schuster et al., 1996). Fasciclin II is thought to affect synapse formation and stabilization at neuromuscular junctions by homophilic transsynaptic binding followed by intracellular interaction with the PDZ-domain protein Dlg (Drosophila discs large), a PSD-95/SAP90 homolog (Thomas et al., 1997). However, NCAM lacks a consensus sequence for PDZ-domain binding. It is therefore likely that the function of fasciclin II is not completely conserved in mammals, but NCAM is supposed to be necessary for synaptogenesis and functional plasticity (Walsh and Doherty, 1997). Addition of PSA (polysialic acid) residues, long polymers of negatively-charged sialic acids, to NCAM interferes with homophilic binding interactions through steric interference.
It is thought that PSA-NCAM represents a less-adhesive form of NCAM, one that would be appropriate for facilitating the synaptic reorganization that occurs during brain development and under conditions of synaptic plasticity in maturity (Rutishauser, 1998). In support of this, PSA expression is more widespread throughout the brain during development, where it plays an essential role in a variety of events. By adulthood, PSA is largely absent in the brain, but expression remains in certain brain regions characterized by ongoing neuronal and synaptic plasticity. Another study demonstrated that when PSA was removed by enzymatic treatment, NCAM-dependent adhesion was increased and synaptic plasticity was not possible (Muller et al., 1996).

1.4.4.3. Cadherin Superfamily

At least 80 different cadherins are expressed within a single mammalian species. The cadherin superfamily includes classic cadherins, protocadherins, desmosomal cadherins and cadherin-related proteins (figure 1.11). All cadherins are single-pass type I transmembrane proteins mediating strong, Ca²⁺-dependent cell-cell adhesion (Angst et al., 2001; Brusés, 2000). They are defined as proteins that contain multiple EC (extracellular) repeats of about 110 amino acids. This motif consists of seven β strands connected by a short linker sequence, very similar to the Ig motif. The EC repeat most distal from the transmembrane domain mediates the principal adhesion between cadherin molecules. Most interactions are homophilic, but weak heterophilic binding, both with other cadherins or with other adhesion proteins such as integrins, can also occur in selected cases (Higgins et al., 1998). The amino acid sequences of the cytoplasmic domains significantly diverge between the subfamilies. Proteins that bind to the cytoplasmic domain of cadherins interact with cytoskeletal proteins and signal transduction pathways to regulate cell adhesion. Cadherins are expressed by almost all cell types where they are enriched in cell-cell junctions of various types. They are essential for cell adhesion during development as well as in mature tissues.

Cadherins exist in a monomeric form that is weakly adhesive and in a cis strand-dimeric form that is strongly adhesive (Colman, 1997; Hagler and Goda, 1998). The cis dimer orients the adhesive interfaces in the two proteins such that they can interact with identical dimers on the opposite membrane surface, thus creating a linear zipper of tightly adherent cadherin molecules. The two forms are in a dynamic equilibrium. It is speculated that the monomeric form is extensively expressed over the cell surface of outgrowing neurons and growth cones. The weak interaction between the monomers allows dynamic interactions between cells. When appropriate cellular targets are recognized by the growth cone, the monomers provide a rapidly accessible and locally recruitable pool for the cis strand-dimer configuration. The strong adhesion mediated by trans strand-dimers lock in and stabilize pre- and postsynaptic membranes. It may be expected that a variety of different cadherins will be functional at different types of synapses.
Classic cadherins, including neural (N-), epithelial (E-), placental (P-), retinal (R-), and vascular endothelial (VE-) cadherins, are composed of a large N-terminal extracellular domain containing five EC repeats, a single transmembrane domain, and a highly conserved C-terminal cytoplasmic region. With few exceptions classic cadherins interact homophilically, preferentially binding to proteins of the same subtype. Strength of adhesion is modulated by the cytoplasmic tail through regulation of lateral clustering by interactions with catenin p120 and δ-catenin (Yap et al., 1998), and through linkage to actin via β/γ- and α-catenins (Takeichi, 1994). The classic Cdh (cadherin) genes consist of 12-17 exons.

A common feature of the protocadherins are six or seven repeats of the EC domain, but their cytoplasmic regions display no similarity to those of classic cadherins (Sano et al., 1993; Suzuki, 2000). The EC domains of protocadherins can undergo homophilic interactions, as found for those of classic cadherins, but molecules associated with the cytoplasmic region are yet to be identified. CNRs belong to the family of protocadherins and were discovered as direct interactors of Fyn, a non-receptor tyrosine kinase known to be involved in synaptic plasticity and other neural functions (Kohmura et al., 1998). CNRs are composed of six EC domains, a single transmembrane region, and a highly conserved intracellular C terminus that binds to the SH3 domain of Fyn and has no homology with known cadherins. Interestingly, 152 amino acids at the C-terminal tail are
identical in all CNRs. Recent studies have revealed that the protocadherins have a very interesting gene structure (figure 1.12), similar to Ig and T cell receptor genes (Sugino et al., 2000; Wu and Maniatis, 1999, 2000; Yagi and Takeichi, 2000). The protocadherin genes are located in the same region of the genome (chromosome 5 in humans), and they are clustered in three groups namely Pcdha (protocadherin; human CNRs), Pcdhβ and Pcdhγ which contain at least 15, 15, and 22 genes, respectively. Each gene family is composed of multiple large exons which are arrayed in tandem on the chromosome and each of which encodes a similar but non-identical ectodomain (in contrast, the extracellular parts of classic cadherins are composed of more than ten exons). Downstream of this region are three small exons that encode the cytoplasmic domain of the protein, which varies among the protocadherin subfamilies, indicating that the cytoplasmic domains are the same within a particular subfamily, but different between the three groups. To generate mRNA transcripts, one of the large ‘variable’ 5’ exons must be joined to the three small ‘constant’ 3’ exons coding for the cytoplasmic domain (Wu and Maniatis, 1999). The potential diversity of surface receptors provided by the protocadherin family may contribute to the establishment of neuronal connectivity (Shapiro and Colman, 1999; Wu and Maniatis, 1999).

![Figure 1.12: Organization of human protocadherin genes.](image)

In the nervous system, classic cadherins and CNRs are best known for their roles in axon guidance and target recognition. The differential region-specific and cell type-specific expression patterns of certain cadherins was interpreted as a possible molecular basis for the specificity of synaptic connectivity in the brain, where the binding specificity of cadherins expressed on invading growth cones could determine synaptic partner choice (Benson and Tanaka, 1998; Colman, 1997; Serafini, 1997, 1999). Because each cadherin binds primarily to cadherin molecules of the same subtype, the specific combination of cadherins expressed by a neuron may limit its connection to only those target cells expressing an overlapping or identical set of cadherins (Shapiro and Colman, 1999; Uemura, 1998). Several studies have implicated cadherins in synaptogenesis (Brose, 1999; Obst-Pernberg and Redies, 1999). N-cadherin was the first cadherin to be identified within the synaptic cleft (Yamagata et al., 1995). N-cadherin is expressed initially at all synaptic sites but rapidly becomes restricted to a subpopulation of excitatory synaptic sites (Benson and Tanaka, 1998). Interestingly, the cadherin-catenin complexes are not present at the active zone but are localized in areas bordering this zone (Fannon and Colman, 1996; Uchida et al., 1996). In these regions, they exhibit a symmetrical distribution over the pre- and postsynaptic plasma membranes. Moreover, it has been shown that multiple classic cadherins are differentially expressed in
the brain, and specific cadherin expression occurs in the process of delineating restricted neural circuits (Inoue et al., 1998; Suzuki et al., 1997). CNRs were also localized to synaptic junctions, suggesting that they may play another role in establishing neuronal connections or in signal transduction at the synaptic membrane (Kohmura et al., 1998). However, in contrast to classic cadherins, these proteins appear to cover the whole area of synaptic contact including active zones and postsynaptic densities (Kohmura et al., 1998). CNR mRNAs are expressed in various regions of the brain, and different neurons express distinct sets of CNR genes. These properties indicate that CNRs may play a role in the formation of specific neural connections and local circuits in the CNS. Thus, the combinatorial expression of cadherin family members could confer an enormous variation of adhesive affinities on neurons (Fannon and Colman, 1996).

Although cadherins are known to play important roles in cell recognition and adhesion during development, they continue to be expressed in many tissues during adulthood and seem to participate in synaptic plasticity (Benson et al., 2000; Hagler and Goda, 1998). Using antibodies to the extracellular domains of N- and E-cadherins as well as peptides that block cadherin interaction, a striking reduction in LTP in hippocampal slices following disruption of cadherin-mediated cell adhesion has been observed (Tang et al., 1998). Furthermore, high extracellular Ca\(^{2+}\) prevented the block of LTP in these cultures, suggesting that a decrease in Ca\(^{2+}\) and subsequent weakening of cadherin interactions may affect synaptic efficacy. Another recent study has revealed that protein structure and distribution of N-cadherin are changed through synaptic physiology. In the presynaptic membrane, N-cadherin dispersion after presynaptic depolarization parallels the rapid expansion of the presynaptic membrane subsequent to massive SV fusion (Tanaka et al., 2000). Moreover, N-cadherin dimerizes and becomes markedly protease resistant, while other surface molecules are completely degraded. These and other experiments suggest that cadherins are involved in the alteration of synaptic morphology.

1.4.4.4. Neurexins and Neuroligins

All previously described families of CAMs are expressed ubiquitously in almost every organ and tissue studied. In contrast, the neurexins and neuroligins are brain-specific proteins (Missler et al., 1998; Missler and Südhof, 1998). Neurexin I\(\alpha\), the first neurexin identified, was isolated by its ability to bind to immobilized \(\alpha\)-latrotoxin which is a spider venom, and the other neurexins were subsequently discovered by their homologies to neurexin I\(\alpha\) (Ushkaryov and Südhof, 1993; Ushkaryov et al., 1992, 1994). Neurexins are type I membrane proteins encoded by at least three different genes (neurexin I, II, and III), each of which has two independent promoters that generate transcripts for the longer \(\alpha\)-neurexins and the shorter \(\beta\)-neurexins (Geppert et al., 1992). The \(\beta\)-neurexin promoter is nested in an intron of the \(\alpha\)-neurexin gene. This results in the synthesis of six principal neurexin isoforms, called neurexins I\(\alpha\) to III\(\beta\).

All \(\alpha\)- and \(\beta\)-neurexins have the same domain structure (figure 1.13). \(\alpha\)-Neurexins contain an N-terminal signal peptide followed by three overall repeats composed of two similar LNS (laminin, neurexin, sex hormone-binding globulin) domains separated from each other by an EGF (epidermal growth factor)-like sequence. After these three repeats, \(\alpha\)-neurexins contain a sequence that is probably O-glycosylated and a single transmembrane region, followed by a short cytoplas-
mic tail. β-Neurexins are identical with the C-terminal part of α-neurexins but lack the N-terminal five LNS domains and three EGF-like sequences. Instead, the signal peptide of β-neurexin is followed by a short β-neurexin-specific sequence. Consequently, β-neurexins are truncated forms of α-neurexins containing a short unique N-terminal sequence. Most α-neurexins are differentially spliced at five sites (Ullrich et al., 1995; Ushkaryov et al., 1992); only neurexin IIIα does not use splice site 2 (Ushkaryov and Südhof, 1993). The shorter β-neurexins contain only splice sites 4 and 5. Alternative splicing results in the variable addition of extra sequences. At most sites only a few amino acids are added, but some sites (4 and 5) involve insertions of up to 191 residues. Only two or three sequence variants are observed for some splice sites, whereas others occur in more than ten different forms. Interestingly, some of the insert sequences in splice site 5 of neurexins IIIα/β contain stop codons and produce secreted proteins without a transmembrane region (Ushkaryov and Südhof, 1993).

As a consequence of multiple copies of the gene, multiple mRNAs synthesized from independent promoters, and multiple transcripts derived by alternative splicing, neurexins are supposed to occur in thousands of isoforms (Ullrich et al., 1995). Neurexins are detected by Northern blotting only in brain (Ushkaryov and Südhof, 1993; Ushkaryov et al., 1992), and by in situ hybridization only in neurons (Ullrich et al., 1995). The expression pattern of each of the six neurexins is unique, but overlaps with that of other neurexins. The distributions of α- and β-transcripts from the same neurexin gene are also remarkably different, showing that the two promoters in the same gene are regulated independently. Furthermore, at least some sites of alternative splicing are used differentially, suggesting that alternative splicing is regulated at least partly in a manner specific for a given type of neuron (Ichtchenko et al., 1995).

The structures of α- and β-neurexins resemble that of receptors. Their extensive alternative splicing could serve to amplify the number of ligands for neurexins, or to regulate their interactions with different binding partners. Two ligands that exclusively bind to α-neurexins have been discovered: α-Latrotoxin, a neurotoxin that causes massive neurotransmitter release from presynaptic nerve endings (Ushkaryov et al., 1992), and neurexophilin, a small protein which is
expressed in a brain-specific manner only in a subset of neurons and has a possible role as a neuropeptide (Petrenko et al., 1996). Affinity chromatography experiments with total rat brain proteins on immobilized neurexin Iβ led to the discovery of a family of β-neurexin-binding proteins called neuroligins (Ichchenko et al., 1995, 1996). Like neurexins, neuroligins are encoded by at least three genes (neuroligin 1, 2, and 3) and are detectable by Northern blotting only in the brain. In situ hybridization experiments localized them primarily to neurons, but, in contrast to neurexins, the expression patterns of different neuroligin isoforms are overlapping (Scheiffele et al., 2000). All three neuroligins bind to all three β-neurexins, but not to α-neurexins, presumably because the N-terminal sequence unique to β-neurexins is involved in neuroligin binding. Neuroligins, like the neurexins, are subject to alternative splicing, although only at two sites (A and B; splice site B is present only in neuroligin 1). Alternative splicing of neuroligins has no effect on β-neurexin binding (Ichchenko et al., 1995), whereas alternative splicing of β-neurexins tightly regulates their interaction with neuroligins. Neuroligins only interact with β-neurexins that lack an insert in splice site 4, but bind not to β-neurexins that contain an insert in this site (Ichchenko et al., 1995, 1996). Binding of neuroligin to neurexin is Ca²⁺-dependent (Nguyen and Südhof, 1997), and a Ca²⁺-binding EF-hand motif has been identified in the extracellular domain of neuroligin (Tsigelny et al., 2000).

Similar to neurexins, neuroligins form a multigene family of brain proteins that may collaborate with neurexins in mediating cell-cell interactions between neurons. Structurally, neuroligins are type I membrane proteins composed of five domains: An N-terminal cleaved signal peptide, a large extracellular domain homologous to acetylcholinesterase and containing the sites of alternative splicing, a linker domain that may be O-glycosylated, a single transmembrane region, and a short cytoplasmic tail (figure 1.14A). The esterase-like domain carries a substitution of the active site serine to glycine, suggesting that neuroligins are not catalytically active esterases. Instead, this domain is supposed to mediate the interaction to the neurexin proteins. Similar esterase-like domains have been found in neurotactin and gliotactin, Drosophila proteins that are involved in cell-cell interactions (Darboux et al., 1996). The disulfide-bonded cysteine residues in esterases are also conserved in neuroligins. The three rat neuroligin isoforms are similar in their sequences (figure 1.14B). Overall, they share 52% sequence identity at the amino acid level. The extracellular domains (55% identity) and the transmembrane regions (91%) are more conserved than the cytoplasmic sequences (31%). The most conserved sequences between neuroligins are observed in the esterase-like domain, and the most divergent sequences in the linker domain and in parts of the cytoplasmic tail.
The human *neuroligin* 3 gene is localized on chromosome X (Philibert et al., 2000). It spans approximately 30 kb and contains eight exons (figure 1.15). Exon 1 and part of exon 2 encode the 5'-untranslated region. The ATG start codon is located in exon 2, and the TAG stop codon in exon 8. The size of the exons varies from 60 nucleotides for exons 3 and 4 to almost 2 kb for exon 8. Exons 3 and 4 code for two inserts of splice site A. Sequence analysis identified a putative promoter 2.5 kb upstream of exon 1.
Immunogold EM analysis of rat brain sections provided evidence that neuroligin 1 is enriched in PSDs, its extracellular tail protruding into the synaptic cleft (Song et al., 1999). This finding places the β-neurexin/neuroligin junction at the synapse. Unfortunately, it has so far not been possible to localize neurexins in the mammalian CNS at the ultrastructural level, and their synaptic localization therefore remains to be demonstrated. In analogy to GluRs, neuroligin 1 is specifically localized to excitatory, but not inhibitory synapses. This localization is paralleled by the postsynaptic pool of PSD-95/SAP90 which is also selectively targeted to excitatory synapses (Rao et al., 1998). In addition to binding to extracellular ligands (β-neurexins), neuroligins interact intracellularly with synaptic PDZ domain proteins like PSD-95/SAP90 and S-SCAM (Hirao et al., 1998; Irie et al., 1997), suggesting that neuroligins not only serve as adhesion molecules between cells but also affect the assembly of protein complexes involved in signal transduction (figure 1.16). Similar to neuroligins, neurexins also bind to an intracellular PDZ domain protein, namely CASK (Hata et al., 1996). As a result of these interactions, the neurexin/neuroligin junction resembles a tight junction in that it is coated by PDZ domain proteins, but the resulting molecular architecture is asymmetric.

Although homophilic CAMs such as cadherins or CNRs may play a critical role in synaptic recognition and adhesion, the asymmetry of synapses is likely to be mediated in part by homophilic transsynaptic signaling. β-Neurexins and neuroligins seem ideally suited to explain certain

---

Figure 1.15: Organization of the human neuroligin 3 gene. Exonic regions are indicated by filled boxes (1-8). The area of the promoter sequence is denoted by a hatched line. The start and stop codons are delineated by filled triangles. From Philibert et al., 2000.

Figure 1.16: Neuroligins and β-neurexins act as adhesion molecules in the synapse. Binding is regulated by alternative splicing of β-neurexins at splice site 4 (arrows). Intracellularly, neurexins bind to CASK, whereas neuroligins bind to various MAGuKs like PSD-95/SAP90. Other proteins bind also to these scaffolding molecules to establish a functional synapse. From Missler and Südhof, 1998.
aspects of synaptogenesis, such as the specific recruitment of pre- and postsynaptic protein components to their respective subcellular compartments, because they represent the only known heterophilic cell adhesion system present in the synapse. Neuroligins are expressed late in development, with a postnatal peak at the time when most synapses are formed that parallels the expression peak of neurexins (Irie et al., 1997; Song et al., 1999). Therefore it can be suggested that neuroligins function in a late step of synaptogenesis and/or in the modulation of mature synapses (Brose, 1999). A recent study has shed new light on this point (Scheiffele et al., 2000). Pontine explants were co-cultured with non-neuronal HEK293 cells which do not express endogenous neuroligin. Surprisingly, the pontine explants extended axons onto the HEK293 cells and formed presynaptic junctions when the HEK293 cells were previously transfected with mouse neuroligin 1 or 2 cDNAs, but not when mock-transfected or transfected with other neuronal CAM cDNAs. This gives evidence that induction of presynaptic differentiation in CNS axons is a response inherent only to neuroligins. Addition of a soluble neurexin-Ig fusion protein blocked neuroligin-induced SV clustering which is a central feature of the initiation of synaptogenesis, presumably by interfering with the binding of endogenous neurexins on pontine axons with the exogenous neuroligins in HEK293 cells. Using a co-culture system of pontine explants and cerebellar granule cells, their *in vivo* targets, addition of the soluble neurexin-Ig fusion protein decreased SV clustering, indicating that the β-neurexin/neuroligin interaction likely plays a role in synaptogenesis. The conclusion from this study is that a single molecule, neuroligin, might be able to induce an extensive program of presynaptic differentiation.

The widespread and overlapping expression of different neuroligin isoforms and splice variants suggests that these proteins are unlikely to specify neuronal cell types for synapse formation, but are more likely components of a general synaptic machinery. A model proposes that the initial contact between a growth cone and its target cell is mediated through homophilic interactions between members of the cadherin family of CAMs (Brose, 1999). Once this initial contact is made, neuroligins initiate the establishment of a functional synapse at the contact site. They create a transsynaptic link by binding to presynaptic β-neurexins. This interaction serves as a signal to the pre- and postsynaptic compartments to start the recruitment of the necessary protein components. Postsynaptically, neuroligins bind to PSD-95/SAP90 and other homologous proteins (Irie et al., 1997), which in turn recruit transmitter receptors, ion channels, and signal transduction proteins to the site of β-neurexin/neuroligin contact, building a functional PSD. Presynaptically, β-neurexins bind to CASK (Hata et al., 1996) which in turn would then recruit proteins of the presynaptic release machinery to initiate the assembly of a presynaptic active zone. Indeed, CASK links the β-neurexin/neuroligin junction to the SV fusion machinery by binding to Mint1 (Butz et al., 1998). In addition to a role in protein recruitment during synaptogenesis, the transsynaptic β-neurexin/neuroligin junction also creates a functional link between pre- and postsynaptic compartments. This could serve in mature synapses to functionally couple the postsynaptic signal transduction apparatus to the presynaptic release machinery (and vice versa), providing a possibility to regulate synaptic plasticity, either by inducing new presynaptic specializations or by stabilizing preexisting structures. This is a fundamental requirement for brain functioning.
1.5. Scope of this Study

An estimation to quantify neuronal diversity in the vertebrate CNS has given a number of about 500 different neuronal cell types (Serafini, 1999), approximately $10^{10}$-$10^{11}$ neurons and $10^{15}$ synapses. This enormous complexity requires highly selective intercellular recognition systems to guarantee correct target finding of axonal growth cones during synaptogenesis. The current opinion is that members of the cadherin superfamily mediate this specificity (Brose, 1999). Based on the intriguing finding that neuroligins are able to induce presynaptic differentiation (Scheiffele et al., 2000), we hypothesized that additional neuroligins or similar molecules may exist to serve this demand. Supporting this idea is the fact that at present neuroligins have been predominantly assigned to excitatory synapses (Song et al., 1999); therefore it can be speculated that other neuroligin isoforms or similar proteins are targeted to different types of synapses or even are expressed in other cell types.

To test this hypothesis, the following experiments were performed: Firstly, it should be investigated whether proteins homologous to the rat neuroligins can be identified in mouse. Therefore, a library containing cDNAs of mouse brain was screened. Secondly, as the human genome is sequenced now (Lander et al., 2001; Venter et al., 2001), an analysis of DNA database sequences should be performed, again with the aim to identify further neuroligin isoforms or molecules with similar functions. An interesting molecule in this context is gliotactin, a protein which is essential to maintain the barrier between nerves and hemolymph in *Drosophila* (Auld et al., 1995). Gliotactin has the same domain structure as neuroligins, therefore it can be speculated that in vertebrates there are further homologous molecules with functions outside of the synapse. Finally, if such proteins were found, studies as to elucidate their tissue expression pattern, their subcellular localization, or their capability to interact with other molecules should be done.
2. MATERIALS AND METHODS

2.1. Materials

2.1.1. Chemicals and Reagents

acetic acid  
acetone  
N-acetyl-L-alanyl-L-glutamine  
acrylamide  
agar low melting  
agarose  
6-aminocaproic acid  
ammonium peroxodisulfate (APS)  
ampicillin  
Bacto agar  
Bacto tryptone  
Bacto yeast extract  
benzil  
benzoylperoxide  
N,N-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (Bes)  
blocking reagent  
boric acid  
bovine serum albumin (BSA)  
bromophenol blue  
calcium chloride  
chloroform  
complete mini  
$[\alpha^{-35}S]$-dATP  
2'-deoxynucleoside 5'-triphosphates  
1,4-diazabicyclo[2.2.2]octane (DABCO)  
2',3'-dideoxynucleoside 5'-triphosphates  
diethylpyrocarbonate (DEPC)  
DIG easy hyb
dimethyldichlorosilane  
dimethylsulfoxide (DMSO)  
disodium hydrogen phosphate  
disodium 3-(4-methoxySpiro{1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.13,7]decan}-4-yl)phenyl phosphate (CSPD)  
DL-dithiothreitol (DTT)  
Dulbecco's modified Eagle's medium (DMEM)  
ECL Western blotting detection reagent  
ethanol  
ethidiurum bromide  
ethylene-diaminetetraacetic acid (EDTA)  
ethylene glycol-bis(β-aminoethylether) N,N,N',N'-tetraacetic acid (EGTA)  
foetal bovine serum  
formaldehyde  
formamide  
gelatin  
gentamycin  
glutaraldehyde  
glycerol  
glycine  
guanidine thiocyanate (GTC)  
hydrochloric acid  
isopropanol  
kanamycin  
LR gold acrylic resin  
maleic acid  
manganese chloride  
β-mercaptoethanol  
methanol  
N,N'-methylene-bis-acrylamide (bis-acrylamide)  
methyl alcohol  
3-morpholinopropanesulfonic acid (MOPS)  
Mowiol 4-88  
non-essential amino acids (NEAA)  
Nonidet P-40 (NP-40)  
paraformaldehyde  
phenol  
Ponceau S  
potassium acetate  
potassium iodide  
n-propyl-gallate  
5x protein assay reagent
2. MATERIALS AND METHODS

protein G-sepharose  
RNase inhibitor  
rubidium chloride  
skim milk powder  
sodium acetate  
sodium chloride  
sodium citrate  
sodium dodecylsulfate (SDS)  
sodium hydroxide  
sodium N-lauroylsarcosinate  
sodium pyruvate  
N,N,N',N'-tetramethylethlenediamine (TEMED)  
tris[hydroxymethyl]aminomethane (Tris)  
Triton X-100  
Tween-20  
uranyl acetate  
urea  
xylene cyanol

Sigma Chemicals  
Roche Diagnostics  
Fluka Chemicals  
Fluka Chemicals  
Sigma Chemicals  
Fluka Chemicals  
Sigma Chemicals  
Fluka Chemicals  
Siegfried  
Fluka Chemicals and Life Technologies  
Fluka Chemicals  
Sigma Chemicals  
Sigma Chemicals  
Fluka Chemicals  
Fluka Chemicals  
Riedel-de Haën  
Fluka Chemicals

2.1.2. Enzymes

Acc I  
AP (alkaline phosphatase)  
BamHI  
Bsu36I  
DNA polymerase I, large (Klenow) fragment  
EcoRV  
HincII  
HindIII  
HpaI  
KpnI  
lysozyme  
N-glycosidase F  
RNase A  
RNase H  
RQ1 RNase-free DNase  
RsrII  
SalI  
SpeI  
SphI  
superscript II RNase H reverse transcriptase  
T3 RNA polymerase  
T4 DNA ligase  
T4 DNA polymerase

New England Biolabs  
Roche Diagnostics  
New England Biolabs  
New England Biolabs  
New England Biolabs  
New England Biolabs  
New England Biolabs  
New England Biolabs  
Qiagen  
Promega  
New England Biolabs  
New England Biolabs  
New England Biolabs  
Roche Diagnostics  
Life Technologies  
Roche Diagnostics  
New England Biolabs  
New England Biolabs
2. MATERIALS AND METHODS

2.1.3. Kits

- DIG RNA labeling kit: Roche Diagnostics
- jetquick gel extraction spin kit: Genomed
- Plasmid midi kit: Qiagen
- qiaquick spin miniprep kit: Qiagen
- superscript preamplification system kit: Life Technologies
- T7 sequenase v2.0 DNA sequencing kit: Amersham Pharmacia Biotech

2.1.4. Plasmid Vectors

- pBS/hNL1 (KIAA1070): from Dr. T. Nagase, Japan
- pBS/hNL4 (KIAA1260): from Dr. T. Nagase, Japan
- pCE/β-actinT7as: from Dr. L. Rohrer, Zürich
- pcDNAI/Amp: Invitrogen
- pCMV/myc,PSD-95: from Dr. C. Garner, USA
- pEGFP-N2: Clontech Laboratories
- pGEM-2: Promega
- pNKS2/sig,myc: from Dr. S. Gloor, Zürich

2.1.5. Primers

- F5hNL4: Microsynth 5'-ATTCAGTATGGTCAACTAC-3'
- F11hNL4: Microsynth 5'-ATGCAGATTTGAACCATGTCAC-3'
- MBNL12+: Microsynth 5'-ATACTGATGGGAACAAGGAGATC-3'
- R9hNL4: Microsynth 5'-GGAAATAGGGCAAAGCTATACTC-3'
- SGNL6: Microsynth 5'-GGCTAIAAC(GT)IGTGT(ACGT)(CG)(AT)(AG)TG(ACGT)-3'

2.1.6. Bacterial Strain

- TOP10 (E.coli): Invitrogen
2. MATERIALS AND METHODS

2.1.7. Cell Lines

BV-2 (mouse microglia) ATCC
C6 (rat glioma) ATCC
COS-7 (monkey kidney) ATCC
EC219 (rat brain endothelia) ATCC
GP8.3 (rat brain endothelia) ATCC
HUVEC (human umbilical vein endothelia) ATCC
L929 (mouse connective tissue) ATCC
MDCK II (dog kidney) ATCC
N-2a (mouse neuroblastoma) ATCC
PBMEC/C1-2 (pig brain endothelia) ATCC

2.1.8. Primary Cells

astrocytes (mouse) from Dr. V. Otto, Zürich
choroid plexus epithelial cells (mouse) from Dr. B. Engelhardt, Germany
microglial cells (mouse) from Dr. K. Frei, Zürich
microvessel endothelial cells (mouse) from Dr. H. Ishihara, Zürich
umbilical vein endothelial cells (human) from Dr. M. Wachtel, Zürich

2.1.9. Antibodies

goat \(\alpha\) rat PSD-95 IgG Santa Cruz Biotechnology
mouse \(\alpha\) human c-myc IgG (clone 9E10) Zymed Laboratories
mouse \(\alpha\) rat NL1/3 IgG (clone 4F9) Synaptic Systems
mouse \(\alpha\) human ZO-1 IgG (clone 1A12) Zymed Laboratories
rabbit \(\alpha\) human ZO-1 Ig Sigma Laboratories
\(\alpha\) DIG-AP conjugate Roche Diagnostics

2.1.10. Additional Materials

human brain polyadenylated RNA (female, age 15) Clontech Laboratories
human tissue polyadenylated RNA blot Clontech Laboratories
medical X-ray films Fuji
mouse brain (P0-P14) cDNA library from Dr. R. Hillenbrand, Basel
protran BA 83 nitrocellulose membrane Schleicher & Schuell
SDS-PAGE standards, broad range Bio-Rad Laboratories
2. MATERIALS AND METHODS

2.11. Solutions and Buffers

2.11.1. Molecular Biology

2.11.1.1. Nucleic Acid Purification and Electrophoresis

**TE (Tris-EDTA) buffer**
- 10 mM Tris-Cl, pH 8.0
- 1 mM EDTA
- autoclave

**mini-mini lysis buffer**
- 50 mM Tris-Cl, pH 8.0
- 50 mM EDTA
- 1 mg/ml lysozyme
- 1 mg/ml RNase A

**4 M GTC solution**
- 50 mM Tris-Cl, pH 7.6
- 4 M GTC
- 10 mM EDTA
- stir overnight at RT (room temperature), heat at 60°C for 10 min
- 2% Na N-lauroylsarcosinate
- store in the dark at 4°C up to 4 month
- 1% β-mercaptoethanol

**50x TAE (Tris-acetic acid-EDTA)**
- 2 M Tris-Cl, pH 8.0
- 1 M acetic acid
- 50 mM EDTA
- autoclave

**10x DNA loading buffer**
- 0.5% bromophenol blue
- 0.5% xylene cyanol
- 50% (v/v) glycerol

**10x MOPS buffer**
- 200 mM MOPS, pH 7.0
- 50 mM Na acetate
- 10 mM EDTA
- filter sterilize, store in the dark at RT

**RNA loading buffer**
- 1x MOPS buffer
- 6% (v/v) formaldehyde
- 50% (v/v) formamide, deionized
- 10% (v/v) glycerol
- 0.01% bromophenol blue
- always prepare fresh
2. MATERIALS AND METHODS

10x TBE (Tris-boric acid-EDTA)  
900 mM Tris-HCl, pH 8.0  
900 mM boric acid  
20 mM EDTA  
autoclave

40% acrylamide mix  
39% (w/v) acrylamide  
1% (w/v) bis-acrylamide

2.1.11.1.2. Enzyme Reactions

10x NEBuffer 1  
100 mM bis Tris propane-HCl, pH 7.0  
(New England Biolabs)  
100 mM MgCl₂  
10 mM DTT

10x NEBuffer 2  
100 mM Tris-HCl, pH 7.9  
(New England Biolabs)  
100 mM MgCl₂  
500 mM NaCl  
10 mM DTT

10x NEBuffer 3  
500 mM Tris-HCl, pH 7.9  
(New England Biolabs)  
100 mM MgCl₂  
1 M NaCl  
10 mM DTT

10x NEBuffer 4  
200 mM Tris-acetate, pH 7.9  
(New England Biolabs)  
100 mM Mg acetate  
500 mM K acetate  
10 mM DTT

10x NEBuffer BamH I  
100 mM Tris-HCl, pH 7.9  
(New England Biolabs)  
100 mM MgCl₂  
1.5 M NaCl  
10 mM DTT

10x NEBuffer Sal I  
100 mM Tris-HCl, pH 7.9  
(New England Biolabs)  
100 mM MgCl₂  
1.5 M NaCl  
10 mM DTT

100x BSA stock solution  
10 mg/ml BSA  
(New England Biolabs)
2. MATERIALS AND METHODS

10x T4 DNA polymerase buffer
(New England Biolabs)
100 mM Tris-HCl, pH 7.9
100 mM MgCl₂
500 mM NaCl
10 mM DTT

10x Klenow buffer
(New England Biolabs)
100 mM Tris-HCl, pH 7.5
50 mM MgCl₂
75 mM DTT

10x PCR buffer
(Promega)
100 mM Tris-HCl, pH 9.0
500 mM KCl
1% Triton X-100

MgCl₂ stock solution
(Promega)
25 mM MgCl₂

dNTP mix (10 mM each)
10 mM dATP
10 mM dCTP
10 mM dGTP
10 mM dTTP

5x T7 sequenase reaction buffer
(Amersham Pharmacia Biotech)
200 mM Tris-HCl, pH 7.5
100 mM MgCl₂
250 mM NaCl

DTT solution
(Amersham Pharmacia Biotech and Life Technologies)
100 mM DTT

5x labeling mix
7.5 µM dCTP
7.5 µM dGTP
7.5 µM dTTP

enzyme dilution buffer
(Amersham Pharmacia Biotech)
10 mM Tris-HCl, pH 7.5
5 mM DTT

ddNTP termination mix
80 µM dATP
80 µM dCTP
80 µM dGTP
80 µM dTTP
8 µM ddNTP
50 mM NaCl

2x stop solution
95% (v/v) formamide
20 mM EDTA
0.05% bromphenol blue
0.05% xylene cyanol
### 2. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>5x first strand buffer</td>
<td>250 mM Tris-HCl, pH 8.3</td>
</tr>
<tr>
<td>(Life Technologies)</td>
<td>15 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>375 mM KCl</td>
</tr>
<tr>
<td>10x transcription buffer</td>
<td>400 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td>(Roche Diagnostics)</td>
<td>60 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>100 mM DTT</td>
</tr>
<tr>
<td></td>
<td>20 mM spermidine</td>
</tr>
<tr>
<td>10x NTP labeling mix</td>
<td>10 mM ATP, pH 7.5</td>
</tr>
<tr>
<td>(Roche Diagnostics)</td>
<td>10 mM CTP</td>
</tr>
<tr>
<td></td>
<td>10 mM GTP</td>
</tr>
<tr>
<td></td>
<td>6.5 mM UTP</td>
</tr>
<tr>
<td></td>
<td>3.5 mM DIG-11-UTP</td>
</tr>
<tr>
<td>10x dephosphorylation buffer</td>
<td>500 mM Tris-HCl, pH 8.5</td>
</tr>
<tr>
<td>(Roche Diagnostics)</td>
<td>1 mM EDTA</td>
</tr>
<tr>
<td>10x T4 polynucleotide kinase buffer</td>
<td>700 mM Tris-HCl, pH 7.6</td>
</tr>
<tr>
<td>(New England Biolabs)</td>
<td>100 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>50 mM DTT</td>
</tr>
<tr>
<td>10x T4 DNA ligase buffer</td>
<td>500 mM Tris-HCl, pH 7.5</td>
</tr>
<tr>
<td>(New England Biolabs)</td>
<td>100 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>100 mM DTT</td>
</tr>
<tr>
<td></td>
<td>10 mM ATP</td>
</tr>
<tr>
<td></td>
<td>250 µg/ml BSA</td>
</tr>
<tr>
<td>10x DNase buffer</td>
<td>400 mM Tris-HCl, pH 7.9</td>
</tr>
<tr>
<td></td>
<td>60 mM MgCl₂</td>
</tr>
<tr>
<td></td>
<td>100 mM CaCl₂</td>
</tr>
<tr>
<td></td>
<td>100 mM NaCl</td>
</tr>
</tbody>
</table>

#### 2.1.11.1.3. Northern Blotting

- **DEPC-treated H₂O**
  - 0.1% DEPC in H₂O
  - shake for at least 60 min, then autoclave
  - or heat at 65°C for 20 min

- **20x SSC (standard saline citrate)**
  - 300 mM Na citrate, pH 7.0
  - 3 M NaCl
  - autoclave

- **2x washing solution**
  - 2x SSC
  - 0.1% SDS
2. MATERIALS AND METHODS

0.5x washing solution

maleic acid buffer

10x blocking reagent stock solution

washing buffer

blocking buffer

detection buffer

CSPD substrate solution

2.1.11.1.4. Bacteria Manipulations

LB (Luria-Bertani) medium

transformation buffer 1

transformation buffer 2
2.1.11.2. Cell Biology

culture medium

1x DMEM
10% (v/v) foetal bovine serum
(inactivate at 56°C for 30 min)
2 mM L-glutamine
1 mM Na pyruvate
1x NEAA
40 mg/l gentamycin

10x PBS (phosphate-buffered saline)
(Life Technologies)
80 mM Na$_2$HPO$_4$, pH 7.4
15 mM KH$_2$PO$_4$
27 mM KCl
1.37 M NaCl

10x DPBS (Dulbecco's PBS)
(Life Technologies)
80 mM Na$_2$HPO$_4$, pH 7.4
15 mM KH$_2$PO$_4$
27 mM KCl
1.37 M NaCl
9 mM CaCl$_2$
5 mM MgCl$_2$

10x trypsin-EDTA solution
(Life Technologies)
1x PBS
0.5% trypsin
7 mM EDTA
145 mM NaCl

2x BBS (Bes-buffered saline)
50 mM Bes, pH 6.95
1.5 mM Na$_2$HPO$_4$
280 mM NaCl
adjust to pH 6.95 with NaOH,
filter sterilize

Lisbeth's embedding medium
30 mM Tris-HCl, pH 9.5
50 mg/ml n-propyl-gallate
70% (v/v) glycerol
store in aliquots at -20°C

Mowiol embedding medium
2.4 g Mowiol 4-88
4.8 ml glycerol
6 ml H$_2$O
stir at 50-70°C for 3 hrs, centrifuge to
remove undissolved material
2.5% DABCO
2.5% KI
store at 4°C
### 2.1.11.3. Biochemistry

**Lysis buffer**
- 20 mM Tris-Cl, pH 7.5
- 150 mM NaCl
- 1% Triton X-100
- 1x complete

**GlycoF lysis buffer**
- 50 mM Tris-Cl, pH 7.5
- 150 mM NaCl
- 0.5% NP-40
- 0.1% SDS
- 1x complete

**Immunoprecipitation lysis buffer**
- 50 mM Tris-Cl, pH 7.5
- 150 mM NaCl
- 1% Triton X-100
- 1x complete

**5x Lämmli loading buffer**
- 250 mM Tris-Cl, pH 6.8
- 500 mM DTT (or β-mercaptoethanol)
- 10% (w/v) SDS
- 0.2% bromophenol blue
- 50% (v/v) glycerol

**30% acrylamide mix**
- 29% (w/v) acrylamide
- 1% (w/v) bis-acrylamide

**5x Electrophoresis buffer**
- 125 mM Tris-Cl, pH 8.3
- 1.25 M glycine
- 0.5% SDS
- do not adjust pH!

**Cathode buffer**
- 25 mM Tris-Cl, pH 9.4
- 40 mM 6-aminocaproic acid
- 20% (v/v) methanol

**Anode buffer 1**
- 300 mM Tris-Cl, pH 10.4
- 20% (v/v) methanol

**Anode buffer 2**
- 25 mM Tris-Cl, pH 10.4
- 20% (v/v) methanol

**Ponceau S solution**
- 0.2% Ponceau S
- 3% acetic acid
## 2. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th>Solution Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TBS (Tris-buffered saline)</td>
<td>100 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>1.5 M NaCl</td>
</tr>
<tr>
<td>blotto solution</td>
<td>1x TBS</td>
</tr>
<tr>
<td></td>
<td>5% (w/v) skim milk powder</td>
</tr>
<tr>
<td></td>
<td>0.1% Tween-20</td>
</tr>
<tr>
<td>wash buffer TBS-T (TBS-Tween)</td>
<td>1x TBS</td>
</tr>
<tr>
<td></td>
<td>0.1% Tween-20</td>
</tr>
<tr>
<td>stripping buffer</td>
<td>200 mM glycine-HCl, pH 2.2</td>
</tr>
<tr>
<td></td>
<td>1% Tween-20</td>
</tr>
<tr>
<td></td>
<td>0.1% SDS</td>
</tr>
<tr>
<td>TEN (Tris-EDTA-NaCl)</td>
<td>50 mM Tris-HCl, pH 8.0</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>1 mM EDTA</td>
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</table>

### 2.1.11.4. Electron Microscopy

<table>
<thead>
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<th>Solution Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>fixation solution</td>
<td>1x PBS</td>
</tr>
<tr>
<td></td>
<td>4% (w/v) paraformaldehyde</td>
</tr>
<tr>
<td></td>
<td>(depolymerize at 70°C)</td>
</tr>
<tr>
<td></td>
<td>0.1% glutaraldehyde</td>
</tr>
<tr>
<td>PBGG (PBS-BSA-gelatin-glycine) buffer</td>
<td>1x PBS</td>
</tr>
<tr>
<td></td>
<td>0.2% gelatin</td>
</tr>
<tr>
<td></td>
<td>heat to dissolve</td>
</tr>
<tr>
<td></td>
<td>0.5% BSA</td>
</tr>
<tr>
<td></td>
<td>50 mM glycine</td>
</tr>
</tbody>
</table>
2. MATERIALS AND METHODS

2.2. Methods

2.2.1. Molecular Biology

Molecular biology is the science of the molecular structure and function of nucleic acids and proteins (i.e. enzymes).

2.2.1.1. Nucleic Acid Purification

Purification of nucleic acids is the first step in most molecular biology studies and all recombinant DNA techniques. Methods for purifying nucleic acids are often combinations of extraction/precipitation, chromatography, centrifugation, electrophoresis, and affinity separation.

2.2.1.1.1. DNA Purification

For plasmid isolations, the 'qiaprep spin miniprep kit' and the 'plasmid midi kit' from Qiagen were used, following the unmodified standard protocols. A single bacterial colony is inoculated into LB medium containing the appropriate antibiotic (100 µg/ml ampicillin or 30 µg/ml kanamycin), and grown with vigorous shaking for 12-16 hrs at 37°C. After harvesting and resuspension, the bacterial cells are lysed under alkaline conditions in the presence of RNase A. SDS solubilizes the phospholipid and protein components of the cell membrane, leading to lysis and release of the cell contents, while the alkaline conditions denature the chromosomal but hardly the plasmid DNA, as well as proteins. The lysate is subsequently neutralized and adjusted to high-salt binding conditions in one step. The high-salt concentration causes denatured proteins, chromosomal DNA, cellular debris, and SDS to precipitate, while the smaller plasmid DNA renatures and stays in solution. After centrifugation, the DNA is adsorbed onto the silica-gel membrane and separated by washing steps from degraded RNA, protein, other cellular components, and salts. The plasmid DNA is eluted from the membrane by low-salt buffer.

A convenient way to analyze plasmid DNA from a single bacterial colony is the mini-mini preparation method (e.g. to check presence of inserts in low-efficiency cloning procedures).

- colonies to be analyzed should be grown to about 2 mm in diameter
- suck colonies with sterile tip and rinse into a tube containing 10 µl of mini-mini lysis buffer
- let react for at least 5 min at RT
- add 1 µl of H2O-saturated phenol and mix
- centrifuge for 1 min at full speed
- take supernatant (8 µl), add 10x DNA loading buffer and load on an agarose gel

DNA fragments ranging from 100 bp to 10 kb from PCR (polymerase chain reaction) and other enzymatic reactions can be purified from residual reaction components such as primers, unincorporated nucleotides, enzymes, mineral oil, and salts using the 'qiaquick PCR purification kit' from Qiagen. The unmodified standard protocol of the manufacturer was used.
2.2.1.1.2. Extraction and Precipitation of DNA

The extraction procedure separates proteins and lipophilic molecules (in organic phase) from DNA, RNA and nucleic acid-similar molecules like sugars (in aqueous phase).

- fill DNA solution up to a final volume of 200 µl with TE buffer
- add same volume of mixture phenol:chloroform 1:1 (200 µl)
- vortex for 30-60 sec until an emulsion forms
- centrifuge for 2 min at full speed
- transfer supernatant (aqueous phase with DNA) to a fresh tube

A salt is necessary for dehydration of nucleic acids (charge-neutralization) so that they are able to precipitate in ethanol.

- add 1/10 volume of 1 M NaCl (20 µl)
- add 2.5 volumes of 100% ethanol -20°C (500 µl)
- shake and incubate on ice for 15 min
- centrifuge for 30 min at full speed and 4°C
- remove supernatant
- carefully wash pellet with 2.5 volumes of 70% ethanol -20°C (500 µl; to remove salts)
- centrifuge for 5 min at full speed and 4°C
- remove supernatant
- air-dry pellet until the last traces of fluid have evaporated
- resuspend pellet in TE buffer

2.2.1.1.3. RNA Isolation

To obtain good preparations of eukaryotic RNA from cells grown in tissue cultures, it is necessary to minimize the activity of RNases liberated during cell lysis by using inhibitors of RNases or methods that disrupt cells and inactivate RNases simultaneously. In 4 M GTC, sodium N-lauroylsarcosinate, and reducing agents such as β-mercaptoethanol, cellular structures disintegrate and proteins are denatured (Chomczynski and Sacchi, 1987). Under the slightly acidic pH of the extraction step, DNA and proteins are extracted into the interphase and/or the organic phase, whereas the RNA is in the aqueous phase.

- remove medium in culture flask
- wash cells twice with ice-cold PBS
- add 500 µl 4 M GTC solution per 25 cm² culture flask
- remove cells with scraper and fill into a tube
- homogenize carefully with a syringe fitted to a 21 gauge needle
- add 50 µl (1/10 volume) 3 M Na acetate pH 5.2, vortex
- add 500 µl (1 volume) H₂O-saturated phenol, vortex
- add 500 µl (1 volume) chloroform, vortex for 15 sec
- cool on ice for 15 min
• centrifuge for 20 min at 10000 g and 4°C (RNA is in aqueous phase, DNA and proteins are in interphase and organic phase)
• mix upper (aqueous) phase with 500 µl isopropanol (1 volume)
• leave at -20°C for at least 60 min (to precipitate RNA)
• centrifuge for 20 min at 10000 g and 4°C
• dissolve pellet in 150 µl 4 M GTC solution (1/3 of original volume)
• precipitate RNA with 500 µl isopropanol at -20°C for 60 min (1 volume)
• centrifuge for 10 min at 10000 g and 4°C
• optional: dissolve pellet in 100 µl DNase buffer, add 1-2 U RQ1 RNase-free DNase and incubate at 37°C for 30 min
• extract with phenol:chloroform (add 100 µl TE buffer, add 200 µl phenol:chloroform, vortex 45 sec, centrifuge for 2 min at full speed)
• extract with chloroform (to remove traces of phenol)
• add 20 µl (1/10 volume) 3 M Na acetate pH 5.2
• add 500 µl (2.5 volumes) 100% ethanol -20°C and precipitate at -70°C for at least 20 min
• centrifuge for 15 min at 10000 g and 4°C
• remove supernatant and carefully wash pellet with 500 µl 70% ethanol -20°C
• centrifuge for 5 min at 10000 g and 4°C
• air-dry pellet
• dissolve pellet in DEPC-treated H2O

2.2.1.2. Nucleic Acid Gel Electrophoresis and Gel Extraction

Nucleic acid fragments are separated by electrophoresis on an agarose gel until the fragment of interest is resolved. Nucleic acids, which are negatively charged at neutral pH, migrate through the gel toward the anode. Larger molecules migrate more slowly because of greater frictional drag. The loading buffer increases the density of the sample, ensuring that the nucleic acid drops evenly into the well; its color simplifies the loading process. Bromophenol blue migrates through agarose gels approximately 2.2x faster than xylene cyanol. The location of the DNA/RNA within the gel can be determined directly by staining with low concentrations of the fluorescent intercalating dye ethidium bromide; bands containing as little as 1-10 ng of DNA can be detected by direct examination of the gel in UV light. If necessary, these bands of DNA can be recovered from the gel. Agarose, which is extracted from seaweed, is a linear polymer (figure 2.1).

Figure 2.1: Basic structure of agarose.

The concentration of agarose in the gel determines the size range of DNA molecules which can be resolved (table 2.1).
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<table>
<thead>
<tr>
<th>Agarose (w/v)</th>
<th>Range of Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4%</td>
<td>2500 – 30000 bp</td>
</tr>
<tr>
<td>0.8%</td>
<td>1000 – 15000 bp</td>
</tr>
<tr>
<td>1.0%</td>
<td>500 – 10000 bp</td>
</tr>
<tr>
<td>1.2%</td>
<td>250 – 6000 bp</td>
</tr>
<tr>
<td>1.5%</td>
<td>250 – 5000 bp</td>
</tr>
<tr>
<td>2.0%</td>
<td>100 – 2500 bp</td>
</tr>
</tbody>
</table>

Table 2.1: *Separation range of linear DNA in agarose gels.*

- add 1% (w/v) agarose to 1x TAE (30 ml)
- melt agarose in microwave oven until a clear, transparent solution is achieved
- add H$_2$O to compensate evaporated liquid
- add 0.5 µg/ml ethidium bromide
- fill gel into form, insert comb
- let polymerize the gel, then remove comb
- load DNA samples (mixed with 10x DNA loading buffer) onto gel (100 ng DNA per slot; 1 µg DNA per cm slot for preparative gels)
- let run the gel in 1x TAE running buffer at 100 V (preparative gels: 1-5 V/cm length of gel)
- analyze gel under UV lamp (365 nm or 312 nm)

For extraction of DNA from agarose gels, the standard protocol of the 'jetquick gel extraction spin kit' from Genomed was used. Extraction and purification of DNA fragments (40 bp to 50 kb) from agarose gels are based on solubilization of agarose in the presence of a concentrated chaotropic salt. The high concentration of the chaotropic salt disrupts hydrogen bonding between sugars in the agarose polymer, whereas the high salt concentration dissociates DNA binding proteins from the DNA fragments. The next step is selective, quantitative adsorption of nucleic acids to the highly specific silica membrane of the jetquick micro spin column under the same high-salt conditions. All non-nucleic acid impurities such as agarose, proteins, salts, and ethidium bromide are removed during a washing step. Elution of the DNA is accomplished with a low-salt solution such as Tris buffer (10 mM Tris-HCl, pH 8.0) or H$_2$O.

Because RNA is able to make a high degree of secondary structures, it has to be denatured for gel electrophoresis. For this purpose, formamide and formaldehyde are used.

- for a 1% (w/v) gel, add 0.3 g agarose to 21.7 ml H$_2$O
- melt agarose in microwave oven until a clear, transparent solution is achieved
- add H$_2$O to compensate evaporated liquid and let cool to 60°C
- add 3 ml 10x MOPS buffer, 5.3 ml formaldehyde (37% = 12.3 M; 2.2 M final concentration) and 0.5 µg/ml ethidium bromide
- fill gel into form, insert comb and let polymerize the gel
- prepare RNA loading buffer and dilute RNA (10 µg total RNA or 2 µg polyadenylated RNA)
- incubate samples for 15 min at 65°C, immediately chill on ice
- prerun gel for 5 min at 50 V, rinse slots with pipette and load samples
- run gel in 1x MOPS buffer at 50-80 V
- analyze gel under UV lamp (365 or 312 nm)
2.2.1.3. Determination of Nucleic Acid Concentration

If the sample is pure, the concentration of the nucleic acid can be measured spectrophotometrically, because the bases absorb UV light. If there is not sufficient nucleic acid to assay spectrophotometrically, or if the nucleic acid is contaminated with substances that absorb UV light, the amount of nucleic acid can be estimated using the UV-induced fluorescence emitted by ethidium bromide molecules intercalated between the bases. Because the fluorescence is proportional to the total mass of nucleic acid, the quantity of nucleic acid in the sample can be estimated by comparing the fluorescent yield of the sample with that of standards.

- dilute e.g. 1 µl DNA solution in 220 µl H₂O
- measure light emission (OD) at λ = 260 nm

| dsDNA | 1 OD₂₆₀ ≈ 50 ng/µl H₂O |
| ssRNA | 1 OD₂₆₀ ≈ 40 ng/µl H₂O |
| ssDNA | 1 OD₂₆₀ ≈ 33 ng/µl H₂O |
| oligonucleotide | 1 OD₂₆₀ ≈ 20-30 ng/µl H₂O |

- calculate: OD₂₆₀ x 220 x 50 ng/µl = y ng DNA/µl
- pure DNA: OD₂₆₀/OD₂₈₀ ≥ 1.8 and OD₃₂₀ → 0 (an OD₂₆₀/OD₂₈₀ < 1.8 indicates that the preparation is contaminated with proteins and aromatic substances, an OD₂₆₀/OD₂₈₀ > 2 indicates a possible contamination with RNA)
- pure RNA: OD₂₆₀/OD₂₈₀ ≥ 2.0 (an OD₂₆₀/OD₂₈₀ < 2.0 means that the preparation is contaminated with proteins and aromatic substances)

2.2.1.4. Enzymes for Molecular Biology

The primary tools used by the molecular biologist in manipulating DNA are restriction enzymes, polymerases and DNA/RNA-modifying enzymes.

2.2.1.4.1. Restriction Endonucleases

Restriction endonucleases are bacterial enzymes that recognize specific DNA sequences and cleave it at distinct positions. Nearly 3000 restriction endonucleases have been found exhibiting over 200 different specificities. Enzymes isolated from different sources that recognize the same sequence are called isoschizomers (may have different cleavage sites). One unit of restriction endonuclease activity is defined as the amount of enzyme required to completely digest 1 µg substrate DNA (or fragments) in 60 min at the appropriate temperature under optimal assay conditions as stated for each restriction endonuclease. Some enzymes require BSA for optimal activity because proteins rapidly denature in dilute solutions (<100 µg/ml) and/or adsorb to plastic. Under extreme non-standard conditions, restriction enzymes are capable of cleaving sequences which are similar but not identical to their defined recognition sequence (star activity). Certain enzymes can be heat inactivated by incubating at 65°C or 80°C for 20 min.
2. MATERIALS AND METHODS

Rules of thumb (in practice): 1-3 U enzyme/µg DNA in 2 hrs
0.5 U enzyme/µg DNA overnight

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>5 µl (2 µg)</td>
</tr>
<tr>
<td>SalI (20 U/µl)</td>
<td>0.3 µl</td>
</tr>
<tr>
<td>NEBuffer SalI (10x)</td>
<td>1 µl</td>
</tr>
<tr>
<td>BSA stock solution (10 mg/ml)</td>
<td>0.1 µl (100 µg/ml)</td>
</tr>
<tr>
<td>H2O</td>
<td>3.6 µl</td>
</tr>
<tr>
<td>90 min at 37°C</td>
<td>10 µl (200 ng DNA/µl)</td>
</tr>
</tbody>
</table>

Table 2.2: Components for digestion.

2.2.1.4.2. T4 DNA Polymerase

Bacteriophage T4 DNA polymerase possesses 5’→3’ polymerization and 3’→5’ exonuclease activities, but no 5’→3’ exonuclease activity, and therefore is capable of filling-in 5’-overhangs and removing 3’-overhangs (its 3’→5’ exonuclease activity is more than 200x that of the Klenow fragment). This enzyme is used for cloning of PCR fragments because Taq DNA polymerase has a terminal transferase activity that preferentially adds an adenine nucleotide to the 3’-ends of PCR products. T4 DNA polymerase can be heat inactivated by incubating at 75°C for 10 min.

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>DNA</td>
<td>10 µl (3 µg)</td>
</tr>
<tr>
<td>T4 DNA polymerase (3 U/µl)</td>
<td>0.3 µl (1-5 U/µg DNA)</td>
</tr>
<tr>
<td>T4 DNA polymerase buffer (10x)</td>
<td>5 µl</td>
</tr>
<tr>
<td>BSA stock solution (10 mg/ml)</td>
<td>0.25 µl (50 µg/ml)</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>0.5 µl (100 µM)</td>
</tr>
<tr>
<td>H2O</td>
<td>33.95 µl</td>
</tr>
<tr>
<td>20 min at 12°C</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Table 2.3: Components for T4 DNA polymerase reaction.

2.2.1.4.3. DNA Polymerase I, Large (Klenow) Fragment

Klenow fragment lacks the 5’→3’ exonuclease activity of intact *E.coli* DNA polymerase I but retains the 5’→3’ polymerase, the 3’→5’ exonuclease and strand displacement activities. This enzyme does not degrade but fills-in 5’-termini to make blunt ends (for removing 3’-overhangs use T4 DNA polymerase, because it is more effective than Klenow fragment; for filling-in 5’-overhangs use either enzyme). The Klenow fragment reaction can be stopped by adding EDTA to 10 mM final concentration and heating at 75°C for 10 min. Klenow fragment can be heat inactivated by incubating at 75°C for 20 min.
2. MATERIALS AND METHODS

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>4 µl (1 µg)</td>
<td></td>
</tr>
<tr>
<td>Klenow fragment (5 U/µl)</td>
<td>1 µl (1 U/µg DNA)</td>
<td></td>
</tr>
<tr>
<td>Klenow buffer (10x)</td>
<td>2 µl</td>
<td></td>
</tr>
<tr>
<td>dNTP mix (2 mM each)</td>
<td>0.33 µl (33 µM)</td>
<td></td>
</tr>
<tr>
<td>H₂O</td>
<td>12.67 µl</td>
<td></td>
</tr>
<tr>
<td>15 min at 25°C</td>
<td>20 µl</td>
<td></td>
</tr>
</tbody>
</table>

Table 2.4: Components for Klenow fragment reaction.

2.2.1.4.4. Taq DNA Polymerase

_Taq_ DNA polymerase (from _Thermus aquaticus_) is a thermostable enzyme that replicates DNA at 74°C (60 bases/sec) and exhibits a half-life of 40 min at 95°C. This enzyme catalyzes the polymerization of nucleotides into DNA in the 5’→3’ direction in the presence of magnesium.

Reaction mixture: see section ‘2.2.1.5. Polymerase Chain Reaction’.

2.2.1.4.5. T7 Sequenase v2.0 DNA Polymerase

T7 sequenase v2.0 DNA polymerase is a genetically engineered form of bacteriophage T7 DNA polymerase. It has no 3’→5’ exonuclease activity as compared to native T7 DNA polymerase. It is highly processive, polymerizes DNA rapidly and incorporates useful nucleotide analogues (ddNTPs, dITP). This enzyme is used for DNA sequencing.

Reaction mixture: see section ‘2.2.1.6. DNA Sequencing’.

2.2.1.4.6. Superscript II RNase H- Reverse Transcriptase

Superscript II RNase H- reverse transcriptase (from Moloney murine leukemia virus) is a RNA-dependent DNA polymerase requiring a DNA primer and a RNA template to synthesize a complementary DNA strand. This enzyme is genetically engineered by the introduction of point mutations in the RNase H active center. It is used for first- and second-strand synthesis of cDNA from mRNA for cloning.

Reaction mixture: see section ‘2.2.1.5.3. RT-PCR’.
2.2.1.4.7. T3 and T7 RNA Polymerases

Bacteriophage T3 and T7 RNA polymerases are DNA-dependent RNA polymerases which exhibit extremely high specificity for T3/T7 promoter sequences and produce large quantities of RNA. These enzymes are applied for the synthesis of RNA transcripts which are used as hybridization probes.

Reaction mixture: see section ‘2.2.1.7.2. Preparation of RNA Probes’.

2.2.1.4.8. Alkaline Phosphatase

AP (from calf intestinal mucosa) catalyzes the hydrolysis of 5’-phosphate groups from DNA, RNA, dNTPs and NTPs. This prevents religation of linearized cloning vector DNA. AP can be heat inactivated by incubating at 65°C for 10 min in the presence of 20 mM EGTA.

Reaction mixture:

<table>
<thead>
<tr>
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<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>20 µl (10 µg)</td>
</tr>
<tr>
<td>AP (1 U/µl)</td>
<td>3 µl (1 U/pmol DNA ends)</td>
</tr>
<tr>
<td>dephosphorylation buffer (10x)</td>
<td>20 µl</td>
</tr>
<tr>
<td>H₂O</td>
<td>158 µl</td>
</tr>
<tr>
<td></td>
<td>60 min at 37°C 200 µl (50 ng DNA/µl)</td>
</tr>
</tbody>
</table>

Table 2.5: Components for AP reaction.

2.2.1.4.9. T4 Polynucleotide Kinase

Bacteriophage T4 polynucleotide kinase catalyzes the transfer of the γ-phosphate from ATP to the 5’-terminus of polynucleotides. This enzyme also possesses a 3’-phosphatase activity. It is used because PCR primers are not phosphorylated and therefore cannot be ligated into a cloning vector. T4 polynucleotide kinase can be heat inactivated by incubating at 65°C for 20 min.

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA</td>
<td>20 µl</td>
</tr>
<tr>
<td>T4 polynucleotide kinase (10 U/µl)</td>
<td>0.2 µl</td>
</tr>
<tr>
<td>T4 polynucleotide kinase buffer (10x)</td>
<td>5 µl</td>
</tr>
<tr>
<td>ATP (10 mM)</td>
<td>5 µl (1 mM)</td>
</tr>
<tr>
<td>H₂O</td>
<td>24.3 µl</td>
</tr>
<tr>
<td>60 min at 37°C (for blunt ends and 3’-overhangs: 5 min at 70°C, then on ice, then add kinase)</td>
<td>50 µl</td>
</tr>
</tbody>
</table>

Table 2.6: Components for T4 polynucleotide kinase reaction.
2.2.1.4.10. T4 DNA Ligase

Bacteriophage T4 DNA ligase catalyzes the joining of two strands of DNA between the 5'-phosphate and 3'-hydroxyl groups of adjacent nucleotides in the presence of ATP in either a blunt end or cohesive end configuration. Single-stranded nicks in dsDNA are also closed. T4 DNA ligase can be heat inactivated by incubating at 65°C for 10 min (heat inactivation will decrease the efficiency of transformation).

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>vector + insert DNA</td>
<td>2 µl (0.1-1 µM in 5'-termini)</td>
</tr>
<tr>
<td>T4 DNA ligase (400 U/µl)</td>
<td>1 µl</td>
</tr>
<tr>
<td>T4 DNA ligase buffer (10x)</td>
<td>2 µl</td>
</tr>
<tr>
<td>H2O</td>
<td>15 µl</td>
</tr>
<tr>
<td>for cohesive ends: 10 min at 25°C</td>
<td>20 µl</td>
</tr>
<tr>
<td>for blunt ends: 120 min at 25°C</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Table 2.7: Components for T4 DNA ligase reaction.

2.2.1.4.11. RQ1 RNase-Free DNase

RQ1 RNase-free DNase (from bovine pancreas) is a preparation of DNase I that degrades ss- or dsDNA to produce 3'-hydroxyl oligonucleotides. This preparation is qualified for use in applications where maintaining the integrity of RNA is critical (e.g. in isolation of RNA). DNase I can be heat inactivated by incubating at 65°C for 15 min in the presence of 2.5 mM EDTA.

Reaction mixture:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>total RNA</td>
<td>50 µl (100 µg)</td>
</tr>
<tr>
<td>RQ1 RNase-free DNase (1 U/µl)</td>
<td>2 µl</td>
</tr>
<tr>
<td>DNase buffer (10x)</td>
<td>10 µl</td>
</tr>
<tr>
<td>DTT solution (100 mM)</td>
<td>10 µl (10 mM)</td>
</tr>
<tr>
<td>H2O (DEPC-treated)</td>
<td>28 µl</td>
</tr>
<tr>
<td>15-30 min at 37°C</td>
<td>100 µl (1-2 µg RNA/µl)</td>
</tr>
</tbody>
</table>

Table 2.8: Components for DNase reaction.

2.2.1.4.12. RNase A

Ribonuclease A (from bovine pancreas) is an endoribonuclease that specifically attacks ssRNA 3’ to pyrimidine residues and cleaves the phosphate linkage to the adjacent nucleotide. RNase A can be made DNase-free by boiling for 10 min and is used in DNA purification procedures.

Reaction mixture: see section '2.2.1.1.1. DNA Purification'.
2. MATERIALS AND METHODS

2.2.1.4.13. RNase H

_E.coli_ ribonuclease H is an endoribonuclease that degrades the RNA portion of DNA-RNA hybrids. It does not degrade single-stranded nucleic acids, duplex DNA or dsRNA. RNase H is used for the removal of the mRNA during second-strand cDNA synthesis.

Reaction mixture: see section ‘2.2.1.5.3. RT-PCR’.

2.2.1.4.14. Lysozyme

Lysozyme (from hen egg white) is used with EDTA to break down the cell wall and outer membrane of bacteria in plasmid DNA preparation.

Reaction mixture: see section ‘2.2.1.1.1. DNA Purification’.

2.2.1.5. Polymerase Chain Reaction

The PCR technique is an _in vitro_ method for enzymatically synthesizing defined sequences of DNA.

2.2.1.5.1. Guidelines of Primer Design

The approach to the selection of efficient and specific primers remains somewhat empirical. Primers can be specific to a particular DNA sequence or degenerated to anneal to DNA sequences of homologous genes. Designing primers with the lowest possible degeneracy has high priority so that the portion of fitting primers is high. It is extremely important that the primer fits exactly with its 3'-end to the template DNA, because _Taq_ DNA polymerase is unable to elongate if the last base does not fit. To reduce the degeneracy of the primer, an inosine can be inserted at positions where all four bases are possible (inosine forms stable basepairs with all four conventional bases). It is recommended to insert inosines only at the 5'-end of the primer, because at the 3'-end the base pairing has to be exactly. Sequences not complementary to the template can be added to the 5'-end of the primers (e.g. restriction sites or regulatory elements like promoters). Primer sequences should have the following general characteristics:

- primer length around 20-25 bases
- GC content around 40-60%
- primers are not complementary to each other at the 3'-ends (so primer-dimers will not form)
- primers should not have three or more G or C at the 3'-end
- primers should not have a T at the 3'-end, because such primers would have a greater tolerance of mismatch (use G or C at 3'-end, last six bases at 3'-end: about 50% G or C)
- primers should not contain internal secondary structures
- primers have a melting temperature T_m that allows annealing temperatures of 55-65°C (for maximum specificity use temperatures of 62-65°C)
Whenever possible, primer pairs should be designed to have similar $T_m$ values. The melting temperature $T_m$ of the primers and the annealing temperature for the PCR can be estimated with the 'nearest neighbour' algorithm or with the following simple formula:

$$T_m = 2^\circ C(A+T) + 4^\circ C(G+C)$$

annealing temperature = $T_m - 5^\circ C$

2.2.1.5.2. Setting up PCR

In PCR, a DNA sample is combined with two oligonucleotide primers, dNTPs, and the thermostable Taq DNA polymerase in a suitable buffer (table 2.9). The mixture is incubated at three temperatures for several cycles. Because the products of one round of amplification serve as templates for the next, each successive cycle essentially doubles the amount of the desired DNA product. With PCR, less than 10 pg of template DNA can be detected. To prevent evaporation of the PCR sample inside the tube leading to changes in the concentration of the reaction components, the sample must be overlaid with a drop of mineral oil.

<table>
<thead>
<tr>
<th>Component</th>
<th>Final Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR buffer (10x)</td>
<td>1x</td>
<td>2.5 µl</td>
</tr>
<tr>
<td>MgCl$_2$ stock solution (25 mM)</td>
<td>1.5 mM</td>
<td>1.5 µl</td>
</tr>
<tr>
<td>dNTP mix (10 mM each)</td>
<td>200 µM</td>
<td>0.5 µl</td>
</tr>
<tr>
<td><em>Taq</em> DNA polymerase (0.5 U/µl)</td>
<td>0.5 U</td>
<td>1 µl</td>
</tr>
<tr>
<td>template</td>
<td>10 pg – 500 ng</td>
<td>-</td>
</tr>
<tr>
<td>primers (30 µM)</td>
<td>500 nM</td>
<td>0.4 µl</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>-</td>
<td>fill up to 25 µl</td>
</tr>
</tbody>
</table>

Table 2.9: Standard conditions for PCR.

*Taq* DNA polymerase activity is sensitive to the concentration of Mg$^{2+}$ ions as well as to the nature and concentration of monovalent ions. MgCl$_2$ is not included in the PCR buffer, because it can have a profound effect on the specificity and yield of an amplification. Excess Mg$^{2+}$ will result in the accumulation of non-specific amplification products, and insufficient Mg$^{2+}$ will reduce the yield. As dNTPs appear to quantitatively bind Mg$^{2+}$ ions, the amount of dNTPs present in a reaction will determine the amount of free Mg$^{2+}$ available.

Initial heating of the PCR mixture for some minutes at 94°C is enough to completely denature DNA so that the primers can anneal to their complementary sequences of the template as the reaction mix is cooled. The choice of the primer annealing temperature is a critical factor in designing a high-specificity PCR. If the temperature is too high, no annealing occurs, but if it is too low, non-specific annealing will increase dramatically. Primer extension is carried out at 72°C, the temperature optimum of *Taq* DNA polymerase. The next step is then denaturation at 94°C. This cycle is repeated up to 40 times (table 2.10; figure 2.2). After the last cycle, the reaction tubes are held at 72°C for some min to promote completion of partial extension products. 'Hot start' means that the *Taq* DNA polymerase is omitted during initial heating and added at the annealing temperature in the first cycle. This prevents polymerizing from unspecific annealed primers during the heat-up to the initial heating temperature. A simplified hot start can be performed by starting
the PCR program and once the thermal cycler has reached the initial 94°C, the PCR tubes are added.

<table>
<thead>
<tr>
<th>Step</th>
<th>Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial denaturation</td>
<td>94°C/5 min</td>
</tr>
<tr>
<td>Primer annealing</td>
<td>T_m/45 sec</td>
</tr>
<tr>
<td>Primer extension</td>
<td>72°C/90 sec</td>
</tr>
<tr>
<td>Denaturation during cycling</td>
<td>94°C/30 sec</td>
</tr>
<tr>
<td>Final primer extension</td>
<td>72°C/6 min</td>
</tr>
</tbody>
</table>

Table 2.10: Cycling parameters for PCR.

2.2.1.5.3. RT-PCR

Sensitive methods for the detection and analysis of rare mRNA transcripts or other RNAs present in low abundance are an important aspect of most cell and molecular biology studies. RNA cannot serve as a template for PCR, so it must first be reverse transcribed into cDNA. Synthesis of single-stranded cDNA was performed using the 'superscript preamplification system kit' from Life Technologies. First, RNAs are primed using either random hexamers, oligo(dT), or gene-specific primers. From these priming sites, superscript II RNase H- reverse transcriptase is able to synthesize first strand cDNA. To increase sensitivity of PCR amplification from cDNA targets, the RNA template from the cDNA:RNA hybrid molecule can be removed by digestion with RNase H. Following use of this system, target cDNA can be amplified with specific oligonucleotide primers by the PCR method, and RT-PCR (reverse transcription-PCR) products can be cloned into an appropriate vector for subsequent characterization procedures. Frequently, RNA preparations contain small amounts of genomic DNA that may subsequently be amplified in the PCR along with the target cDNA. As a precaution, a control experiment can be performed without reverse transcriptase. Products generated in the absence of reverse transcriptase are of genomic origin. Such RNA preparations should be treated with DNase I.
• add the following components to a 0.5 ml tube:
  - 1-5 µg total RNA or 50-500 ng polyadenylated RNA
  - 500 ng oligo(dT)$_{12-18}$ primer
  - fill up to 12 µl with H$_2$O
• incubate at 70°C for 10 min, chill on ice and briefly centrifuge
• add the following components:
  - 4 µl 5x first strand buffer
  - 2 µl 100 mM DTT solution
  - 1 µl 10 mM dNTP mix
• incubate at 42°C for 2 min
• add 1 µl superscript II RNase H$^-$ reverse transcriptase (200 U), mix
• incubate at 42°C for 50 min
• inactivate at 70°C for 15 min
• optional: add 1 µl E.coli RNase H (2 U) and incubate at 37°C for 20 min

2.2.1.6. DNA Sequencing

The sequencing technique was developed to determine the nucleotide sequence of DNA. This made possible the analysis of the structure and function of genes. In addition to the procedure described below, the sequencing service of Microsynth was used.

2.2.1.6.1. Sequencing Reaction

The Sanger dideoxy-mediated chain termination sequencing method involves the synthesis of a DNA strand by the T7 sequenase v2.0 DNA polymerase using a ssDNA template. DNA synthesis is initiated at the site where a primer anneals to the template and is carried out in two steps to ensure efficient incorporation of radioactive precursors in the newly synthesized DNA (figure 2.3). The first is the labeling step and the second is the chain termination step using ddNTPs. In the first step, the primer is extended using limiting concentrations of the dNTPs, including radioactively labeled dATP ($[^{35}$S]-dATP). This step continues to virtual complete incorporation of labeled nucleotides into DNA chains which are distributed randomly in length from several nucleotides to hundreds of nucleotides. The longer extensions are much less common, but they contain proportionately more label so they still appear on gels. In the second step, the concentration of all the dNTPs is increased (what results in the further elongation of each chain at a very rapid rate) and a ddNTP is added (dNTP:ddNTP = 10:1). Processive DNA synthesis occurs until all growing chains are terminated by a ddNTP. During this step, the chains are extended on the average only several dozen nucleotides. The reactions are terminated by the addition of EDTA and formamide (stop solution), denatured by heating and run on electrophoresis gels. The labeled chains of various lengths can be visualized by autoradiography. The 'T7 sequenase v2.0 DNA sequencing kit' from Amersham Pharmacia Biotech has been used.
### 2. MATERIALS AND METHODS

#### A. Annealing

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA1 (1-8 µg)</td>
<td>3.7</td>
<td>3.5</td>
<td>3.7</td>
<td>3.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DNA2, up to 5 µl</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primer (10 ng)</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 M NaOH</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

Inoculate for 10 min at 37°C (meanwhile, prepare B and C1)

#### B. Labeling

1. Add 5.5 µl to each tube of A
2. Incubate for 5 min at RT, prepare C1 and C2

#### C. Termination

1. Fill 2.5 µl of ddG, ddA, ddT or ddC termination mix into tubes and prepare them opened in centrifuge
2. Transfer 3.5 µl of solution A+B on the wall of the tubes
3. When the 5 min of B are over: centrifuge down all drops
4. Incubate for 5 min at 37°C

#### D. Stop

1. Add 4 µl of 2x stop solution to each tube
2. Heat for 3 min at 75°C, put on ice and load 3 µl onto sequencing gel, or store at -20°C

Figure 2.3: Protocol for sequencing.

---

### 2.2.1.6.2. Denaturing Gel Electrophoresis and Autoradiography

The four sequencing reactions are separated by high-resolution electrophoresis. The quality of the gel electrophoresis is often the factor which limits the extend of sequence information that can be determined in a single sequencing experiment. The length of time the gel is run will determine the region of sequence that is readable. Under optimal conditions, 300 or more bases can be read starting at the bottom of a gel of about 35 cm length.

Polyacrylamide gels are, compared with agarose gels, more effective for separating small fragments of DNA. Their resolving power is extremely high, and fragments of DNA that differ in size by as little as one base can be separated from one another. Acrylamide (figure 2.4) is a monomer. In the presence of free radicals, which are usually supplied by APS and stabilized by TEMED, a chain reaction is initiated in which monomers of acrylamide are polymerized into long chains. When the bifunctional agent bis-acrylamide (figure 2.4) is included in the polymerization reaction, the chains become crosslinked to form a gel, whose porosity is determined by the length of the chains and the degree of crosslinking.
2. MATERIALS AND METHODS

The length of the chains is determined by the concentration of acrylamide in the polymerization reaction (table 2.11).

<table>
<thead>
<tr>
<th>Acrylamide (w/v)</th>
<th>Range of Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.5%</td>
<td>1000 – 2000 bp</td>
</tr>
<tr>
<td>5%</td>
<td>80 – 500 bp</td>
</tr>
<tr>
<td>8%</td>
<td>60 – 400 bp</td>
</tr>
<tr>
<td>12%</td>
<td>40 – 200 bp</td>
</tr>
<tr>
<td>15%</td>
<td>25 – 150 bp</td>
</tr>
<tr>
<td>20%</td>
<td>6 – 100 bp</td>
</tr>
</tbody>
</table>

Table 2.11: Separation range of linear DNA in polyacrylamide gels.

For the analysis of the products of DNA sequencing reactions, denaturing polyacrylamide gels are necessary. These gels are polymerized in the presence of an agent (urea) that suppresses base pairing in nucleic acids.

- wash the two glass plates with soap and dry them
- treat the smaller plate with dimethyldichlorosilane
- clean the plates with ethanol
- bring two spacers between the two plates and fix the whole 'sandwich' with clamps
- seal the lower part of the 'sandwich' with adhesive tape
- prepare the sequencing gel (6%, 50 ml):
  - fill 21 g urea, 5 ml 10x TBE and 7.5 ml 40% acrylamide mix to 50 ml with H₂O and dissolve (eventually in 37°C waterbath), then cool (chilled solution polymerizes slower) and add 50 µl TEMED and 214 µl 10% APS
  - immediately fill gel into the 'sandwich' (avoid air bubbles)
  - fix combs (inverted)
- let gel polymerize (put a weight on the upper plate)
- use gel directly or store it at 4°C (cover upper end with wetted paper)
- remove combs, clamps and adhesive tapes
- insert the 'sandwich' into the sequencing apparatus
- fill in 1 l of running buffer (1x TBE)
- let prerun the gel for at least 15 min (1200 V)
- remove salts from slots with pipette and carefully insert combs
- load samples (in the order GATC)
- run gel (1800 V)
  - short run: bromophenol blue on lower end of gel (about 75 min)
  - long run: xylene cyanol on lower end of gel (about 150 min)
- remove 'sandwich' from apparatus, remove spacers
2. MATERIALS AND METHODS

- carefully remove smaller plate (which is siliconized)
- cover gel on larger plate with blot paper
- lift paper with gel on it and cover gel with Saran wrap
- insert gel into dryer for at least 2 hrs (80°C) or 4 hrs (60°C)
- remove Saran wrap
- eventually check radioactivity with Geiger counter
- expose radioactive gel on medical X-ray film for at least one day
- develop film and read sequence

2.2.1.7. Northern Blotting

The transfer of RNA from a gel to a membrane is called 'Northern blot'. Northern hybridization may be used to quantitate and measure the size of specific mRNA molecules in cellular RNA preparations. Throughout the Northern blot experiment, one should be careful to avoid the introduction of RNases, as RNA is susceptible to degradation even after its immobilization on a nylon membrane.

2.2.1.7.1. The DIG-System for Filter Hybridization

The non-radioactive DIG system from Roche Diagnostics is based on digoxigenin, a steroid isolated from digitalis plants. The blossoms and leaves of these plants are the only natural sources of digoxigenin. The steroid is coupled to a dUTP via an alkali-labile esterbond (figure 2.5), or to UTP. DIG-nucleotides are used as substrates for DNA polymerases (such as E.coli DNA polymerase I, T4 DNA polymerase, T7 DNA polymerase, reverse transcriptase, and Taq DNA polymerase), as well as RNA polymerases (SP6, T7, or T3 RNA polymerases), and terminal transferase.

![Structure of alkali-labile DIG-11-dUTP](image)

Figure 2.5: Structure of alkali-labile DIG-11-dUTP.

After hybridization of the labeled probe to DNA or RNA on a membrane, the blot is submitted to immunological detection using \(\alpha\) DIG antibody conjugated to AP. The detection of DIG-labeled probe can be performed either with colorimetric or chemiluminescent substrates. If a colorimetric substrate is used, the signal develops directly on the membrane. CSPD is a chemiluminescent substrate for AP that enables extremely sensitive and fast detection of biomolecules by producing visible light which is recorded on X-ray films. Enzymatic dephosphorylation of CSPD
by AP leads to the metastable phenolate anion which decomposes and emits light at a maximum wavelength of 477 nm (figure 2.6).

![CSPD reaction catalyzed by AP](image)

Figure 2.6: CSPD reaction catalyzed by AP.

### 2.2.1.7.2. Preparation of RNA Probes

DIG-labeled RNA probes demonstrate stronger signals and less non-specific hybridization than DNA probes on Northern and Southern blots. DIG-labeled ssRNA probes of defined length are generated by *in vitro* transcription using the 'DIG RNA labeling kit' from Roche Diagnostics. DIG-UTP (alkali-resistant) is incorporated by SP6, T7 and T3 RNA polymerases. The plasmid vector should contain a promoter for RNA polymerase. For the synthesis of 'run off' transcripts, the plasmid is linearized by a restriction enzyme (to avoid transcription of undesirable sequences, use an enzyme that leaves 5'-overhangs or blunt ends). For long-term storage, labeled RNA should be precipitated with ethanol and also be stored under ethanol at -20°C or below. Antisense RNA probes have to be generated for analysis of transcription patterns in Northern or *in situ* hybridization experiments.

- digest plasmid vector with appropriate restriction enzyme
- purify linearized vector by gel extraction
- perform *in vitro* transcription using the 'DIG RNA labeling kit' from Roche Diagnostics:
  - add 1 µg linearized vector to DEPC-treated H₂O to a final volume of 13 µl
  - add the following on ice:
    - 2 µl NTP labeling mix
    - 2 µl transcription buffer (10x)
    - 1 µl RNase inhibitor (20 U)
    - 2 µl T3 or T7 RNA polymerase (40 U)
  - incubate for 2 hrs at 37°C
  - optional: add 2 µl RNase-free DNase I (20 U) to remove template DNA, incubate for 15 min at 37°C and stop reaction by adding 2 µl 200 mM EDTA pH 8.0

### 2.2.1.7.3. Hybridization

Prehybridization prepares the membrane for probe hybridization by blocking non-specific nucleic acid binding sites on the membrane with protein. This ultimately serves to lower background. Labeled probes can hybridize non-specifically to sequences that bear homology to the
probe sequence. Such hybrids are less stable than perfectly matched hybrids and can be dissociated by performing washes of various stringency (the lower the salt concentration and the higher the wash temperature, the more stringent the wash).

The appropriate hybridization temperature is calculated according to the equations in table 2.12. The actual hybridization temperature $T_{hyb}$ is 20-25°C below the calculated $T_m$ value (oligonucleotides -10°C). $T_m$ and renaturation of DNA are primarily influenced by four parameters: Temperature, pH, concentration of monovalent cations (higher salt concentrations increase the stability of the hybrid because cations interact with phosphate groups of nucleic acids and decrease therefore electrostatic repulsion between the two strands of the duplex) and presence of organic solvents (reduce thermal stability of the polynucleotides). Mismatching of basepairs results in reduction of both hybridization rates and thermal stability of the resulting duplexes. On the average, the $T_m$ decreases about 1°C per % base mismatch for large probes.

<table>
<thead>
<tr>
<th>System</th>
<th>Formula A</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA-DNA hybrids B</td>
<td>$T_m=81.5^\circ C+16.6\log [Na^+] + 0.41(%GC) - 0.61(%for) - 500/N$</td>
</tr>
<tr>
<td>DNA-RNA hybrids B</td>
<td>$T_m=79.8^\circ C+18.5\log [Na^+] + 0.58(%GC) + 11.8(%GC)^2 - 0.50(%for) - 820/N$</td>
</tr>
<tr>
<td>RNA-RNA hybrids B</td>
<td>$T_m=79.8^\circ C+18.5\log [Na^+] + 0.58(%GC) + 11.8(%GC)^2 - 0.35(%for) - 820/N$</td>
</tr>
<tr>
<td>oligonucleotides C</td>
<td>for oligos 14-25 nucleotides in length: $T_m=2^\circ C(A+T)+4^\circ C(G+C)$ for oligos longer than 25 nucleotides: see formula for DNA-DNA hybrids</td>
</tr>
</tbody>
</table>

$A$ [Na$^+$] between 0.01-0.4 M and %GC between 30-75% (if [Na$^+$] > 0.4 M: no [Na$^+$] term)

$B$ $T_{hyb}=T_m-20$ to -25°C

$C$ $T_{hyb}=T_m-10^\circ C$

Table 2.12: Formulas to calculate melting temperature $T_m$. [Na$^+$]: concentration of Na$^+$ ions, %GC = percentage of G and C nucleotides in the nucleic acid, %for = percentage of formamide in the hybridization solution, N = length of the duplex in bp.

- soak membrane in H$_2$O
- prehybridize in 'DIG easy hyb' solution at 68°C for 2 hrs under gentle agitation
- add RNA probe (not boiled, dissolved in 200 µl H$_2$O) to 'DIG easy hyb' solution and incubate overnight at 68°C (100 ng/ml)
- remove hybridization solution
- wash membrane 2x for 5 min in 2x washing solution at RT
- wash membrane 2x for 15 min in 0.5x washing solution at 68°C
- perform DIG-detection

2.2.1.7.4. Detection

Chemiluminescent detection is a three-step process. In the first step, membranes are treated with blocking reagent to prevent non-specific binding of antibody to the membrane. Then, membranes are incubated with a dilution of α DIG-AP conjugate. In the third step, the membrane carrying the hybridized probe and bound antibody conjugate is reacted with a chemiluminescent substrate and exposed to a X-ray film to record the chemiluminescent signal.
2. MATERIALS AND METHODS

- rinse membrane briefly in washing buffer for 2 min
- allow the chemiluminescent substrate CSPD to come to RT
- incubate membrane for 30 min (or longer) in blocking buffer at RT
- dilute α DIG-AP conjugate 1:10'000 in blocking buffer (centrifuge antibody for 2 min at full speed to remove precipitates)
- incubate membrane for 30 min in antibody solution
- wash 2x for 15 min in washing buffer
- equilibrate 2 min in detection buffer
- prepare CSPD substrate solution (1 ml per 100 cm² of membrane; dilute CSPD 1:100 in detection buffer)
- place dripped off membrane on a clean plastic sheet (do not use Saran wrap)
- add CSPD substrate solution to membrane and spread liquid over the whole surface
- cover the membrane with a second sheet of plastic foil
- incubate for 15 min at 37°C to enhance the luminescent reaction
- expose at RT to medical X-ray film (30 min as starting point, luminescence continues for at least 48 hrs and signal intensity increases during the first hrs)

2.2.1.7.5. Stripping

Due to the stability of RNA-RNA hybrids, stripping of Northern blots is not always successful. When stripping and rehybridization of blots is planned, the membrane should not dry off at any time.

- wash blot for 2 min in washing buffer
- heat 100 ml of 0.5% SDS solution in a 500 ml beaker to 90-100°C
- shortly before the SDS solution starts to boil, transfer the membrane to a clean tray (make sure that exposure to air is minimal)
- pour the boiling SDS solution over the membrane
- incubate for 10 min on a rocking platform
- start with prehybridization or air-dry until blot is dry enough to be slipped into a plastic bag (store at -20°C)

2.2.1.8. Bacteria Manipulations

Bacteria are used in molecular biology as tools to produce large amounts of plasmid DNA and to isolate a single plasmid DNA out of a mixture (cloning).

2.2.1.8.1. Determination of Bacteria Concentration

The concentrations of bacterial liquid cultures are measured spectrophotometrically at wavelength 600 nm.
2. MATERIALS AND METHODS

- dilute bacterial liquid culture 1:10 in LB medium
- measure light emission (OD) at \( \lambda = 600 \text{ nm} \)

\[
1 \text{ OD}_{600} = 8 \times 10^8 \text{ cells/ml}
\]

- calculate: \( \text{OD}_{600} \times 10 \times 8 \times 10^8 = y \text{ cells/ml} \)
- \( \sim 1-5 \mu\text{g DNA/ml} \) (varies between different strains and plasmids) → calculate: \( z \text{ g DNA/cell} \)

2.2.1.8.2. Freezing Bacteria

Bacteria can be stored for indefinite time in cultures containing glycerol (to avoid formation of crystal water).

- add 150 µl sterile glycerol to 850 µl of bacterial culture to give a final glycerol concentration of 15% (v/v)
- vortex culture
- freeze culture in liquid N\(_2\) (to avoid sedimentation of bacteria) and store at -80°C

2.2.1.8.3. Production of LB Agar Plates

Clones are streaked onto agar plates containing the appropriate selective agent so that single colonies can be isolated. This procedure should then be repeated to ensure that a single colony of an antibiotic-resistant clone can be picked.

- add 1.5% (w/v) Bacto agar to LB medium and melt in microwave oven
- allow medium to cool until touchable without pain (–50°C)
- add ampicillin (100 µg/ml) or kanamycin (30 µg/ml)
- pour into sterile 90 mm plates (remove air bubbles with flame of Bunsen burner)
- when medium has hardened completely, invert the plates and store them at 4°C until needed
- before use, incubate plates at 37°C in the inverted position for about 1 hr to remove condensation within the plates

2.2.1.8.4. Transformation of Bacteria

Bacteria can be treated to induce a transient state of 'competence' during which they are able to take up DNA (membrane becomes permeable). How such agents act is unknown, as is the mechanism by which plasmid DNA enters competent \textit{E.coli} cells.
2.2.1.8.4.1. Production of Competent Cells

This treatment prepares the bacteria so that they are able to take up DNA derived from a variety of sources during CaCl$_2$-transformation.

- inoculate a single colony of *E.coli* cells in 5 ml LB medium overnight (without antibiotics)
- add 2 ml of the culture to 200 ml LB medium in a 2 l flask
- grow at 37°C to an OD$_{550}$ = 0.48 under vigorous shaking (2-2.5 hrs)
- chill cells in ice water (to stop growth of cells)
- fill cells into 250 ml prechilled centrifuge beaker
- centrifuge cells for 10 min at 3000 rpm and 4°C
- pour off supernatant and resuspend pellet in 60 ml precooled transformation buffer 1 on ice
- incubate the culture for at least 2 hrs on ice
- centrifuge cells for 10 min at 3000 rpm and 4°C
- pour off supernatant and resuspend pellet in 8 ml precooled transformation buffer 2 on ice
- aliquot the competent cells (e.g. 300 µl) and freeze in liquid N$_2$, store at -80°C

2.2.1.8.4.2. Transformation

Transformation is called the delivery of DNA to bacteria cells. There are two different methods to transfer DNA into *E.coli*: CaCl$_2$-transformation and electroporation. Below is shown the procedure for CaCl$_2$-transformation.

- thaw cells (-80°C) on ice
- add 5 µl of ligation reaction to 45 µl cells (100 pg pGEM-2 as positive control), mix on ice
- incubate for 15-30 min on ice
- incubate for 5 min at 37°C (or 2 min at 42°C)
- incubate for 2 min on ice
- add 250 µl LB medium (without antibiotics)
- incubate for 45 min at 37°C (turn tube every 15 min)
- plate e.g. 100 µl (of total 300 µl) on LB agar plate (with antibiotic), invert plate and incubate overnight at 37°C

2.2.1.8.4.3. Evaluation of Transformation Efficiency

Depending on the transformation technique used, there are expectation values for the transformation frequency (table 2.13).

- count colonies per plate → x
- calculate amount of DNA [µg] per plate (only vector) → y
- calculate colonies per µg DNA → x/y
- expectations (colonies per µg DNA):


2. MATERIALS AND METHODS

<table>
<thead>
<tr>
<th></th>
<th>CaCl₂-Transformation</th>
<th>Electroporation</th>
</tr>
</thead>
<tbody>
<tr>
<td>vector (linear, dephos.) + insert</td>
<td>$10^4$</td>
<td>$10^6 - 10^7$</td>
</tr>
<tr>
<td>only vector (linear, dephos.)</td>
<td>$10^3 - 10^4$</td>
<td>$10^5$</td>
</tr>
<tr>
<td>+ control (vector circular)</td>
<td>$10^7$</td>
<td>$1x10^8 - 5x10^8$</td>
</tr>
</tbody>
</table>

Table 2.13: Expectation data for transformations.

2.2.2. Cell Biology

Cell biology involves techniques used for growing and analyzing of cells in culture. Freshly from a piece of organ or tissue isolated cells are known as 'primary cultures'. A primary culture may be regarded as such until it is subcultured for the first time. It then becomes a 'finite' cell line which can be subcultured for a limited number of passages. An 'established' or 'continuous' cell line, in contrast, has a nearly unlimited lifespan in culture. Such cells originate from spontaneous immortalized primary cells or from transformed cells.

All media used in cell culture have as a basis a synthetic mixture of inorganic salts to maintain proper pH, to maintain ideal osmotic pressure, and to provide a source of energy. Mammalian cells can survive over a wide pH range (6.6-7.8), but the optimal growth of cells is obtained at pH 7.2-7.4. As cells in culture produce acidic products which act to lower the pH of the medium, a buffer is included in the medium. The most commonly used buffer is sodium bicarbonate. The bicarbonate ion can be converted to gaseous CO₂ and lost from the medium, resulting in a rise in the pH. It is possible to control this by artificially supplying CO₂ to the atmosphere and preventing the gas from leaving the liquid.

2.2.2.1. Cell Passage

Cells grown in monolayer lay down ECM and attachment proteins that permit them to adhere to the growth surface. Cells to be passaged or harvested are typically detached by treatment with trypsin, a proteolytic enzyme from bovine pancreas that disrupts the matrix and attachment proteins (trypsin causes cellular damage and therefore time of exposure should be kept to a minimum). The term 'passage' denotes the transfer of cells from one culture vessel to another.

- remove spent culture medium from flask
- wash cells with PBS
- add 1 ml 1x trypsin-EDTA solution per 75 cm² flask, incubate at 37°C until the cells loose contact with the flask (check under microscope)
- add 2 ml culture medium (contains trypsin inhibitors) and prepare single cell suspension with fire-polished glass pipette
- plate cells in new culture vessels, add fresh culture medium
- distribute cells inside flasks (no circle movements) and incubate at 37°C and 5% CO₂
2. MATERIALS AND METHODS

2.2.2.2. Cell Freezing

While short-term preservation of cell lines using mechanical freezers (-80°C) is possible, storage in liquid N\textsubscript{2} (-196°C) is much preferred because of almost infinite storage times. Cellular damage induced by freezing and thawing is generally believed to be caused by intracellular ice crystals and osmotic effects. The addition of a cryoprotective agent, such as DMSO, and the selection of suitable freezing and thawing rates minimizes cellular injury.

- prepare culture medium with 10% (v/v) DMSO, filter sterilize and incubate at 4°C
- trypsinize adherent cells
- resuspend cells in e.g. 4 ml culture medium with 10% (v/v) DMSO
- make 1 ml aliquots in cryotubes
- incubate aliquots in polystyrene box at -80°C for 2-3 days
- transfer aliquots to liquid N\textsubscript{2} refrigerator at -196°C

2.2.2.3. Cell Reconstitution from DMSO Cultures

Rapid thawing of the cell suspension is essential for optimal recovery. The DMSO has to be removed because it is toxic to the cells at high concentrations.

- remove cryotube from frozen storage and quickly thaw cells in a 37°C waterbath
- transfer the contents to a tube containing about 10 ml of culture medium
- centrifuge at 700 rpm for 5 min
- remove supernatant
- add 5 ml of fresh culture medium and resuspend cells
- plate cells in a culture flask

2.2.2.4. Coating of Coverslips

Normal attachment, growth, and development of many cell types are dependent on attachment factors and ECM components. While some cells are able to synthesize these components, others require an exogenous source. Examples are collagen type I, fibronectin, gelatin or laminin. Artificial poly-L-lysine increases the number of positively charged sites available for cell binding.

- eventually treat coverslips with HCl and NaOH, air-dry and autoclave them
- drop several times about 30 μl coating solution on a dish (50 μg/ml collagen type 1, 10 μg/ml fibronectin, 1% gelatin, 20 μg/ml laminin, or 250 μg/ml poly-L-lysine in H\textsubscript{2}O)
- put 12 mm coverslips on drops
- incubate for at least 30 min at RT
- wash coverslips with PBS (free poly-L-lysine may be cytotoxic)
- lift coverslips and transfer them with their coated side up into a 24 well plate
- eventually cover with PBS to avoid drying up
2. MATERIALS AND METHODS

2.2.2.5. Plating Cells

Accurate enumeration of cell density is an important aspect of cell culture. It is comfortable to plate cells on coverslips which can be removed from the 24 well plate and analyzed under the microscope.

- trypsinize cells
- add 2 ml culture medium and prepare single cell suspension with fire-polished glass pipette
- count cells in a Neubauer counting chamber (count three large squares: average e.g. 73, then concentration is $73 \times 10^4$ cells/ml)
- dilute cells in culture medium to $6.5 \times 10^4$ cells/ml
- add 3 ml of the suspension to each 60 mm plate or 300 µl to each well of a 24 well plate ($2 \times 10^5$ cells/60 mm plate, $2 \times 10^4$ cells/well of 24 well plate); the cell number seeded should produce 40-60% confluence on the day of transfection
- incubate plate for about 18 hrs at 37°C and 5% CO₂

2.2.2.6. Transient Transfection

Transfection is the delivery of foreign molecules such as DNA into eukaryotic cells. The transfection efficiency is influenced by a variety of parameters including quality of the cell culture, character of the cell line, choice of the transfection method and quality of the plasmid DNA used. Of the variety of different transfection methods described in the literature, the DEAE-dextran method, the calcium phosphate method, electroporation and liposome-mediated transfection are the most commonly used. When cells are transiently transfected, the DNA is introduced into the nucleus of the cell, but does not integrate into the chromosome. This means that many copies of the gene of interest are present, leading to high levels of expressed protein. With stable or permanent transfection, the transfected DNA is integrated into the chromosomal DNA.

In calcium phosphate-mediated transfection, calcium phosphate is used as a carrier to deliver DNA into cells (Chen and Okayama, 1987). Plasmid DNA is mixed with CaCl₂ and 2x BBS at pH 6.95. This mixture is added directly to medium in a dish containing cells and incubated overnight under a low CO₂ atmosphere (at 3.5% CO₂ the medium becomes slightly basic after overnight incubation, pH 7.6-7.7 compared to pH 7.2-7.4 under normal conditions, because the sodium bicarbonate buffer system releases CO₂ leading to increase of pH). Because of the high pH, the calcium phosphate/DNA complex forms gradually in the medium and precipitates on cells. This slow development of the calcium phosphate/DNA complex in the medium seems to be the major factor contributing to high transformation efficiency. The calcium phosphate is thought to facilitate the binding of the DNA to the cell surface. It is believed that the DNA then enters the cell by endocytosis.
2. MATERIALS AND METHODS

- remove spent culture medium from plate
- add 2.8 ml fresh culture medium per 60 mm plate (for 24 well plate always use 1/10 volumes)
- return plate to incubator, reduce [CO₂] to 3.5%
- mix transfection sample (mix 4 µg DNA with 100 µl 0.25 M CaCl₂ and vortex, then add 100 µl 2x BBS and vortex)
- leave sample at RT for 15 min
- add 200 µl of this precipitate dropwise to each 60 mm plate while gently moving the plate (1-3 µg DNA/ml medium)
- incubate cells overnight at 37°C and 3.5% CO₂
- remove spent culture medium from wells
- wash cells 2x with PBS and add fresh culture medium
- incubate for 24-60 hrs at 37°C and 5% CO₂

2.2.2.7. Fluorescent Antibody Staining

The indirect fluorescent antibody staining technique involves two general steps. Firstly, a primary antibody is used to label cells expressing an epitope. Then a secondary antibody, which is specific to the primary antibody type and is coupled to a fluorescent dye, is applied. By virtue of the fluorescence, the antibody-antigen complexes can be visualized under the fluorescence microscope. Fluorescence is an optical phenomenon in which UV or short wavelength visible light energy is absorbed by a molecule (fluorophore) and rapidly emitted as light of a longer wavelength.

- remove spent culture medium from 24 well plate
- wash cells 3x with DPBS
- eventually fix cells:
  - fix cells with 3% (w/v) paraformaldehyde in PBS (depolymerized at 70°C) for 15 min at RT
  - wash cells with DPBS
  - incubate cells for 5 min with 100 mM glycine in PBS at RT (to reduce non-reactive aldehyde groups)
  - wash cells with DPBS
- eventually permeabilize cells:
  - incubate cells for 15 min with 0.2% Triton X-100 in PBS at RT
  - wash cells 2x with DPBS
- or fix and permeabilize cells in one step:
  - immerse coverslips in 300 µl methanol:acetone 1:1 -20°C
  - incubate for 10 min at -20°C
  - air-dry coverslips at RT
- add blocking solution (2% (w/v) skim milk powder in PBS) and incubate for 10 min at RT on a rocking platform
- wash cells with DPBS
- add 300 µl of primary antibody in blocking solution and incubate for at least 60 min at RT
- wash cells 3x for 5 min with DPBS
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- add 300 µl of secondary antibody in blocking solution and incubate for 60 min at RT (centrifuge solution to remove antibody precipitates)
- wash cells 3x for 5 min with DPBS
- mount coverslips in Lisbeth's embedding medium:
  - let aliquot of Lisbeth's embedding medium come to RT
  - drop 7 µl of Lisbeth's embedding medium onto a microscope slide
  - remove coverslips from 24 well plate, drain off excess liquid on a paper towel
  - mount coverslips on drop, fix coverslips with 3 drops of nail-varnish and let dry
  - seal with nail-varnish
- or mount in Mowiol embedding medium:
  - drop 5 µl of Mowiol embedding medium onto a microscope slide
  - remove coverslips from 24 well plate
  - dip coverslips into H2O (to remove salts), drain off excess liquid on a paper towel
  - mount coverslips on drop
  - allow Mowiol embedding medium to dry for at least 60 min in the dark
- incubate slides at 4°C and check preparations under fluorescence microscope or confocal laser scanning microscope

2.2.3. Biochemistry

Biochemistry is the chemistry of life and involves the techniques used for the identification and analysis of proteins.

2.2.3.1. Preparation of Protein Extracts

Mammalian cells in culture may be lysed gently with detergents. The target antigen should stay in a form that is immunoreactive and undegraded. The efficiency of extraction is often affected by the pH and ionic strength of the lysis buffer, the concentration and type of detergent used, and the presence or absence of chelating agents such as EDTA and EGTA. To minimize proteolytic activity in cell lysates, they should be kept cold, and protease inhibitors should be included in the lysis buffer (mix contains several protease inhibitors with broad inhibitory specificity).

- remove spent culture medium from dish
- wash cells twice with ice cold PBS
- add 160 µl of ice cold lysis buffer per 60 mm dish
- remove cells with scraper and fill into a chilled tube
- homogenize carefully with pipette (yellow tip) or with a syringe fitted to a 21 gauge needle (to shear the chromosomal DNA)
- remove aliquot for determination of protein concentration
- mix extract with 5x Lämmli loading buffer and boil for 3 min (to denature proteins and dissolve SDS which might have been precipitated)
- store samples at -20°C
Protein extracts can also be prepared using tissues. Mammalian tissues are usually dispersed mechanically and then dissolved in Lämmli loading buffer.

- freeze tissue in liquid N₂
- weigh piece of tissue (≈15%)
- transfer tissue in dounce homogenisator and add 85% lysis buffer
- homogenize carefully
- remove aliquot for determination of protein concentration
- mix extract with 5x Lämmli loading buffer and boil for 3 min
- store samples at -20°C

### 2.2.3.2. Determination of Protein Concentration

Protein concentrations of extracts are measured by a Bradford assay. This assay is based on the observation that the absorbance maximum for an acidic solution of Coomassie brilliant blue G-250 shifts from 465 nm to 595 nm when binding to protein occurs. Beer's law may be applied for accurate quantitation of protein by selecting an appropriate ratio of dye volume to sample concentration.

- prepare several dilutions of protein standard (0, 1, 2, 4, 8, 16 and 32 µg BSA in 0.8 ml H₂O)
- dilute 8 µl of each sample in 0.8 ml H₂O (place 0.8 ml H₂O in 'blank' tube)
- add 0.2 ml 5x protein assay reagent
- vortex and incubate for 5 min to 1 hr at RT
- measure OD₅₉₅ versus reagent blank
- plot OD₅₉₅ versus concentration of standards (µg) and read unknowns from the standard curve (x µg/8 µl = y µg/µl)

### 2.2.3.3. SDS-PAGE and Western Blotting

PAGE (polyacrylamide gel electrophoresis) is a widely used technique for the identification and characterization of proteins (Laemmli, 1970). Visualization of the proteins in electrophoresis gels involves the use of one or more of the many staining procedures which have been developed. In Western blotting, the samples to be assayed are solubilized with detergents and reducing agents, separated by SDS-PAGE and transferred to a membrane which may be stained (figure 2.7). The filter is then exposed to antibodies specific for the target protein. Finally, the bound antibody is detected by a labeled secondary antibody. As little as 1-5 ng of an average-sized protein can be detected by Western blotting.
2. MATERIALS AND METHODS

Figure 2.7: Steps in Western blotting. (1) SDS-PAGE of proteins. (2) Transfer of proteins from gel to membrane. (3) Immunological detection of target proteins with antibodies. (4) Visualization of antibody-antigen complexes.

2.2.3.3.1. SDS-PAGE of Proteins

Gel electrophoresis separates biomolecules in complex mixtures according to their physical properties of mass and charge. Protein electrophoresis is generally performed in polyacrylamide gels which are composed of long linear polyacrylamide chains crosslinked with bis-acrylamide (figure 2.4) to create a network of pores (table 2.14).

<table>
<thead>
<tr>
<th>Acrylamide (w/v)</th>
<th>Range of Separation</th>
</tr>
</thead>
<tbody>
<tr>
<td>6%</td>
<td>60 – 220 kDa</td>
</tr>
<tr>
<td>8%</td>
<td>40 – 100 kDa</td>
</tr>
<tr>
<td>10%</td>
<td>20 – 70 kDa</td>
</tr>
<tr>
<td>12%</td>
<td>20 – 60 kDa</td>
</tr>
<tr>
<td>15%</td>
<td>10 – 40 kDa</td>
</tr>
</tbody>
</table>

Table 2.14: Separation range of proteins in SDS-polyacrylamide gels.

In SDS-PAGE, proteins are denatured by heating them in buffer containing the strong anionic detergent SDS and a thiol reducing agent to break disulfide bonds, such as β-mercaptoethanol or DTT. The denatured polypeptides bind SDS (1.4 g SDS/g protein) and become negatively charged. Because the amount of SDS bound is proportional to the molecular mass of the polypeptide, SDS-polypeptide complexes migrate through polyacrylamide gels in accordance with the size of the polypeptide.

The gel has two parts: An upper stacking and a lower running gel. The samples and the stacking gel contain Tris-HCl pH 6.8, the electrophoresis buffer contains Tris-glycine pH 8.3, and the running gel contains Tris-HCl pH 8.8. All components of the system contain 0.1% SDS. The denatured samples are loaded into the wells, and current is applied. The Cl⁻ ions already present in the gel run faster than the SDS-proteins and form an ion front. The glycine molecules flow in from the electrophoresis buffer and form a front behind the proteins. A voltage gradient is created between Cl⁻ ions and glycine molecules which concentrates the proteins in between. On the surface of the running gel, the higher pH favors the ionization of glycine, and the resulting glycine ions migrate through the stacked polypeptides and travel through the running gel immediately behind the Cl⁻ ions. Freed from the moving boundary, the SDS-polypeptide complexes move through the running gel (smaller pore size than stacking gel) and are separated according to size.
2. MATERIALS AND METHODS

- prepare one or two 'sandwiches' containing cleaned glass plates, alumina plates and two spacers (1.5 mm), in gel caster
- mark the position of the stacking gel (length of teeth of comb plus 1 cm)
- mix running gel (15 ml for two gels) in the following order:

<table>
<thead>
<tr>
<th>Components</th>
<th>8%</th>
<th>10%</th>
<th>12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>6.9 ml</td>
<td>5.9 ml</td>
<td>4.9 ml</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>4 ml</td>
<td>5 ml</td>
<td>6 ml</td>
</tr>
<tr>
<td>1.5 M Tris-HCl pH 8.8</td>
<td>3.8 ml</td>
<td>3.8 ml</td>
<td>3.8 ml</td>
</tr>
<tr>
<td>10% SDS solution</td>
<td>150 µl</td>
<td>150 µl</td>
<td>150 µl</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>150 µl</td>
<td>150 µl</td>
<td>150 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>9 µl</td>
<td>6 µl</td>
<td>6 µl</td>
</tr>
</tbody>
</table>

- immediately pour gel into the 'sandwich' up to the mark (avoid air bubbles)
- overlay the gel with isopropanol (gel ≥10%) or 0.1% SDS (gel ≤8%) to prevent oxygen from diffusing into the gel and inhibiting polymerization
- let gel polymerize (about 30 min)
- pour off the overlay and wash the top of the gel with H₂O (to remove unpolymerized acrylamide), then remove H₂O completely with a paper towel
- clean comb with H₂O and dry with ethanol
- mix stacking gel (8 ml for two gels) in the following order:

<table>
<thead>
<tr>
<th>Components</th>
<th>5%</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>5.5 ml</td>
</tr>
<tr>
<td>30% acrylamide mix</td>
<td>1.3 ml</td>
</tr>
<tr>
<td>1 M Tris-HCl pH 6.8</td>
<td>1 ml</td>
</tr>
<tr>
<td>10% SDS solution</td>
<td>80 µl</td>
</tr>
<tr>
<td>10% APS solution</td>
<td>80 µl</td>
</tr>
<tr>
<td>TEMED</td>
<td>8 µl</td>
</tr>
</tbody>
</table>

- immediately pour gel onto the surface of the polymerized running gel (avoid air bubbles)
- insert comb into stacking gel solution
- let gel polymerize (about 30 min)
- remove comb carefully and rinse slots with H₂O (to remove unpolymerized acrylamide)
- remove gel 'sandwiches' from the gel caster
- mount the 'sandwiches' against the core with the alumina plate against the gasket using the clamps
- position the central core across the center of the buffer chamber and push down gently
- add about 300 ml of electrophoresis buffer to the top and bottom reservoirs (do not prerun the gel since this will destroy the discontinuity of the buffer systems)
- load samples onto the gel (up to 100 µg total cellular protein; load an equal volume of 1x Lämmli loading buffer into any slots that are unused)
- place the safety lid on the unit and attach the leads to the power supply
- run gels at 40 mA (20 mA per gel) until the bromophenol blue reaches the bottom of the running gel (about 2 hrs)
- remove the side clamps and lift each gel 'sandwich' off the core
2.2.3.3.2. Western Transfer

In the blotting step, electrophoretically separated proteins are transferred from a gel to a solid support. The gel and its attached nitrocellulose membrane are sandwiched between pieces of blot paper that have been soaked in transfer buffers. The ‘sandwich’ is then placed between plate electrodes with the nitrocellulose filter on the anode side (figure 2.8).

- **cathode**
  - cathode buffer soaked
  - Whatman 3MM papers (2x)
  - gel
  - nitrocellulose filter
  - anode buffer 2 soaked
  - Whatman 3MM papers (2x)
  - anode buffer 1 soaked
  - Whatman 3MM paper
  - mylar mask

- **anode**

Figure 2.8: Arrangement in semi-dry electrotransfer unit.

- cut five pieces of blot paper and one piece of nitrocellulose filter to the exact size of the SDS-polyacrylamide gel (5 cm x 8.5 cm; wear gloves)
- float the nitrocellulose filter on the surface of a tray of H₂O and allow it to wet, then submerge it and heat to 70°C (to remove air inside the filter), incubate membrane for at least 10 min in heated H₂O
- soak the pieces of blot paper:
  - 2 papers in cathode buffer
  - 1 paper in anode buffer 1
  - 2 papers in anode buffer 2
- remove glass plate
- remove alumina plate
- put one paper with cathode buffer onto rough side (alumina plate side) of gel (use glass pipette as a roller and squeeze out any air bubbles)
- cut gel to fitting size (remove stacking gel)
- turn gel
- put nitrocellulose filter onto 'sandwich' (smooth side of gel; never remove filter from gel because protein transfer starts immediately)
- mark one corner of the filter
- put papers with anode buffer 2 onto 'sandwich'
- put paper with anode buffer 1 onto 'sandwich'
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- turn 'sandwich' and place onto anode
- put second paper with cathode buffer onto 'sandwich'
- place the upper electrode (cathode) on top of the stack
- place a weight on the cathode
- connect the electrical leads
- apply a current of 1.25 mA/cm² of gel (50 mA per gel) for 60 min
- turn off the electric current and disconnect the leads
- disassemble the transfer apparatus from the top downward

2.2.3.3.3. Ponceau S Staining

Staining of the nitrocellulose filter with Ponceau S is used to provide visual evidence that electrophoretic transfer of proteins has taken place and to locate molecular weight markers, whose positions on the filter are then marked. Staining with Ponceau S is completely compatible with all methods of immunological probing because the stain is transient and is washed away during processing of the Western blot.

- if the nitrocellulose filter has been dried, float it on the surface of a tray of H₂O and allow it to wet, then submerge it for at least 5 min
- transfer the filter to a tray containing Ponceau S solution
- incubate the filter for some sec with gentle agitation
- when the bands of protein are visible, rinse the membrane in H₂O, mark the positions of marker bands and wash the nitrocellulose filter in several changes of H₂O
- air-dry filter (eventually overnight)

2.2.3.3.4. Immunological Detection of Target Proteins

In specific antigen detection, unoccupied protein-binding sites on the nitrocellulose filter are saturated to prevent non-specific binding of antibodies. The blot is then probed for the proteins of interest with specific, primary antibodies. Secondary antibodies, specific for the primary antibody type and conjugated to detectable reporter groups, such as enzymes or radioactive isotopes, are then used to label the primary antibodies. Finally, the labeled protein bands are made visible by the bound reporter groups acting on a substrate, or by radioactive decay.

ECL (enhanced chemiluminescence) Western blotting is a light-emitting non-radioactive method for detection of immobilized specific antigens conjugated indirectly with peroxidase-labeled antibodies. Peroxidase/hydrogen peroxide catalyze the oxidation of luminol in alkaline conditions (figure 2.9). Immediately following oxidation, the luminol is in an excited state which then decays to ground state via a light-emitting pathway. Enhanced chemiluminescence is achieved by performing the oxidation of luminol by the peroxidase in the presence of chemical enhancers such as phenols. This has the effect of increasing the light output about 1000x. The emission can be detected by a short exposure to autoradiography films.
2. MATERIALS AND METHODS

- block membrane in blotto solution for 45 min at RT on a rocking platform
- incubate membrane with primary antibody diluted in blotto solution for 90 min at RT
- briefly rinse filter in TBS-T and wash 3x for 5 min
- incubate membrane with secondary antibody (IgG-peroxidase conjugate) diluted in blotto solution for 45 min at RT
- briefly rinse filter in TBS-T and wash 3x for 5 min
- wash filter for 5 min in TBS
- mix an equal volume of solution 1 and solution 2 of ECL Western blotting detection reagent (65 µl/cm² membrane)
- in the dark room, drain the excess buffer from the washed membranes and place them on a piece of Saran wrap, protein side up
- add the detection solution on the membrane
- incubate for precisely 1 min at RT without agitation
- drain off excess detection reagent
- wrap filter in fresh Saran wrap, gently smooth out air pockets
- place the blot, protein side up, in a film cassette and expose it to a medical X-ray film for 2 min
- remove film, immediately replace with a fresh film and reclose film cassette
- develop first piece of film immediately, and on the basis of its appearance estimate how long to continue the exposure of the second piece of film
- wash away detection solution from membrane with TBS-T
- store membrane wet wrapped in Saran wrap at 4°C

2.2.3.3.5. Stripping of Membranes

The complete removal of primary and secondary antibodies from membranes is possible. The membranes may be stripped of bound antibodies and reprobed several times.

- wash membrane with TBS-T
- incubate membrane with stripping buffer 2x for 40 min at RT
- briefly rinse filter in TBS-T and wash 3x for 5 min
- wash filter for 5 min in TBS
- eventually perform detection (check)
- wash away detection solution from membrane with TBS-T

Figure 2.9: ECL reaction catalyzed by peroxidase.
2. MATERIALS AND METHODS

2.2.3.4. Glycoprotein Analysis

Glycoproteins and other glycoconjugates play important roles in the cell. These molecules have been implicated in cellular immunity, viral infection, and development. To gain insight into the crude structure of the carbohydrate side chains attached to a protein, a number of glycosidases can be used. N-glycosidase F (from *Flavobacterium meningosepticum*) cleaves all types of asparagine-bound N-glycans from glycoproteins unless they carry $\alpha(1-3)$-linked core fucose residues (present in insect and plant glycoproteins). The reaction products are ammonia, aspartic acid (in the peptide chain) and the complete oligosaccharide. The enzyme deglycosylates a number of glycoproteins in their native form, but denaturation (e.g. by heating at 100°C in the presence of SDS) increases the deglycosylation rate considerably.

- remove spent culture medium from dish
- wash cells twice with ice cold PBS
- add 200 $\mu$l of ice cold GlycoF lysis buffer per 60 mm dish
- remove cells with scraper and fill into a chilled tube
- homogenize carefully with a pipette (yellow tip) or with a syringe fitted to a 21 gauge needle (to shear the chromosomal DNA)
- remove aliquot for determination of protein concentration
- eventually store samples at -20°C
- dilute cell lysate (40 $\mu$g) in GlycoF lysis buffer up to 48 $\mu$l
- boil for 10 min, put on ice and briefly centrifuge
- add N-glycosidase F (2 $\mu$l = 2 U), mix
- incubate overnight at 37°C
- analyze by Western blotting

2.2.3.5. Immunoprecipitation

Immunoprecipitation is used to detect and quantitate target antigens in mixtures of proteins. This technique allows to concentrate and purify small amounts of a protein in one step. Lysis procedures must be optimized to ensure maximal cell disruption with minimal damage to the protein. Mammalian cells in tissue culture may be lysed gently with detergents (Triton X-100 maintains protein-protein interactions). To reduce background caused by non-specific adsorption of irrelevant cellular proteins to protein G-sepharose, the cell lysates are first treated only with the protein G-sepharose in absence of the antibody (preclearing). After the addition of specific antibody directed against the target protein to the precleared cell lysate, the antigen-antibody complex is precipitated by addition of protein G-sepharose which binds to the $F_c$ region of the antibodies. The optimal level of antibody should be determined experimentally for each application.

- remove spent culture medium from dish
- wash cells twice with ice cold PBS
- add 200 $\mu$l of ice cold immunoprecipitation lysis buffer per 60 mm dish
- remove cells with scraper and fill into a chilled tube
• incubate the lysate on ice for 20 min and shake from time to time (solubilization of membrane proteins)
• homogenize carefully with pipette (yellow tip) or with a syringe fitted to a 21 gauge needle (to shear the chromosomal DNA)
• centrifuge at 10000 g and 4°C for 10 min (to remove debris), remove supernatant
• remove aliquot for determination of protein concentration
• mix extract (400 µl) with 20 µl protein G-sepharose (washed 3x in TEN, 1:1 in TEN) and incubate at 4°C for 45 min (on rotating wheel)
• centrifuge at 12000 g and 4°C for 2 min and collect supernatant
• add 30 µl protein G-sepharose and incubate at 4°C for 45 min (on rotating wheel)
• centrifuge at 12000 g and 4°C for 2 min (collect supernatant)
• wash pellet 3x with 1 ml immunoprecipitation lysis buffer (resuspend, rotate 15 min, centrifuge)
• resuspend pellet in 60 µl 2x Lämml loading buffer
• boil samples for 4 min and put on ice
• remove sepharose by centrifugation (2x) and analyze by Western blotting

2.2.4. Electron Microscopy

There are two principal phenomena involved in image formation (contrast generation) in the transmission EM that relate to how electrons in the beam interact with the atoms in the specimen: They are referred to as elastic and inelastic scattering. The image in the EM arises from that part of the scattered electrons which are deflected through relatively small angles without loss of energy. Electrons that have been elastically scattered in wide angles are blocked by apertures. Through this selective operation, differences in brightness (contrast) are generated between differently scattering objects. Because most atoms of biological samples scatter only weak, contrast is improved by introduction of heavy metal atoms.

2.2.4.1. Chemical Fixation of Single Cells

The purpose of tissue fixation is to retain the tissue in a state as near to life as possible. In immuno-EM, suboptimal fixation must often be employed in order to retain antigenicity. The most widely used fixatives are chemical crosslinkers such as aldehydes, e.g. formaldehyde, glutaraldehyde, and acrolein. These compounds react with primary amine groups and stabilize proteins by crosslinking them into a gel. For easier handling, cell suspensions are usually embedded in a matrix such as agar prior to dehydration and embedding.

• remove culture medium from dish
• wash cells with PBS
• detach cells either in PBS/2 mM EDTA or trypsinize
• centrifuge at 1000 g for 3 min
2. MATERIALS AND METHODS

- wash cells with PBS, incubate for 5 min at 37°C (to remove medium components that might crosslink with the cells)
- fix cells in fixation solution for 60 min at 37°C
- wash cells 2x for 5 min with PBS at 4°C
- add cells into a 1.5 ml tube, add solution of 2% (w/v) low melting agar in PBS (cooled to 20°C) and centrifuge immediately
- allow agar to solidify at 4°C
- use a razor blade to remove tip of tube containing the cells, and transfer into H₂O

2.2.4.2. Dehydration

With few exceptions, most of the useful resins for EM embedding are not miscible with H₂O. Therefore, before infiltration with plastic resin, tissue must be dehydrated in an organic solvent.

- incubate sample in 70% ethanol for 15 min at 4°C
- incubate sample in 99.8% ethanol for 15 min at 4°C

2.2.4.3. Embedding in LR Gold Resin

Before sectioning, tissue is usually embedded in plastic to provide support during sectioning and stability under the electron beam. LR gold resin is an acrylic embedding medium that polymerizes either at low temperatures under UV light in the presence of the light-activated catalyst benzil, or at higher temperatures (RT or 50°C) in the presence of the catalyst benzoylperoxide. Low temperature embedding media reduce lipid extractions and other ultrastructural changes.

- incubate sample in 33% (v/v) monomer (LR gold + 0.1% benzil) in ethanol at -20°C for 4 hrs
- incubate sample in 66% (v/v) monomer (LR gold + 0.1% benzil) in ethanol at -20°C overnight
- incubate sample in 100% monomer (LR gold + 0.1% benzil + 0.1% benzoylperoxide) at -20°C for 2 hrs
- incubate sample in 100% monomer (LR gold + 0.1% benzil + 0.1% benzoylperoxide) at -20°C for 2 hrs
- polymerize resin at -18°C for 24 hrs under UV light in a 0.5 ml tube
- polymerize resin at RT for 4 days under UV light

2.2.4.4. Ultramicrotomy

Once the specimen is embedded, it must be sectioned thin enough (usually in the order of 50-100 nm) to be viewed under the EM. Ultrathin sections are collected on copper grids (200 mesh, 3.05 mm in diameter) which are coated with an electron-transparent support film.
2.2.4.5. Postembedding Immunoelectron Microscopy

In preembedding immuno-EM, all fixation, permeabilization, and antibody labeling steps are performed prior to embedding cells or tissue. Postembedding immuno-EM involves antibody labeling of thin sections of embedded cells and tissues on EM grids. To reduce non-reactive aldehyde groups from the fixation step which will bind antibodies non-specifically, the PBGG buffer contains the reducing agent glycine. To prevent antibodies from binding non-specifically to cellular or tissue components, the PBGG buffer also contains BSA for blocking. Colloidal gold, conjugated to secondary antibodies, can be easily visualized after staining with lead and uranyl ions.

- block grids for 10 min at RT on 50 µl drops of PBGG buffer, then remove buffer
- incubate grids for 60 min at RT on drops containing primary antibody in PBGG buffer
- remove buffer, rinse grids 6x for 2 min in PBGG
- incubate grids for 60 min at RT on drops containing secondary antibody in PBGG buffer (centrifuge solution to remove antibody precipitates)
- remove buffer, rinse grids 3x for 2 min in PBGG
- rinse grids 3x for 2 min in H₂O, then remove H₂O

2.2.4.6. Post-Staining

Biological specimens are made up mostly of low-atomic-weight elements that do not effectively stop electrons and thus possess very low contrast under the EM in their native states. The specimens are therefore stained with electron-dense substances to impart contrast to cellular structures, most often with uranyl acetate and lead citrate (Reynolds, 1963). In addition to providing contrast, such substances serve to protect the sections from beam damage and to impart clarity to the fine structure of cells. Uranyl acetate is particularly good for DNA-containing structures and membranes, lead stains improve contrast of ribosomes, glycogen, and cytoplasmic ground substance.

- incubate grids on drops of uranyl acetate solution (2% in H₂O) for 5 min at RT
- rinse grids 2x for 30 sec on drops of H₂O, then remove H₂O
- incubate grids on drops of lead citrate solution (Reynolds, 1963) for 5 min at RT
- rinse grids 4x on drop of H₂O, then remove H₂O
- check grids under the EM

2.2.5. Database and Protein Structure Analysis

For database analysis, the public sequences available from the 'National Center for Biotechnology Information' at http://www.ncbi.nlm.nih.gov/blast/ were screened using the nucleotide sequence databases non-redundant, htgs (unfinished high throughput genomic sequence), human ESTs (expressed sequence tag), mouse ESTs and other ESTs. For crystal structure analysis, the program Chain v5.2 on a Silicon Graphics computer was used.
3. RESULTS

3.1. Isolation of a cDNA Coding for Mouse Neuroligin 1

The first aim of this study was to examine whether molecules identical or similar to the three rat neuroligins are expressed in mice. Starting from an *E.coli* library containing cDNAs of mouse brain, a clone was isolated using the RC-PCR (rapid cloning of cDNAs by screening pools of cDNA by PCR) method combined with colony hybridization (Bolliger, 1997). For sequencing, the cDNA was removed from the library vector using restriction enzyme *Sal* I and ligated into the cloning vector pGEM-2 (digested with the same enzyme and dephosphorylated), giving construct SC1. The cDNA contains approximately 500 bp of 5'-untranslated sequences and 75% of the coding sequence of mouse neuroligin 1 (covering almost the whole esterase-like sequence), but the 3'-end containing the transmembrane region and the cytoplasmic part was missing.

The lacking 3'-terminal fragment should be generated by PCR. Two primers were chosen which amplify a fragment of 1378 bp. Primer MBNL12+ is specific for mouse neuroligin 1 and anneals within the known coding sequence, primer SGNL6- is degenerated and contains the TAG stop codon (for exact positions of primers and restriction sites see figure 3.1). With the exception of the MgCl2 concentration which was increased to 2.5 mM, standard PCR conditions were applied. As template, 100 ng cDNA from the mouse brain library were used, and the annealing step was performed at 55°C. The resulting fragments were recovered from an agarose gel and phosphorylated with T4 polynucleotide kinase. The cloning vector pGEM-2 was digested with *Hinc* II which generates blunt ends, purified by gel extraction and dephosphorylated using alkaline phosphatase. Then a ligation reaction was performed using the linear pGEM-2 vector and the PCR fragments. After transformation into *E.coli*, three clones containing inserts were sequenced. Comparing these sequences among each other and with the rat neuroligin 1 sequence made clear that the PCR-generated 3'-fragment contained some errors. The next step was to assemble the 3'-end PCR fragment with the 5'-part of the neuroligin sequence in the cDNA. A double digestion was performed with clone SC1 and the three pGEM-2 vectors containing the PCR inserts using restriction enzymes *Eco* R V, which cuts within the coding region, and *Xba* I, which cuts after the 3'-end of the cDNA in the vector. The digested vector SC1 and the three fragments were purified by gel extraction, and SC1 was dephosphorylated using alkaline phosphatase. Vector SC1 was then ligated separately with each fragment, and from each of the three reactions one clone was sequenced (clones NL1a, NL1b and NL1c). Due to the errors in the PCR-generated fragments, the part between restriction sites *Eco* R V and *Kpn* I in the coding region of clone NL1a was replaced by the corresponding region of NL1c, and the new construct was named pGEM-2/mNL1.
Compared to the rat sequence, 96.9% of the bases are identical in mouse neuroligin 1 (figure 3.1). Most nucleotide changes are variations at position 3 of codons, consequently most are silent at the protein level. In fact, the amino acid sequence differs only at two positions (317 and 773), an A→T amino acid replacement found in the cDNA and a V→I exchange found in all three PCR-generated fragments, giving an identity of 99.8% between rat and mouse neuroligin 1 proteins. 

For transfection of cell lines, the mouse neuroligin 1 cDNA had to be transferred from the cloning vector pGEM-2 into a mammalian expression vector. For this purpose, pcDNAI/Amp was double digested with Hind III and Xba I. The vector was then dephosphorylated by alkaline phosphatase and purified from the small polylinker insert by agarose gel extraction. The vector pGEM-2/mNL1 was digested with the same restriction enzymes, and the insert containing the whole coding sequence and the 500 bp 5'-untranslated sequences of the mouse neuroligin 1 cDNA was recovered from an agarose gel. Vector and insert were ligated to each other by T4 DNA ligase, and DNA sequencing confirmed the correctness of construct pcDNAI/mNL1 proteins. 

Figure 3.1: Coding sequence of the mouse neuroligin 1 cDNA. Nucleotides that differ from the rat neuroligin 1 cDNA are boxed on a black background. The predicted signal sequence is shown in blue, the esterase-like sequence in red, and the transmembrane sequence in green. The asterisk denotes the position of splice site A (no insert was found in the mouse cDNA), splice site B is in brackets.  The predicted signal sequence is shown in blue, the esterase-like sequence in red, and the transmembrane sequence in green. The asterisk denotes the position of splice site A (no insert was found in the mouse cDNA), splice site B is in brackets.
At that time, no neuroligin antibody was commercially available. For this reason, a c-myc tag should be cloned to the 5’-end of the coding sequence of the mouse neuroligin 1 cDNA. As neuroligins are membrane proteins, they contain at the N terminus a signal peptide which is cleaved away after translocation through the ER membrane. Therefore, a construct was generated containing a signal sequence at the 5’-terminus followed by the c-myc tag and the neuroligin sequence. For practical reasons the vector pNKS2/sig,myc was chosen which contains the signal sequence of *X.laevis* NCAM followed by the c-myc tag. This vector was digested with *Sal*I which cleaves right after the c-myc tag, then made blunt ended using Klenow fragment and dephosphorylated using alkaline phosphatase. Construct pGEM-2/mNL1 was double digested with *Acc*I, which cleaves after the signal sequence of mouse neuroligin 1, and *Eco*RV, which cleaves within the coding region. The fragment of interest was recovered from an agarose gel and made blunt ended using T4 DNA polymerase. This neuroligin fragment was then ligated into the linear pNKS2/sig,myc vector, and DNA sequencing confirmed the correctness of the new construct pNKS2/sig,myc,mNL1 (figure 3.2). The sig,myc,mNL1 cassette was then removed from the pNKS2 vector by digestion with *Hind*III which cleaves in front of the signal sequence and *Hpa*I which cuts within the coding sequence of mouse neuroligin 1. Vector pGEM-2/mNL1 was digested with the same enzymes which cleave at corresponding positions. Both vector and sig,myc,mNL1 fragment were purified by gel extraction, and the vector was dephosphorylated with alkaline phosphatase. The ligation reaction gave a correct clone with the designation pGEM-2/sig,myc,mNL1. Again, this construct was transferred into the mammalian expression vector pcDNAI/Amp. Therefore, the whole sig,myc,mNL1 cDNA was removed from the pGEM-2/sig,myc,mNL1 vector using *Hind*III and *Xba*I, purified by gel extraction and ligated into the previously described vector pcDNAI/Amp which was digested with the same enzymes. DNA sequencing confirmed that construct pcDNAI/sig,myc,mNL1 is correct.

MALPRCMWNYWVRAMMADVACVVRGSAFLTLCLGCCLQTPVRVLSQKLDVPLUTTFNGKIRGIKKEL... native mNL1
MLHIKDLIWTLYFPGTAVALKLISBEDLVEPVTINFGKIRGIKKEL... sig,myc,mNL1

Figure 3.2: N termini of native mouse neuroligin 1 and of construct sig,myc,mNL1. Native mouse neuroligin 1 has a predicted signal peptide of 45 amino acids (blue). Construct sig,myc,mNL1 has the signal peptide of *X.laevis* NCAM (green), the c-myc tag (yellow), then two amino acids that arose by cloning, followed by the mouse neuroligin 1 sequence (red).

To study the mouse neuroligin 1 protein without an artificial N terminus, a construct was generated having the GFP (green fluorescent protein) at the C terminus of mouse neuroligin 1. For this reason, the mammalian expression vector pEGFP-N2 was digested with restriction enzyme *Bam*HI, which cleaves in the polylinker in front of the GFP gene, and was made blunt ended using T4 DNA polymerase. This linear vector was then digested with *Hind*III which also recognizes a restriction site in the polylinker, and then the vector was dephosphorylated using alkaline phosphatase. Construct pcDNAI/mNL1 was digested with *Rsr*II, a restriction enzyme that cleaves in the coding sequence at the 3’-end of the neuroligin 1 cDNA (in the protein, the 67 C-terminal amino acids will therefore be lacking). After a T4 DNA polymerase reaction to make blunt ends, this vector was digested with *Hind*III which cleaves in front of the 5’-end of the cDNA. Both DNAs were extracted from agarose gels and used for a ligation reaction using T4 DNA ligase. DNA sequencing confirmed the presence of a correct clone, pEGFP/mNL1,GFP, which is a construct of the mouse neuroligin 1 cDNA having the GFP at its 3’-terminal end.
3. RESULTS

3.2. Expression of Mouse Neuroligin 1 in COS-7 Cells

A good way to express a cloned gene in mammalian cells is to use a transient expression system such as that provided by the COS-7 cell line. In addition to functional SV40 large T antigen, COS-7 cells produce the permissivity factors required for replication of DNAs that contain the SV40 origin of replication, resulting in an increase in plasmid copy number (>10⁵ per cell) and subsequently high levels of protein expression. To examine whether the mouse neuroligin 1 cDNA encodes a mature protein, COS-7 cells plated on coverslips were transiently transfected with the vector pcDNAI/sig,myc,mNL1 using the calcium phosphate-mediated transfection method. At 24 h after transfection, the cells were fixed and stained with the α c-myc antibody. No specific immunolabeling was observed from these cells (data not shown). When the cells were fixed and also permeabilized prior to antibody staining, immunofluorescence microscopy analysis revealed that most protein was not delivered to the plasma membrane as expected, but accumulated within the cell (figure 3.3A). A clear staining was observed surrounding the nucleus, presumably showing the ER and/or the Golgi apparatus, whereas the membrane was only faintly colored. Mock-transfected cells showed weak background staining (data not shown).

![Figure 3.3: Immunofluorescence microscopy analyses of different cell lines transfected with the mouse neuroligin 1 cDNA. All cells were analyzed at 24 h after transfection and were permeabilized.](image)

The question was why the neuroligin 1 protein is not targeted to its expected destination, the plasma membrane. One possible explanation is that the time between transfection and analysis is too short for proper delivery of the protein. To verify this point, immunofluorescence microscopy analyses were performed at 20, 44, 94 and 136 h after transfection into COS-7 cells. An expression maximum was observed after approximately 40 h, but even after five days the charac-
teristic ER/Golgi apparatus staining still persisted, whereas the membrane showed no significant labeling (data not shown). Another possibility for intracellular protein accumulation could be that the protein synthesis machinery of the COS-7 cell is overloaded. Therefore, several cell lines not expressing the SV40 large T antigen were transfected with the mouse neuroligin 1 cDNA. All cells tested (HUVEC, EC219, L929) indeed showed reduced expression of mouse neuroligin 1, but the distribution pattern of the protein inside the cell was identical to that observed from COS-7 cells (figures 3.3B-C and data not shown). In addition, reducing the amount of plasmid DNA in the transfection experiment had no quantitative influence on protein expression in all cell lines tested. Instead, less DNA resulted in a lower transfection efficiency (data not shown).

Now the suspicion came up that the artificial N terminus of the sig,myc,mNL1 construct containing the NCAM signal sequence and the c-myc tag might cause the problems. The question was whether the signal peptide could not be cleaved, a process occurring after translocation into the ER membrane, or if the c-myc tag leads to misfolding of the protein, both possibilities resulting in retardation or stop of delivery of the protein to the membrane. Therefore, vector pEGFP/mNL1,GFP was generated which has the native signal sequence. Transfection experiments revealed that this construct showed an identical staining pattern of ER/Golgi apparatus as observed with plasmid pcDNAI/sig,myc,mNL1 (figure 3.3D). Extending the time between transfection and sample processing to 88 h did not result in delivery of the protein to the plasma membrane (data not shown). When the α NL1/3 antibody became available which was generated against the extracellular domain of rat neuroligin 1 and recognizes neuroligins 1 and 3 (Song et al., 1999), experiments using the native mouse neuroligin 1 cDNA showed the same phenomenon (figure 3.3E). In contrast, examination of primary mouse astrocytes which were positive on the Western blot (see below) gave a weak dot-like staining of the cell surface, whereas no internal structures were detected in permeabilized cells (data not shown). For additional illustration, figure 3.4 shows a confocal laser scanning microscope image of a COS-7 cell expressing mouse neuroligin 1.

![Figure 3.4: Confocal laser scanning micrograph of a mouse neuroligin 1-expressing COS-7 cell.](image)

At 24 h after transfection with plasmid pcDNAI/sig,myc,mNL1, the cells were fixed/permeabilized and stained with the α c-myc antibody. The square picture shows a COS-7 cell from the top, the two other pictures show side views. Arrowheads depict focus planes. The scale bar is 10 µm.
The conclusion from these experiments is that the problem seems to be of biological rather than of technical origin. For unknown reasons, the mouse neuroligin 1 accumulates probably in the ER and/or the Golgi apparatus instead of being transported to the plasma membrane. A Western blot with extracts from COS-7 cells independently transfected with one of the three cDNA constructs is shown in figure 3.5A. In the extract from untagged mouse neuroligin 1-transfected cells, the $\alpha$ NL1/3 antibody reacted with a band of approximately 90 kDa, whereas the rat homolog has an apparent mass of approximately 118 kDa (Ichtchenko et al., 1995). The calculated mass of mouse neuroligin 1 is 92.0 kDa with or 87.0 kDa without the signal peptide, respectively; therefore it could be supposed that the 90 kDa band refers to the unglycosylated protein (see below). In some experiments, in addition to the strong 90 kDa band, a second fine band was visible on the Western blot at 118 kDa (see below). The calculated mass of construct mNL1,GFP is 112.3 kDa (107.3 kDa without the signal peptide); this protein runs as expected from the size. No reactivity of the antibody was observed with extracts from mock-transfected cells. To confirm that the bands detected in figure 3.5A are really of neuroligin origin, an immunoprecipitation was performed using the $\alpha$ NL1/3 antibody (figure 3.5B).

![Figure 3.5](image-url)

**Figure 3.5: Overexpression of mouse neuroligin 1 in transfected COS-7 cells and immunoprecipitation.** The cell extracts were prepared at 24 h after transfection, and the $\alpha$ NL1/3 antibody was used for the Western blot detections. Molecular mass markers in kDa are shown on the left. (A) Western blot of extracts from mouse neuroligin 1-expressing COS-7 cells. (1) 30 µg extract of cells transfected with plasmid pcDNAI/sig,myc,mNL1. (2) 30 µg extract of cells transfected with plasmid pEGFP/mNL1,GFP. (3) 30 µg extract of cells transfected with plasmid pcDNAI/mNL1. (4) 30 µg extract of cells transfected with plasmid pcDNAI/Amp. (B) Immunoprecipitation of mouse neuroligin 1 from transfected COS-7 cells. (1) 30 µg extract of cells transfected with plasmid pcDNAI/mNL1. (2) 30 µg extract of cells transfected with plasmid pcDNAI/Amp. (3) Immunoprecipitation from 300 µg extract (1) using the $\alpha$ NL1/3 antibody. (4) Immunoprecipitation from 300 µg extract (2) using the $\alpha$ NL1/3 antibody.

To corroborate the finding that mouse neuroligin 1 is held back in the ER/Golgi apparatus and fails to be delivered to the plasma membrane, transiently transfected COS-7 cells were investigated by EM. At 24 h after transfection the cells were fixed, dehydrated and subsequently embedded in LR gold resin at -20°C. Ultrathin sections were stained by the $\alpha$ NL1/3 antibody and a secondary antibody conjugated to gold particles, and the sections were contrasted. Although sub-optimal fixation was applied in order to preserve antigenicity, some ultrastructure details were retained in the sections (nucleus, mitochondria, ER/Golgi apparatus-like structures). In accordance to immunofluorescence microscopy experiments described before, a specific staining was observed in dark structures in the cytoplasm surrounding the nucleus, likely to be ER and/or Golgi apparatus, whereas no gold particles were specifically enriched at the plasma membrane (figure 3.6).
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Figure 3.6: Electron photomicrographs of a COS-7 cell transfected with the mouse neuroligin 1 cDNA. For immunostaining, the α NL1/3 antibody was used. Gold particles (10 nm in diameter) are marked by circles. The scale bars are 200 nm. (A) Overview of a COS-7 cell with a sector of the nucleus in the upper left corner and the plasma membrane at the right side of the image. A specific staining can be observed in dark structures throughout the cytoplasm, presumably the ER and/or the Golgi apparatus. Some unspecific labeling is seen in the nucleus and outside of the cell. (B) Detailed image of a dark structure showing the specific neuroligin staining.

Eukaryotic membrane proteins are integrated into the ER membrane during synthesis, and glycosylation starts even before completion of synthesis. The composition and structure of the oligosaccharide side chains are determined by a battery of enzymes in the ER and the Golgi apparatus. The side chains of oligosaccharides can only be covalently bound to protein by one of two possible linkages. In the N-glycosidic linkage, oligosaccharides are attached enzymatically via an N-acetyl glucosamine residue to asparagine residues in the polypeptide chain (consensus sequence -N-X-S/T-). In the S/T-glycosidic linkage, N-acetyl galactosamine, galactose, xylose or N-acetyl glucosamine are covalently bound to the hydroxyl groups of either serine or threonine in the protein. In rat and mouse neuroligin 1 proteins, five consensus sequences for N-linked glycosylation are present. To clarify whether the 90 kDa band observed in figure 3.5A is unglycosylated protein, COS-7 cell extracts were treated for 16 h with the enzyme N-glycosidase F to remove N-linked sugars. As observed on the Western blot shown in figure 3.7A, the mass of mouse neuroligin 1 shifted from 90 kDa to about 80 kDa, therefore the protein has attached sugars. It is unclear why
the deglycosylated protein has a lower apparent mass than calculated (87.0 kDa). For comparison, the same experiment was performed using primary mouse astrocytes which express endogenous neuroligins. As seen on the Western blot, the mass of neuroligins is clearly higher compared to that in transfected COS-7 cells (figure 3.7B). N-glycosidase F treatment failed to reduce the mass to 87.0 kDa. Uncomplete deglycosylation was excluded by lengthening the enzyme reaction time up to 68 h (data not shown). Taken together, as mouse neuroligin 1 is glycosylated, it seems plausible that the stained structures observed in immunofluorescence microscopy and EM really are ER and/or Golgi apparatus. No explanation can be given about the fact that the deglycosylated mouse neuroligin 1 protein has a lower apparent mass than calculated.

Figure 3.7: Deglycosylation of mouse neuroligins. Molecular mass markers in kDa are shown on the left. (A) Western blot of COS-7 cell extracts incubated either with or without N-glycosidase F. The cell extracts were prepared at 24 h after transfection, and the α c-myc antibody was used for the Western blot detection. (1) 60 µg extract of COS-7 cells transfected with plasmid pcDNAI/Amp. (2) 60 µg deglycosylated extract (1). (3) 60 µg extract of COS-7 cells transfected with plasmid pcDNAI/sig,myc,mNL1. (4) 60 µg deglycosylated extract (3). (B) Western blot of primary mouse astrocyte extracts incubated either with or without N-glycosidase F, using the α NL1/3 antibody for the detection. (1) 60 µg extract of primary mouse astrocytes. (2) 60 µg deglycosylated extract (1).

3.3. DNA Database Analysis and Identification of Human Neuroligins

Based on the three known rat neuroligin sequences it should be investigated if, in addition to neuroligin 1, there are further homologous genes in the mouse. In fact, a database search on currently available mouse EST sequences identified 13 sequences coding for mouse neuroligin 2 and four sequences encoding mouse neuroligin 3. All these sequences could be assigned unequivocally, having approximately 97% identity to the rat neuroligins at the cDNA level and almost 100% at the protein level.

Recently, the challenge to sequence the human genome was solved (Lander et al., 2001; Venter et al., 2001). Therefore, it was of special interest to investigate if neuroligin genes can be identified among the 30000-40000 genes expected in the human genome. Searching the human EST database resulted in seven entries that could be assigned to neuroligin 1, 21 sequences which encode neuroligin 2, and five sequences identifiable as neuroligin 3. Analysis of the cDNA database identified three sequences having more than 98% amino acid and approximately 92% nucleotide sequence identity to the three rat neuroligins and thus are probably the human homologs (figure 3.8). Clone KIAA1070 encodes human neuroligin 1 with an insert in splice site B, but not in
splice site A (Kikuno et al., 1999). Sequences encoding two possible inserts in splice site A were found in the chromosomal sequence AC069531 (insert A1) and by RT-PCR analysis (insert A2), respectively. KIAA1366 encodes an N-terminally truncated human neuroligin 2 (Nagase et al., 2000a). Two ESTs (AI337820, AW205858) allowed to unequivocally complete this sequence. Only one insert was found for splice site A. KIAA1480 is also a partial sequence (Nagase et al., 2000b). It is identical to cDNA AF217411 and gene AF217413 which were already identified as human neuroligin 3 (Philibert et al., 2000).

Figure 3.8: Protein sequence alignment of human neuroligins 1-4. Residues shared by at least three of the four human neuroligins are boxed on a black background. The predicted signal peptides are in blue and boxed on a white background. The esterase-like domains in neuroligin 1 is also labeled. Amino acids are numbered at the right.

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et al., 1999). Sequences encoding two possible inserts in splice site A were found in the chromosomal sequence AC069531 (insert A1) and by RT-PCR analysis (insert A2), respectively. KIAA1366 encodes an N-terminally truncated human neuroligin 2 (Nagase et al., 2000a). Two ESTs (AI337820, AW205858) allowed to unequivocally complete this sequence. Only one insert was found for splice site A. KIAA1480 is also a partial sequence (Nagase et al., 2000b). It is identical to cDNA AF217411 and gene AF217413 which were already identified as human neuroligin 3 (Philibert et al., 2000).

Figure 3.8: Protein sequence alignment of human neuroligins 1-4. Residues shared by at least three of the four human neuroligins are boxed on a black background. The predicted signal peptides are in blue and boxed on a white background. The esterase-like domains in neuroligin 1 is also labeled. Amino acids are numbered at the right.
In addition to the 33 human EST sequences which were unequivocally assignable to neuroligins 1-3, 14 EST sequences showed high homologies to neuroligins but could not be allocated to a particular isoform. These sequences have highest homology to a further entry in the cDNA database, KIAA1260 (Nagase et al., 1999a). Alignment of the protein sequence with the compiled sequences of the putative human neuroligins 1-3 demonstrated that this clone codes for a fourth neuroligin which was not found in rodents (figure 3.8). Human neuroligin 4 is composed of an N-terminal signal peptide with a predicted length of 43 amino acids (Nielsen et al., 1997), an acetylcholinesterase-like domain encompassing almost the entire extracellular part, a short linker just in front of the transmembrane domain, and a cytosolic tail (figure 1.14A). As observed with the rat neuroligins 1-3 (Ichtchenko et al., 1996), highest sequence conservation is found in the esterase-like domain, whereas the linker to the transmembrane region is completely different among all known neuroligins (figure 3.8). Pairwise neuroligin comparison within the human proteins indicates closest homology between neuroligins 3 and 4 (table 3.1). This is reflected by the similar lengths of the parts following the esterase-like domains.

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</tr>
<tr>
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<td>62.6%</td>
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Table 3.1: Identity numbers of human neuroligin protein sequences. The amino acid sequences of human neuroligins 1-4, excluding the inserts in splice site A, were pairwise aligned. The numbers shown represent the percent identities.

Based on the neuroligin 4 sequence, RT-PCR was performed on human brain polyadenylated RNA. cDNA was synthesized using primer R9hNL4 (encompassing the stop codon). A full-length product of 2.5 kb and a fragment of 658 bp were amplified on the cDNA by PCR using primers F11hNL4 (encompassing the start codon) and R9hNL4, and primers F5hNL4 and R9hNL4, respectively (data not shown). PCR was performed using standard conditions and an annealing temperature of 60°C. The PCR products were purified by agarose gel extraction, and the 658 bp fragment was directly sequenced using primer F5hNL4. Sequence analysis revealed virtually a 100% match to KIAA1260, but an additional weak signal was found at twelve positions (figure 3.9). In addition, in the course of the DNA database analyses two classes of neuroligin 4-related sequences were discovered: Class 1 with usually 100% identity to human neuroligin 4, and class 2 with, at most, 95% identity. Sequences of the latter class, however, consistently revealed 100% identity to cDNA KIAA0951 (Nagase et al., 1999b) and to those twelve positions within the 658 bp PCR fragment. Further analysis of these sequences allowed the compilation of a complete open reading frame (N-terminally truncated clone KIAA0951 completed with chromosomal sequence AC010979) which could code for a fifth human neuroligin with approximately 97% identity to neuroligin 4 on the protein level (figure 3.10). This neuroligin would differ from neuroligin 4 only by 19 amino acids. Most of these residues are clustered in the region in front of splice site A and at the C terminus. Greater divergence was, however, observed when comparing length and identity of available intron sequences of genomic neuroligin sequences. Between corresponding introns, sequence identity drops to about 70-80% (data not shown). Further evidence for two distinct genes comes from the chromosomal assignment of the neuroligin genes (table 3.2), revealing that human neuroligin 4 is positioned on chromosome X, whereas neuroligin 5 was assigned to chromosome 6 (Y was excluded as RT-PCR was performed on RNA from a female individual).
Figure 3.9: Sequence readout of the 658 bp PCR fragment. The twelve positions that diverge between neuroligins 4 and 5 are marked by arrows. Note that at these positions the readout is not clear, but the two highest peaks always code either for neuroligin 4 or 5. The two arrows marked with asterisks show positions where the neuroligin 5 signal is stronger, at the other ten positions the neuroligin 4 signal is stronger. Marked is also the TAG stop codon (positions 907-909).
3. RESULTS

Figure 3.10: Protein sequence of the putative human neuroligin 5. Residues identical to human neuroligin 4 are boxed on a black background. Insert A2, which was not found in neuroligin 4, is given in brackets. The predicted signal peptide is shown in blue, the esterase-like domain in red, and the transmembrane sequence in green. Amino acids are numbered at the right.

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Table 3.2: Chromosomal assignment of human neuroligin genes. Chromosomal assignments were taken from the references indicated (done by RHM, radiation hybrid mapping) or deduced from database analyses. The assignments of neuroligins 1-4 are without contradictions, whereas inconsistent data exist for neuroligin 5.

The human neuroligin gene consists of eight exons (figure 1.15; Philibert et al., 2000). Analysis of currently available human genomic sequences revealed that all neuroligins have the same exon/intron structure, but the lengths of corresponding introns vary between isoforms (data not shown). Exons 3 and 4 encode the alternatively spliced inserts A1 and A2. Alternative sequences in splice site B were described for rat and mouse neuroligin 1 (Ichtchenko et al., 1995; Scheiffele et al., 2000). Examination of human genomic sequences covering region B showed that the corresponding nine amino acids are not encoded by an individual exon, but would arise from the usage of a second downstream splice donor site at the end of exon 6. In human neuroligins 2-4, such a second splice donor site was not found, and in human neuroligin 3 the donor site is immediately followed by an in-frame stop codon (figure 3.11). Furthermore, within this region the usually high sequence identity among all neuroligins is not maintained. From these findings it is suggested that splice site B does not exist in human neuroligin 1 and that the extra amino acids in rodents might arise from erroneous or rare usage of a downstream splice donor site, while in humans the upstream site might be non-effective so that insert B is always present.

Figure 3.11: Analysis of the putative splice site B. Nucleotides and amino acids shared by at least three of the four neuroligins are boxed on a black background. In the upper gene alignment, red letters mark nucleotides of the exon. In all sequences, the splice consensus sequence AG/GT is found. In the lower protein alignment, green residues are derived from exonic sequences. In region B, the high sequence identity among all neuroligins is not maintained. Asterisks in the neuroligin 3 protein sequence denote in-frame stop codons in the corresponding DNA sequence.
The esterase-like domain of neuroligins has been shown to mediate the extracellular interaction with the neurexin proteins (Scheiffele et al., 2000). Similar domains were identified in the *Drosophila* proteins neurotactin and gliotactin, molecules involved in cell-cell interactions (Darboux et al., 1996). The presently available protein structure with most homology to the esterase-like domain of neuroligins is the mouse acetylcholinesterase (Bourne et al., 1999), having about 35% identical amino acids with neuroligins. Analysis of this crystal structure revealed that it consists of a 12-stranded central mixed β-sheet surrounded by 14 α-helices. The protein has a globular shape with its N and C termini on opposite sides of the molecule (figure 3.12). Therefore one could imagine that a ligand would probably bind to or near to the N terminus of neuroligin, whereas the C terminus is oriented against the plasma membrane. About the function of the inserts in splice sites A and B only little is known. Alternative splicing of neuroligins seems to have no effect on β-neurexin binding (Ichtchenko et al., 1995). Both alternative splice sites, which are absent in esterases, are positioned on the surface of the globular domain. Furthermore, splice site A is close to the N terminus of the protein, raising the possibility that the splice inserts of neuroligins, nevertheless, have a function in ligand binding, maybe in interactions with proteins other than β-neurexins. Interestingly, all neuroligins contain in their extracellular domains a repeat of the tripeptide -L-R-E- which was previously identified as the adhesive site of s-laminin for motor neurons (Hunter et al., 1989). This tripeptide is not conserved in the mouse acetylcholinesterase, but the corresponding amino acids are located on the surface of the molecule near the C terminus. Therefore it can be speculated that this tripeptide offers a binding site for additional ligands, in analogy to the -R-G-D- sequence which is found in many matrix proteins and is recognized by integrins (Ruoslahti and Pierschbacher, 1987).

![Figure 3.12: Crystal structure of mouse acetylcholinesterase. Marked are amino acids where in the neuroligin sequence the splice inserts are positioned (A and B). Also shown are the N- and C-terminal regions of the molecule. White spheres are carbon atoms, red spheres are oxygen atoms, blue spheres are nitrogen atoms, and green spheres are sulfur atoms. This image was generated using the program Chain 5.2.](image)
3. RESULTS

3.4. Analysis of Human Neuroligin 4

Neuroligins from rat were previously shown to be predominantly, if not exclusively, expressed in brain tissue (Ichtchenko et al., 1995, 1996). To investigate the expression pattern of neuroligin 4, a human tissue polyadenylated RNA blot was hybridized with a 3 kb RNA probe containing the last 0.5 kb of the coding sequence of human neuroligin 4 and the 2.5 kb of the 3’-untranslated region (since this part has the lowest homology to the other neuroligins). This RNA probe was generated by transcribing the Bsul36 I-digested vector pBS/hNL4 by T3 RNA polymerase. Neuroligin 4 mRNA with an approximate size of 5.5 kb was found with the highest relative expression in heart. Lower expression levels were detected in liver, skeletal muscle and pancreas, whereas in brain, placenta, lung and kidney, neuroligin 4 mRNA was hardly detectable, if at all (figure 3.13A). The same blot was stripped and reprobed with a human β-actin RNA probe of 0.75 kb (vector pcβ/β-actinT7as digested with Spe I and transcribed with T7 RNA polymerase). Signals of similar intensities were detected in all lanes at 2 kb, and also the additional β-actin transcripts of about 1.6-1.8 kb in heart and skeletal muscle were visible (figure 3.13B).

![Figure 3.13: Northern blot analysis of human neuroligin 4.](image) Size markers in kb are shown on the left. (A) A human tissue polyadenylated RNA blot was hybridized with a 3 kb RNA probe of human neuroligin 4. (1) Heart. (2) Brain. (3) Placenta. (4) Lung. (5) Liver. (6) Skeletal muscle. (7) Kidney. (8) Pancreas. (B) β-actin expression in human polyadenylated RNA. The blot used in (A) was stripped and reprobed with a human β-actin RNA probe.

For eukaryotic expression, cDNA KIAA1260 was transferred from the pBluescript II SK+ vector (pBS/hNL4) into an expression vector. Therefore, vector pcDNAI/Amp was digested with restriction enzymes Xho I and Sph I and dephosphorylated using alkaline phosphatase. Plasmid pBS/hNL4 was digested with the same enzymes to remove the coding region of human neuroligin 4 flanked by 480 bp of 5’- and 970 bp of 3’-untranslated sequences. For comparative studies described below, cDNA KIAA1070 (pBS/hNL1) was cloned into the same expression vector. Plasmid pBS/hNL1 was digested with Xba I to remove the cDNA containing the coding region as well as 450 bp of 5’- and 900 bp of 3’-untranslated sequences. Vector pcDNAI/Amp was also digested with restriction enzyme Xba I and was dephosphorylated. All vectors and fragments were purified.
from agarose gels, and ligation reactions were performed using T4 DNA ligase. Control digestions confirmed the correctness of plasmids pcDNAI/hNL4 and pcDNAI/hNL1.

To investigate whether the neuroligin 4 cDNA encodes a mature protein, COS-7 cells were transiently transfected with vectors pcDNAI/hNL1 and pcDNAI/hNL4. At 24 h after transfection cell extracts were prepared and used for Western blot analysis (figure 3.14). To detect neuroligin proteins, the monoclonal α NL1/3 antibody was used. No reactivity of the antibody was found with extracts from mock-transfected cells. Human neuroligin 1 was produced as a protein with an apparent mass of approximately 118 kDa in COS-7 cells. In cell extracts from neuroligin 4-transfected cells, the antibody reacted with a band of approximately 110 kDa, which is likely to be the respective neuroligin 4 protein. The signal detected for neuroligin 4 is clearly stronger than that detected for neuroligin 1; this seems on the one hand to be an attribute of the α NL1/3 antibody (Song et al., 1999), on the other hand the human neuroligin 1 cDNA has a lower transfection efficiency (observed from immunofluorescence microscopy analyses), resulting in less protein in the cell extract. For comparison, extract of COS-7 cells transfected with the mouse neuroligin 1 cDNA was loaded onto the gel, showing the known band at about 90 kDa. Interestingly, a second weak band, having the same mass as the human neuroligin 1 protein, was observed on this blot.

![Figure 3.14: Overexpression of human neuroligins in transfected COS-7 cells.](image)

Figure 3.14: Overexpression of human neuroligins in transfected COS-7 cells. The cell extracts were prepared at 24 h after transfection, and the α NL1/3 antibody was used for the Western blot detection. Molecular mass markers in kDa are shown on the left. (1) 30 µg extract of cells transfected with plasmid pcDNAI/hNL1. (2) 30 µg extract of cells transfected with plasmid pcDNAI/hNL4. (3) 30 µg extract of cells transfected with plasmid pcDNAI/mNL1. (4) 30 µg extract of cells transfected with plasmid pcDNAI/Amp.

Treatment of the COS-7 cell extracts with N-glycosidase F before electrophoresis reduced the apparent mass of both human proteins to approximately 102 kDa (figure 3.15). The mass shift of about 8 kDa after N-glycosidase F treatment is compatible with the presence of two consensus sequences for N-linked glycosylation in the human neuroligin 4 sequence (figure 3.8). Remarkably, human neuroligin 1, with four consensus sites, shows the same phenomenon. It is presently unclear why the deglycosylated proteins have a higher apparent mass than calculated (for both proteins 87.0 kDa without the signal peptide). Since endogenous neuroligins from mouse astrocytes show the same phenomenon (figure 3.7B), the mass difference is probably caused by an unusual migration behaviour of the proteins, rather than by incomplete deglycosylation. Interestingly is also the fact that on the Western blot a 90 kDa band is clearly visible in extracts from COS-7 cells transfected with the human neuroligin 1 cDNA (figure 3.15), similar to the situation found with mouse neuroligin 1 where sometimes also two bands can be observed (figure 3.14). In addition, deglycosylation of human neuroligin 1 leads also to a second weak band of approximately 80 kDa. Nevertheless, the question about the origin of this 80 kDa band remains unanswered.
3. RESULTS

Figure 3.15: Deglycosylation of human neuroligins. COS-7 cell extracts prepared at 24 h after transfection were incubated either with or without N-glycosidase F before electrophoresis. Western blot detection was performed using the α NL1/3 antibody. Molecular mass markers in kDa are shown on the left. (1) 60 µg extract of cells transfected with plasmid pcDNAI/hNL1. (2) 60 µg deglycosylated extract (1). (3) 60 µg extract of cells transfected with plasmid pcDNAI/hNL4. (4) 60 µg deglycosylated extract (3). (5) 60 µg extract of cells transfected with plasmid pcDNAI/Amp. (6) 60 µg deglycosylated extract (5).

Immunofluorescence microscopy analysis of transiently transfected COS-7 cells seeded on coverslips resulted in detection of the neuroligin 4 protein at the plasma membrane in a pattern indistinguishable from the distribution of human neuroligin 1 (figures 3.16A-C). The cells showed a regular surface staining, and even the most distant processes were labeled. Substantial quantities of the protein were also detectable intracellularly in fixed and permeabilized COS-7 cells, but no ER/Golgi apparatus-like structures as those observed from cells transfected with the mouse neuroligin 1 cDNA were identifiable.

Figure 3.16: Immunofluorescence microscopy analyses of different cell lines transfected with human neuroligin cDNAs. All cells were analyzed at 24 h after transfection and were fixed/permeabilized. The scale bar in (A) is 10 µm. (A) COS-7 cell transfected with plasmid pcDNAI/hNL1 and stained with the α NL1/3 antibody. (B-C) COS-7 cells transfected with plasmid pcDNAI/hNL4 and stained with the α NL1/3 antibody. (D) L929 cells transfected with plasmid pcDNAI/hNL4 and stained with the α NL1/3 antibody. (E) COS-7 cell transfected with plasmid pCMV/myc,PSD-95 and stained with the α c-myc antibody.
An identical staining showing the cell surface was observed in transfected L929 cells (figure 3.16D). For comparison, in figure 3.16E a COS-7 cell is shown which was transfected with a PSD-95/SAP90 cDNA; the staining of this cytoplasmic protein is concentrated within the cell, no fine processes and no cell borders are visible. All these data indicate that the neuroligin 4 cDNA encodes a protein which is glycosylated and integrated into the plasma membrane of COS-7 and L929 cells like human neuroligin 1. Control stainings of mouse neuroligin 1-expressing cells showed the known ER/Golgi apparatus staining, whereas mock-transfected cells showed only weak background labeling (data not shown).

To further investigate basic properties of neuroligin 4, its ability to interact with PSD-95/SAP90 was tested, since rat neuroligins interact with this protein via their C-terminal ends (figure 1.16; Irie et al., 1997) and all human neuroligins contain the consensus motif -T/S-X-V (figure 3.8), required for binding to PDZ domains (Songyang et al., 1997). Separate COS-7 cell cultures were transfected with either neuroligin 4 or myc-tagged PSD-95/SAP90 cDNAs. At 24 h after transfection, cell extracts for immunoprecipitation were prepared from each transfectant series, mixed, and immunoprecipitated with α PSD-95 or α c-myc antibodies. Western blot analysis confirmed the presence of both proteins in the extracts. The coprecipitation of neuroligin 4 in the precipitate was probed by Western blot analysis with the α NL1/3 antibody (figure 3.17A). Neuroligin 4 was clearly detectable in the precipitate, providing evidence for its ability to interact with PSD-95/SAP90. The same result was obtained when transfecting COS-7 cells with both cDNAs simultaneously, whereas no neuroligin immunoreactivity was discovered when omitting PSD-95/SAP90 cDNA in the transfection. Reprobing the blot with α c-myc antibodies confirmed the presence of myc-tagged PSD-95/SAP90 in both extracts and precipitates (figure 3.17B). Parallel experiments using endogenous ZO-1 (zonula occludens) instead of PSD-95/SAP90 cDNA failed to coprecipitate neuroligin 4 (figure 3.18), suggesting a selectivity of the interaction between neuroligins and PDZ domain proteins, as had been shown for rat neuroligins 1-3 (Irie et al., 1997).

![Figure 3.17: Association of neuroligin 4 and PSD-95/SAP90 in transfected COS-7 cells.](image)

The cell extracts were prepared at 24 h after transfection. Molecular mass markers in kDa are shown on the left. (A) Western blot detected with the α NL1/3 antibody. (1) 40 µg extract of cells transfected with plasmid pcDNAI/hNL4 mixed with cells transfected with plasmid pCMV/myc,PSD-95. (2) 40 µg extract of cells transfected simultaneously with plasmids pcDNAI/hNL4 and pCMV/myc,PSD-95. (3) 40 µg extract of cells transfected with plasmid pcDNAI/hNL4 mixed with cells transfected with plasmid pcDNAI/Amp. (4) Pool of three sequential immunoprecipitations from 600 µg extract (1) using the α PSD-95 antibody. (5) Pool of three sequential immunoprecipitations from 600 µg extract (2) using the α PSD-95 antibody. (6) Pool of three sequential immunoprecipitations from 600 µg extract (3) using the α PSD-95 antibody. (B) The blot used in (A) was stripped and reprobed with the α c-myc antibody to identify myc-tagged PSD-95/SAP90. The bands above PSD-95/SAP90 in lanes 1-3 are residual signals of neuroligin 4.
3. RESULTS

Figure 3.18: **Neuroligin 4 does not interact with endogenous ZO-1 in transfected COS-7 cells.** The cell extracts were prepared at 24 h after transfection. Shown are Western blots using either the α NL1/3 antibody (A) or the monoclonal α ZO-1 antibody (B) for the detection. Molecular mass markers in kDa are shown on the left. (1) 30 µg extract of cells transfected with plasmid pcDNAI/hNL4. (2) 30 µg extract of cells transfected with plasmid pcDNAI/Amp. (3) Immunoprecipitation from 300 µg extract (1) using the polyclonal α ZO-1 antibody. (4) Immunoprecipitation from 300 µg extract (2) using the polyclonal α ZO-1 antibody.

3.5. Expression of Neuroligins Outside of Neurons

The fact that human neuroligin 4 mRNA is detectable in different tissues on the Northern blot was surprising (figure 3.13), because the rat neuroligins were found exclusively in the brain (Ichtchenko et al., 1995, 1996). Therefore, different tissues and cell types should be investigated in regard to their ability to transcribe neuroligin genes and express neuroligin proteins. This analysis was performed either by RT-PCR, or by Western blotting using the α NL1/3 antibody which at least recognizes neuroligins 1, 3 and 4 in different species. As is shown in figure 3.19 and table 3.3, in addition to neurons several other brain cell types, i.e. astrocytes, endothelial cells and microglial cells, were found to express neuroligins. In accordance with the human Northern blot, the two kidney-derived cell lines COS-7 (monkey) and MDCK II (dog) were negative in the Western blot. In contrast, the rat liver extract was negative, whereas the human liver was positive for neuroligin 4 on the RNA blot. Obviously, rodent neuroligins are expressed predominantly in the brain, whereas human neuroligins have a broader expression profile. Arguments for this interpretation come from the analysis of deposited EST sequences. Most entries encoding rodent neuroligins are derived from different brain regions, although there are few exceptions. In contrast, ESTs coding for human neuroligins were isolated from additional tissues. Taken together it may be speculated that, at least some, human neuroligin proteins may have functions outside of the nervous system.

Figure 3.19: **Detection of neuroligin proteins in different tissues and cells.** In all lanes, 25 µg extract were loaded. Western blot detection was performed using the α NL1/3 antibody. Molecular mass markers in kDa are shown on the left. (1) HUVEC extract. (2) Human umbilical vein extract. (3) Rat brain extract. (4) EC219 cell extract. (5) Primary mouse microvessel endothelial cell extract. (6) Primary mouse astrocyte extract. (7) COS-7 cell extract. (8) MDCK II cell extract. (9) Rat liver extract. (10) L929 cell extract.
### 3. RESULTS

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Table 3.3: Overview of tissues, primary cells and cell lines tested for neuroligin transcription (RNA) or expression (protein). Numbers in parentheses give relative expression levels. + Neuroligin detected, - neuroligin not detected, n.d. not determined.

The only undisputed tissue in regard to neuroligin expression is the brain which was positive in all species tested. The cDNAs encoding human neuroligins 1-5 were isolated from a brain library (KIAA clones), and correct neuroligin 4/5 sequences were found by RT-PCR on human brain polyadenylated RNA (figure 3.9). Unexpectedly, only a weak signal for neuroligin 4 was detected in brain on the Northern blot (figure 3.13). To further investigate expression of neuroligin proteins in human brain, different tumor extracts were tested by Western blotting using the α NL1/3 antibody (figure 3.20). Tumors originating from astrocytes, oligodendrocytes and, of course, neurons showed strong neuroligin expression. Interestingly, some tumors of a particular type, e.g. GBM (glioblastoma multiforme), showed strong neuroligin expression, whereas others of the same type gave no signal. This may reflect deregulated gene expression. Nevertheless, most samples from human brain tumors showed strong expression of neuroligin proteins.

Figure 3.20: Neuroligin expression in human brain tumors. In all lanes, 30 µg extract were loaded. For Western blot detection, the α NL1/3 antibody was used. Molecular mass markers in kDa are shown on the left. (1) Xanthoastrocytoma. (2) Pilocytic astrocytoma. (3) GBM. (4) Oligodendroglioma. (5) Anaplastic astrocytoma. (6) GBM. (7) Dysembryoplastic neuroepithelial tumor. (8) GBM.
4. DISCUSSION

4.1. Neuroligins - More Members, More Missions

Screening of currently available DNA databases using the known rat neuroligins 1-3 as baits resulted in the identification of sequences encoding the homologous proteins of mouse and human, as well as in the identification of sequences encoding one or two further members of the family which were named neuroligins 4 (and 5). These novel sequences were detected exclusively in human and could not be found in rodent databases. A partial neuroligin 1 cDNA isolated from a mouse brain library and completed with a PCR-generated fragment showed an unexpected behaviour: The protein, produced in transfected COS-7 cells and deglycosylated, ran in SDS-PAGE at a lower mass than calculated, and immunofluorescence microscopy analysis demonstrated that the molecule accumulated intracellularly instead of being transported to the plasma membrane. cDNAs encoding human neuroligins 1 and 4, in contrast, were translated into proteins of expected sizes in COS-7 cells and were incorporated into the plasma membrane. Neuroligin 4 was then investigated in more detail: Neuroligin 4 bound with its C-terminal tail to PSD-95/SAP90, but not to ZO-1, another cytoplasmic PDZ domain-containing protein, exactly as previously shown for rat neuroligins by others (Irie et al., 1997). A Northern blot analysis revealed expression of neuroligin 4 in different human tissues, whereas all rat neuroligins were detected only in brain (Ichtchenko et al., 1995, 1996). Together, these data demonstrate that the neuroligin family is composed of at least one additional member, and that the human proteins may follow missions outside of the central nervous system due to their broader expression.

4.2. Mouse Neuroligin 1 - The Fate of a Membrane Protein

Protein produced from the mouse neuroligin 1 cDNA has the attribute to accumulate intracellularly in the ER and/or the Golgi apparatus instead of being transported to the plasma membrane. As the human homolog expressed in COS-7 cells is detected in the membrane, it can be supposed that the mouse cDNA sequence contains a serious error. When comparing the rat and mouse sequences, only two amino acids differ between the rodent neuroligin 1 proteins (figure 3.1); at position 317, the alanine in the rat sequence is exchanged by a threonine, and at position 773 the mouse protein has an isoleucine instead of the valine in rat. In all eight human and rat neuroligins, amino acid 317 is an alanine, whereas amino acid 773 is a valine in all neuroligins 1 and 2 or an isoleucine in all neuroligins 3-5. It could therefore be speculated that the exchange at position 317 in the mouse sequence causes the problems, eventually leading to misfolding of the
protein and subsequent intracellular aggregation. Nevertheless, several pieces of evidence confirm the correctness of the mouse neuroligin 1 cDNA: Firstly, both amino acid replacements are conservative. Secondly, the exchange at position 317 lies within the cDNA clone isolated from the library and therefore does not represent a PCR-generated error. Thirdly, the exchange within the PCR-generated fragment at position 773 is found in half of the human and rat neuroligins. Finally, the mutation at position 773 does not cause the intracellular retention of the protein, as a construct encoding only the extracellular domain of mouse neuroligin 1, which was expressed in *Drosophila* S2 cells, accumulated within the cells, whereas a control protein was secreted (data not shown). To exclude that the intracellular protein accumulation is a consequence of overloading of the COS-7 cell, the following experiments were performed: (i) The cells were stained at different time points after transfection. (ii) The amount of DNA in the transfection experiment was reduced. (iii) Cell lines that do not replicate plasmids containing the SV40 origin of replication were used for transfection. None of these changes resulted in proper delivery of the protein.

Confusing data were obtained from Western blot experiments: Instead of reacting with a band at approximately 118 kDa as found with rat and human neuroligin 1, the α NL1/3 antibody detected a band of approximately 90 kDa in extracts from COS-7 cells transfected with the mouse cDNA (figure 3.5A). Nevertheless, on some blots a second very fine signal was detected at 118 kDa (figure 3.14). Treatment of this cell extract with N-glycosidase F reduced the mass to approximately 80 kDa (figure 3.7A); this is astonishing because the calculated mass of mouse neuroligin 1 is higher (92.0 kDa with or 87.0 kDa without the signal peptide). Interestingly, as observed from the Western blot shown in figure 3.15, the human neuroligin 1 cell extract shows two bands at 118 kDa and 90 kDa of almost comparable intensities. Deglycosylation of this extract resulted in bands of approximately 102 kDa and 80 kDa (figure 3.15). In addition, in some experiments also the rat neuroligin 1 showed two forms (Ichtchenko et al., 1995; Song et al., 1999). Obviously, neuroligin 1 proteins can be detected on the Western blot as 118 kDa or 90 kDa forms, whereas such a situation was not found with the human neuroligin 4 protein. It can only be speculated about what the 90 kDa form represents: As the deglycosylated proteins run at only 80 kDa, the suspicion obtrudes that the molecule is not synthesized to full-length and therefore can not be transported to the plasma membrane, or that the protein is cleaved, or that it has a very unusual migration behaviour on the gel, what is unlikely due to the presence of the correct 118 kDa band on the gel.

4.3. Human Neuroligin 4 - Discovery and Characterization

A database search resulted in human sequences that were homologous to the three rat neuroligins, of which only human neuroligin 3 was described in the literature (Philibert et al., 2000). Analysis of genomic neuroligin sequences revealed that the gene structure is similar for different neuroligin isoforms. For each of the human neuroligins 1 and 3 two inserts for splice site A (encoded by exons 3 and 4) were discovered either by database analysis or by RT-PCR (figure 3.8). For human neuroligin 2 only one insert (A2) was found, and analysis of the intron sequence between exons 2 and 4 failed to identify an additional insert. The same situation was found in rat (figure 1.14B), therefore it can be speculated that there is only one splice insert for site A in neuroligin 2. The sizes of the corresponding introns vary between human isoforms, whereas the length
of particular exons is conserved among all neuroligins with the exception of exon 6 which is extended in the neuroligin 1 gene by 27 bp encoding region B (figure 3.11).

During the DNA database analysis, human sequences were found that have high homologies to neuroligins but could not be allocated to a particular isoform. Alignment of the derived protein sequence with those of neuroligins 1-3 revealed that these sequences encode a further member of the neuroligin family of cell-surface molecules (figure 3.8). Several points support the identity of human neuroligin 4. Firstly, this protein has highest sequence identity in the order of 63-73% with the previously identified members of the family (table 3.1), but only moderate similarity to other proteins, in particular esterases; in addition, the characteristic domain structure of neuroligins is maintained in neuroligin 4 as well (figure 3.11A). Secondly, as in neuroligins 1-3, a serine residue essential for catalytic activity of esterases is absent in neuroligin 4 and replaced by a glycine (figure 3.8). The other two amino acids of the catalytic triad of esterases (S,E/D,H), however, are preserved, as is the case for neuroligins 1-3. The fixed positions of all cysteine residues in neuroligins, some of which are supposed to form disulfide bridges, are also completely maintained in neuroligin 4 (figure 3.8). Thirdly, immunofluorescence microscopy analysis demonstrated that neuroligin 4 is expressed in transfected COS-7 cells and is integrated into the plasma membrane, a prerequisite for a CAM (figure 3.16). Fourthly, neuroligin 4, like the rat neuroligins 1-3 (Irie et al., 1997), binds to PSD-95/SAP90 (figure 3.17). This interaction is specific, as ZO-1, another PDZ domain protein, failed to coprecipitate neuroligin 4 (figure 3.18). Possible binding of neuroligin 4 to neurexin proteins remains to be investigated. Finally, neuroligin 4 mRNA was detected on the Northern blot (figure 3.13). Whether neuroligin 4 is translated from endogenous mRNA can currently not be assessed because the αNL1/3 antibody recognizes at least neuroligins 1, 3 and 4.

4.4. Human Neuroligin 5 - Evidence for its Existence

Sequences with high homology but without identity to the human neuroligin 4 gene were identified during DNA database analysis and RT-PCR. The evidence in support of the existence of a fifth neuroligin is fourfold: (i) Sequences encoding neuroligins 4 or 5 are derived from cDNAs. (ii) These sequences can clearly be assigned to either neuroligin 4 or 5, thus reducing the incidence of random sequencing artefacts. (iii) Intron and untranslated sequences derived from neuroligin 4 and 5 genomic sequences, respectively, show a lower degree of identity. (iv) Although not without contradictions, currently available data assign neuroligin 4 or 5 genomic sequences to different chromosomes (table 3.2). Since neuroligin 5 sequences were discovered after RT-PCR with polyadenylated RNA from a female individual, assignment of the neuroligin 5 gene to the Y chromosome may be excluded. In addition, expression studies in different human tissues with KIAA0951 (neuroligin 5) and KIAA1260 (neuroligin 4) suggested distinct patterns (see below; Nagase et al., 1999a).

About the exact functions of different neuroligin isoforms only little is known, but about their origin can be speculated. The scheme outlined in figure 4.1 presents a working model for the evolution of neuroligin genes. It is very likely that these genes arose from a common ancestral gene which was previously assembled using an acetylcholinesterase gene and further sequences of unknown origin. During evolution, the neuroligin gene was duplicated several times (I-IV). Be-
cause neuroligin 4 and 5 sequences are almost identical, duplication IV must have occurred at a relatively late point of time. It is tempting to speculate that duplications III and IV exclusively happened in human, as neuroligins 4 and 5 were not identified in rat and mouse. Probably, rodent and human genes drifted apart from each other in the time between gene duplications II and III. Alternative splice site A, found in all neuroligin (figures 3.8 and 3.10) but not in esterase genes, will have developed in the ancestral neuroligin gene before the first duplication. Interestingly, for neuroligin 2, which is the protein with lowest homology to the other family members, only one splice insert in site A was identified (A2). It can be assumed either that before gene duplication I only insert A2 was present in site A of the ancestral neuroligin gene, or that neuroligin 2 has lost the second insert, A1, in the course of evolution. In contrast, site B of neuroligin 1, which was found to be alternatively spliced in rodents and is always present in the human protein, was probably created after gene duplication II.

Figure 4.1: Model for the evolution of neuroligin genes. This scheme was generated considering the identity numbers of human neuroligin cDNAs. The ancestral gene was formed using sequences of an acetylcholinesterase (AChE) and of further, unknown genes. The five neuroligin genes likely arose from the ancestor gene by duplications (roman numbers). Neuroligin 4 and 5 are the most closely related genes because only one branch point separates them.

4.5. Human Neuroligins - Expression Outside of the Nervous System

Northern blot analysis demonstrated a tissue distribution of human neuroligin 4 which, unexpectedly, did not show a significant signal in brain polyadenylated RNA (figure 3.13), despite the fact that the sequenced PCR fragment and clone KIAA1260 were derived from human brain RNA. The broad expression profile of human neuroligin 4, in contrast with the nervous system-restricted expression pattern of the rat neuroligins, may be due to different levels of sensitivity. It cannot be excluded, however, that expression of neuroligins may vary among different species. In support of this hypothesis are expression studies of human neuroligin 3 which report mRNA in heart, brain, skeletal muscle, placenta and pancreas (Németh et al., 1999; Philibert et al., 2000), whereas in rat tissues signals were detected exclusively in brain (Ichtchenko et al., 1995, 1996). Also of note in this context is the finding that the human neuroligin 3 probes detected a 7 kb transcript in the non-nervous system samples, but only a 4 kb transcript in brain (Németh et al., 1999; Philibert et al., 2000). In rat brain RNA, in contrast, comparable amounts of both the 4 kb and the 7 kb neuroligin 3 transcripts were found (Ichtchenko et al., 1996).
Additional evidence for a broader expression pattern of human neuroligins come from the observation that deposited human EST sequences are derived from many different tissues including heart, which showed the strongest signal in the Northern blot (figure 3.13), whereas rodent sequences predominantly originate from brain tissue. Another argument are the expression profiles of the KIAA clones (Kikuno et al., 1999; Nagase et al., 1999a, 1999b, 2000a, 2000b); for all five human neuroligins, this analysis resulted in a relatively broad distribution of the corresponding mRNA transcripts (figure 4.2). The profiles of human neuroligins 1-4 show the strongest signal in brain, whereas neuroligin 5 has the highest transcription level in the pancreas. For neuroligin 4, further tissues with strong signals are heart and liver, whereas lung and kidney show only low transcription levels, consistent with the Northern blot shown in figure 3.13.

Figure 4.2: Expression profiles of the five KIAA clones coding for human neuroligins 1-5. The tissue expression levels of the five neuroligin genes were analyzed by using RT-PCR followed by ELISA (enzyme-linked immunosorbent assay). Gene names are given at the left side of each set of color codes. The color conversion panel shown at the bottom is used for displaying mRNA levels as color codes (increasing portion on total polyadenylated RNA from left to right). Besides ten tissues, nine regions of the adult CNS as well as fetal brain and liver were included in the expression profiling: (1) Heart. (2) Brain. (3) Lung. (4) Liver. (5) Skeletal muscle. (6) Kidney. (7) Pancreas. (8) Spleen. (9) Testis. (10) Ovary. (11) Brain amygdala. (12) Brain corpus callosum. (13) Brain cerebellum. (14) Brain caudate nucleus. (15) Brain hippocampus. (16) Brain substantia nigra. (17) Brain subthalamic nucleus. (18) Brain thalamus. (19) Spinal cord. (20) Fetal liver. (21) Fetal brain. From Kikuno et al., 1999, and Nagase et al., 1999a, 1999b, 2000a, 2000b.

4.6. Future Perspective

If neuroligins have a broader tissue distribution than previously anticipated, this raises the question of subcellular structures and possible functions with which non-nervous system neuroligins may be related. An interesting association in this context may be with subcellular structures that are involved in cell-cell interactions and intercellular communication, such as tight, adherens and gap junctions. Although these structures, in contrast to synapses, are symmetrical with respect to molecular organization and information exchange, neuroligins might play a role there which could be analogous to the one assigned in the synapse, i.e. as inducer/positioner or stabilizer of such cellular contact sites. Supporting this notion of a role of neuroligins in junctional functionality are observations that human endothelial cells and mouse choroid plexus epithelial cells express neuroligins (table 3.3). Choroidal epithelial cells have highly developed tight and, presumably, adherens junctions in order to maintain the barrier between blood and cerebrospinal fluid. In this context it is important to mention that gliotactin, a glial protein in Drosophila which may be a homolog of neuroligins, is involved in maintenance of the glia-made insect blood-nerve barrier.
(Auld et al., 1995). Interestingly, mouse astrocytes in culture are able to express neuroligins (figure 3.7B). Supporting these observations are findings made by Gilbert et al. (2001) who reported expression of neuroligin 3 in many classes of glia, including dorsal root ganglia and cultured Schwann cells. Since Schwann cells form the myelin of peripheral nerves, it can be speculated that neuroligins may play a role in myelin compaction and/or stabilization in junctional structures related to endothelial and epithelial junctions. It has been described that oligodendrocytes in the central nervous system form tight junctional structures (Mugnaini and Schnapp, 1974), and that these junctions are lost in animals devoid of the gene encoding the tight junction protein Osp (oligodendrocyte-specific protein)/claudin-11 (Gow et al., 1999). Whether neuroligins are present in such structures remains to be tested. Additionally, putative extracellular binding partners for neuroligins outside the nervous system have to be identified, since neurexins are significantly expressed only in brain (Ushkaryov and Südhof, 1993; Ushkaryov et al., 1992). A homophilic interaction seems unlikely, since Drosophila S2 cells stably overexpressing neuroligin 1 do not form aggregates (Nguyen and Südhof, 1997). Further experiments will be required to elucidate functional properties of non-neuronal neuroligin proteins.

'I succeeded in collecting some original observations that were not without value.'

Santiago Ramón y Cajal, 1917
5. REFERENCES


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