GENERAL INTRODUCTION

1. Synaptic plasticity

Ever since the recognition of the basic organization of the brain during the last century, hypotheses about the mechanisms of information storage have focussed on the connection between neurons as the most likely site of action. The earliest of these connectionistic hypotheses were those of Ramon y Cajal (Ramon y Cayal, 1911) and Tanzi (Tanzi, 1893), who proposed that utilization promoted the formation of new circuits or that existing circuits were strengthened. The more modern formulation, which underlies much of our current thinking, was that of Hebb (Hebb, 1949), who proposed more specific rules for modification of the connection. These rules have now proved to be remarkably prophetic.

The interconnection between neurons is named synapse. The synapse is the most fundamental unit of information transmission in the nervous system. Information storage, including all forms of memory and behavioral adaptation, are thus believed to emerge from changes in neuronal transmission, both in the short-term and the long-term, a property known as synaptic plasticity. Synaptic plasticity is a highly regulated process, emerging from complex interactions not only at the synapse itself. It can also be the result of a specific neuronal interplay at the molecular, cellular and system level. Thus, understanding the mechanisms underlying synaptic plasticity may help to apprehend general learning and memory processes.

Changes in synaptic plasticity are achieved by changes in inhibitory or excitatory neurotransmission or both. The first part of this thesis deals with the modulation of excitatory neurotransmission. The principal excitatory neurotransmitter in the brain is glutamate. The regulation of glutamate-mediated excitatory neurotransmission has been shown to play a critical role in many aspects of synaptic plasticity. The phosphorylation of glutamate receptors has been demonstrated to alter their function, suggesting that they may be targets of various kinases and phosphatases during the induction and maintenance of synaptic plasticity (reviewed in Roche et al., 1994). The aim of this part of the thesis is to investigate brain regional differences in the modulation of one of the members of the glutamate receptor family, namely the N-methyl-D-aspartate (NMDA) receptor. In an expression system the modulation of the NMDA receptor by phosphorylation is studied. Thereafter the possible physiological role of NMDA receptor modulation is assessed *in vivo* by monitoring immediate early gene expression.

The second part of the thesis focuses on the possible correlation between synaptic plasticity and learning and memory. In particular, the significance of the neuropeptide corticotropin-releasing factor (CRF) and of acute stress on hippocampal synaptic plasticity and learning is investigated.

The following paragraphs will provide more background information and a discussion of the existing literature on the topics studied in this thesis.

1.1 Basic principles of neurotransmission (Fig. 1)

Synapses are the elementary building blocks of neuronal communication. There are two types of synapses either electrical or chemical, although electrical synapses are less common than chemical synapses in the brain. At electrical synapses transmission occurs by means of current flow through gap junctions which connect the cytoplasm of the pre- and postsynaptic cells. Transmission across these electrical synapses is extremely rapid.

Small molecules, the neurotransmitters, mediate information transfer between neurons at chemical synapses. Before release, the transmitters are stored in small membraneous organelles, the synaptic vesicles. Upon arrival of an action potential, the synaptic membrane depolarizes causing an opening of presynaptic calcium channels. The resulting rise in intracellular calcium triggers exocytosis of synaptic vesicles, which releases the transmitter into the synaptic cleft. The transmitter molecules diffuse across the synaptic cleft, which separates the transmitting cell from the receiving (postsynaptic) cell. They bind to specific membrane receptors at the presynaptic cell, the autoreceptors, which can regulate the rate of transmitter release, or to receptors on the membrane of the postsynaptic cell. Postsynaptic receptors binding neurotransmitter can open Na+ or Ca2+ channels at the postsynaptic cell, thus causing a depolarization and carrying on the signal to the next cell. Alternatively, the presynaptic cell can inhibit firing of the postsynaptic cell by release of inhibitory neurotransmitters. There are two types of postsynaptic receptors named ionotropic and metabotropic receptors. Ionotropic receptors are directly coupled to ion channels and are responsible for fast chemical transmission. As soon as the neurotransmitter binds to the receptor, the ion channel opens. Common neurotransmitters that mediate signaling of this type are glutamate and acetylcholine, which are usually excitatory neurotransmitters, and glycine and γ-aminobutyric acid (GABA), which are usually inhibitory neurotransmitters. The metabotropic receptors in the postsynaptic membrane that bind neurotransmitters in the slow chemical transmission pathway are not directly coupled to ion channels but affect them or alter the level of intracellular second messengers like adenosine 3',5'-cyclic monophosphate (cAMP), Ca2+ and diacylglycerol (DAG), through intermediary G-proteins.

If the neurotransmitter would remain bound to the receptor, the postsynaptic cell would be in a state of constant depolarization or hyperpolarization. Therefore, neurotransmitters are enzymatically degraded or they can be transported back to the presynaptic cell via transporters (reuptake). The transmitter-receptor complex can also be taken back into the cell via invagination of membrane which pinches off to form a vesicle, a process called internalisation. The vesicle then fuses with the endosome and inside the endosome transmitter dissociates from the complex and is transferred to the lysosome for degradation and the receptor is recycled to the membrane. The receptor itself can also become desensitized. After prolonged exposure to its own transmitter a receptor can become refractory to later application of the same transmitter.

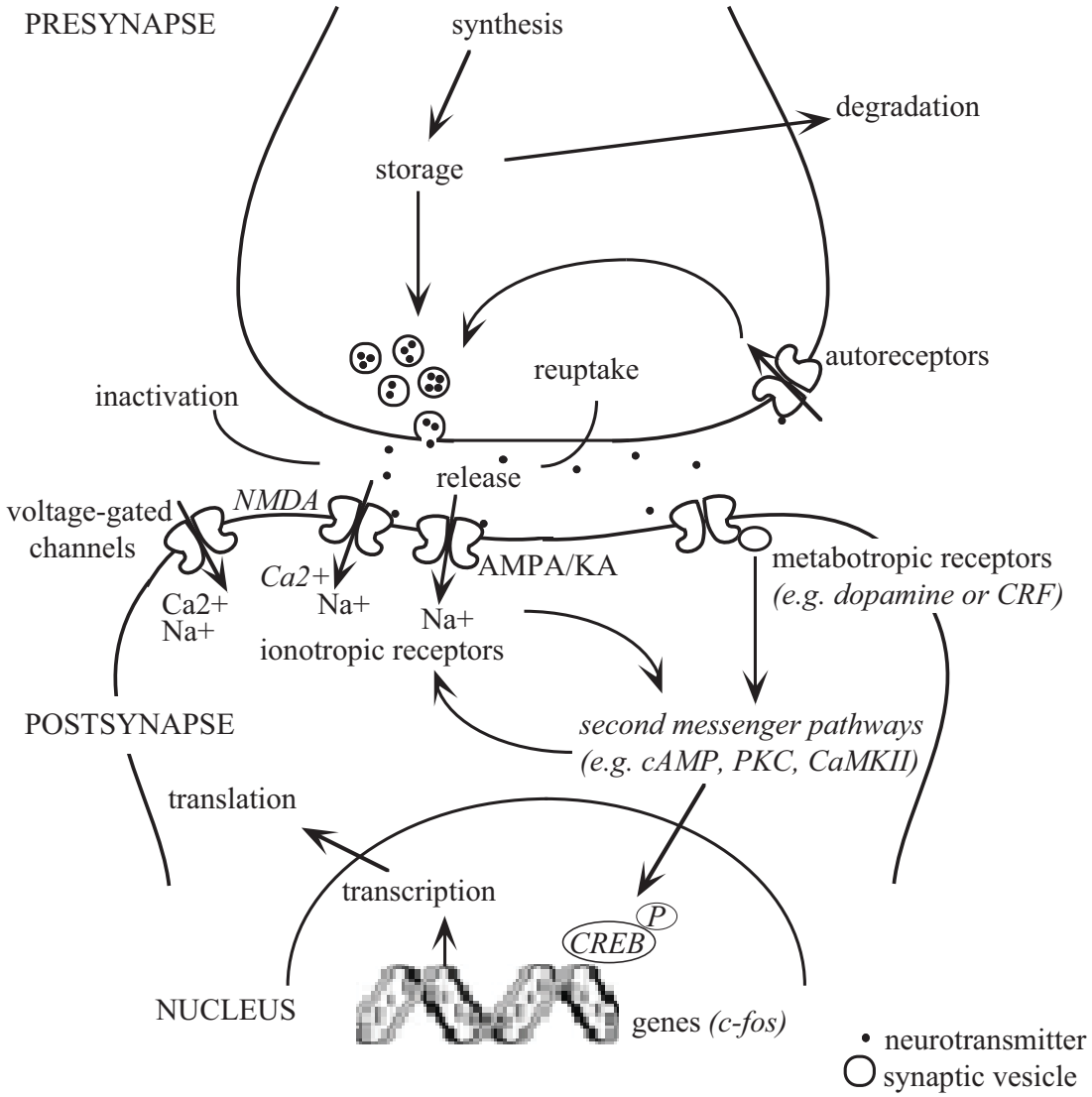

Figure 1. *Overview of the processes involved in synaptic transmission. The elements directly addressed in this thesis are written italic.*

1.2 Second messenger pathways

Changes in the level of second messengers by metabotropic receptors elicit a cascade of events leading, among others, to the activation of enzymes controlling protein phosphorylation. Phosphorylation of proteins is an important mechanism for the modulation of their function, and is thought to play an important role in synaptic plasticity in different brain regions.

Many hormone- and neurotransmitter-stimulated signaling pathways alter the activities of target proteins including receptors, ion channels, synaptic vesicle proteins and nuclear proteins by reversibly phosphorylating serine and threonine residues.

1.2.1 Protein kinases

Target proteins such as the NMDA receptor channel complex, which are involved in synaptic plasticity, can be phosphorylated by serine/threonine protein kinases. cAMP-dependent protein kinase (PKA), protein kinase C (PKC), and calcium/calmodulin-dependent protein kinase (CaMK) are the three principal serine/threonine kinases, which catalyze protein phosphorylation in response to second messengers such as cAMP, Ca2+ and/or DAG.

Mammalian PKA includes four regulatory (RIα, RIβ, RIIα, RIIβ) and three catalytic (Cα, Cβ, Cγ) subunits, each encoded by a unique gene. PKA consists of an inactive heterotetramer of two catalytic subunits bound to two regulatory subunits (Taylor et al., 1990). Together the regulatory and catalytic subunits form the functional enzyme, the holoenzyme. Subunit assembly in the holoenzyme is likely to differ because each subunit shows a distinct expression pattern across the brain regions (Cadd and McKnight, 1989). PKA is activated by cAMP, which is generated by adenylyl cyclase via G protein-coupled receptor activation. When cAMP binds to the regulatory subunit of PKA, the holoenzyme dissociates to yield a regulatory subunit dimer and two activate catalytic subunits.

Increases in intracellular Ca2+ concentrations via influx from the extracellular space and via mobilization from intracellular stores by inositoltriphosphate activate PKC and CaMK. PKC is a group of Ca2+ and phospholipid-dependent serine/threonine kinases. It exists of a family of isozymes that differ in structure, cofactor requirement and substrate specificity (Nishizuka, 1988; Dekker and Parker, 1994). These multiple isoforms of PKC also show distinct patterns of tissue expression and subcellular localization (Tanaka and Saito, 1992). Activation of PKC requires an increase in intracellular Ca2+ and DAG and/or unsaturated fatty acids depending on the isoform involved. Binding of DAG in combination with Ca2+ and phosphatidylserine deinhibits the kinase by producing a conformational change that releases the catalytic site from a pseudosubstrate sequence. Activation of several isoforms is also characterized by a translocation from cytoplasm to membrane.

When the intracellular Ca2+ concentration rises, the concentration of Ca2+/calmodulin (CaM) complex increases. CaM binds to an autoinhibitory domain on the CaM kinases and thereby activates these enzymes. Four different types of CaMK (I, II, III, IV) have been described but main attention concentrates on CaMKII which is also called multifunctional CaMK. CaMKII constitutes up to 2% of the total protein content in certain brain areas such as the hippocampus (Erondu and Kennedy, 1985) and is localized in postsynaptic densities of excitatory synapses as the most abundant protein and as such its participation in mechanisms of synaptic plasticity has been widely discussed (Kennedy, 1989; Soderling, 2000). CaMK is made up of a multiple gene family, in which each of the four distinct classes of CaMK (α, β, γ, δ) is encoded by a separate gene (Tobimatsu and Fujisawa, 1989). Within a class several isoforms have been identified and both homomultimers and heteromultimers exist (Bennett et al., 1983; Kanaseki et al., 1991). The holoenzyme is arranged in a so called hub-and-spoke pattern, which enables unique autoregulatory functions (Kanaseki et al., 1991). Autophosphorylation at threonine-286 (Thr286) increases the affinity of the molecule to calmodulin (Meyer et al., 1992) which makes it Ca2+ independent. Moreover, phosphorylation of the

autonomy site is sufficient to disrupt the autoinhibitory domain, through which the kinase stays partially active even after calmodulin dissociates. Hence, assay of autonomous CaMK activity from control and treated groups gives a direct measure of the fraction of the kinase that has been activated by stimulation of the cells.

1.2.2 Protein phosphatases

Although former studies mainly concentrated on the role of protein kinases in the modulation of target proteins by phosphorylation, it has recently become apparent that dephosphorylation of proteins by phosphatases plays an equally important role in alterations of synaptic transmission. Serine/threonine phosphatases can be divided in two major classes according to similarities in amino-acid sequence. The first class shares a common phosphatase domain and includes phosphatase 1 (PP1), protein phosphatase 2A (PP2A) and calcineurin (PP2B). Recently, molecular cloning identified several more protein serine/threonine phosphatases (PP4, PP5, PP6, and PP7) belonging to this class. The protein phosphatase 2C (PP2C) class consists of several closely related isoforms that have very little sequence homology with the first class (Cohen, 1997).

PP1 has emerged as a prominent regulatory element in synaptic plasticity. It is a multifunctional enzyme that controls the phosphorylation status and activity of a variety of downstream effector molecules that are known to govern synaptic strength (Greengard et al., 1999). These include NMDA (Snyder et al., 1998) and alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) (Yan et al., 1999) glutamate receptors, plus additional components of the calcium signaling cascade, such as CaMKII (Strack et al., 1997) and cAMP response element-binding protein (CREB; Bito et al., 1996). The native structure of PP1 is a 1:1 complex between the catalytic and a number of different regulatory/targeting subunits. Although present throughout the brain, at least four isoforms of the PP1 catalytic subunit are expressed at varying levels in different regions. For example, PP1α and PP1γ1 are abundant in striatum whereas PP1β is much less prevalent (Da Cruz e Silva et al., 1995). Within neurons, PP1 is highly enriched in dendritic spines and is therefore appropriately localized for the regulation of excitatory synaptic transmission (Ouimet et al., 1995). The substrate specificity may be controlled by a number of different regulatory subunits both in different tissues and within the same tissue.

PP2A is composed of a common core enzyme comprising a scaffold subunit that is always associated with the PP2A catalytic subunit. The major native forms of PP2A are heterotrimers in which the core enzyme associates with one of a variety of regulatory subunits that are expressed in a cell- and tissue-specific manner. The precise roles of PP2A in dephosphorylation of brain proteins are not clearly delineated. However, it was shown to dephosphorylate voltage-sensitive sodium channels (Chen et al., 1995) and it selectively dephosphorylated soluble autophosphorylated CaMKII (Strack et al., 1997). On the basis of this finding PP2A was even proposed to be a possible regulator of protein kinases (Barnes et al., 1995; Millward et al., 1999).

PP2B or calcineurin is unique in its requirement for Ca2+ and calmodulin for activation. It is a heterodimeric phosphatase composed of a catalytic subunit and a regulatory subunit. Binding to the CaM complex (Klee et al., 1998) activates the enzyme. It is widely distributed within the brain, with the highest concentrations in

the hippocampus and caudate putamen (Klee et al., 1988). Interestingly, calcineurin becomes activated at much lower Ca2+ concentrations than CaMKII. Once activated, it has a rather restricted substrate specificity. Phosphoproteins and also CaMKII are preferentially dephosphorylated by calcineurin.

Although PP2C is present in the brain (Kasahara et al., 1999; Strack et al., 1997), little is known about the role of this phosphatase in synaptic plasticity, partly because of the lack of appropriate pharmacological tools. One likely substrate is CaMKII, which is dephosphorylated by PP2C at its autophosphorylation site (Fukunaga et al., 1993; Strack et al., 1997).

1.2.3 Phosphoproteins

A number of mechanisms can coordinate the actions of kinases and phosphatases. These include changes in their expression level, their subcellular localization, and phosphorylation of catalytic and regulatory subunits. The action of protein kinases and phosphatases can also be modulated via the intervention of protein phosphatase inhibitors, which are activated by second messenger-regulated protein kinases. Concomitant control of kinases and phosphatases by these endogenous phosphatase inhibitors provides the cell with the capacity to rapidly switch proteins from their phosphorylated to their dephosphorylated state.

Inhibitor-1 was the first such protein to be identified, being found in skeletal muscle (Nimmo and Cohen, 1978). Inhibitor-1 becomes inhibitory to PP1 after phosphorylation on Thr35 by PKA (Endo et al., 1996; Foulkes et al., 1983) and can be dephosphorylated by calcineurin and PP2A (Shenolikar, 1994). It is also phosphorylated at serine-67 (Ser67) by a cyclin-dependent kinase family member (Cdk5) which makes it a less efficient substrate for PKA (Bibb et al., 2001). Inhibitor-1 shows a ubiquitous distribution in the brain (Hemmings et al., 1992; Gustafson et al., 1991; MacDougall et al., 1989).

Dopamine- and cAMP-regulated phosphoprotein with an apparent M_r 32000 (DARPP-32) is a homologue of inhibitor-1 that inhibits PP1 with the same potency upon phosphorylation at Thr34 by PKA. DARPP-32 is also an excellent substrate for phosphorylation by cyclic GMP-dependent protein kinase (PKG) *in vitro* (Hemmings et al., 1984). It differs from inhibitor-1 in that it can also be phosphorylated on Ser45 and 102 by casein kinase II which promotes phosphorylation at Thr34 by PKA (Girault et al., 1989). Furthermore, DARPP-32 can also be phosphorylated by casein kinase I at Ser137 (Desdouits et al., 1995a). Phosphorylation at this site inhibits dephosphorylation of the regulatory Thr34 site (Desdouits et al., 1995b). DARPP-32 is dephosphorylated *in vitro* by calcineurin and PP2A at Thr34 (Hemmings et al., 1984; King et al., 1984; Nishi et al., 1997, 1999) whereas PP2C can dephosphorylate DARPP-32 at Ser137 (Desdouits et al., 1998). Interestingly, Cdk5 can also phosphorylate DARPP-32 at Thr75 (Bibb et al., 1999). Phosphorylation of DARPP-32 at Thr75 inhibits PKA by a competitive mechanism and, thus, decreases the efficacy of dopaminergic signaling. However, dopamine, through the activation of PKA, can also increase the activity of PP2A, leading to the dephosphorylation of Thr75. In this way, activated PKA attenuates its own inhibition. This positive feedback loop will result in amplification of signaling through the dopamine / PKA / Thr34-DARPP-32 / PP1 signal transduction cascade (Nishi et al., 2000). DARPP-32 shows a strong region-specific distribution. It is