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Pharmacological and Developmental Aspects on Neuronal Plasticity

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“There are two mistakes one can make along the road to truth...
not going all the way, and not starting”

Gautama Siddharta ”Buddha”

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PAPERS

This thesis is based on studies presented in the following four papers, referred to in the text by their roman numerals.

- I. Johansson, I. M., Bjartmar, L., Marcusson, J., Ross, S. B., Seckl, J. R., and Olsson, T. (1998) "Chronic amitriptyline treatment induces hippocampal NGFI-A, glucocorticoid receptor and mineralocorticoid receptor mRNA expression in rats." *Brain Res Mol Brain Res*; 62, 92-95.

- II. Bjartmar, L., Johansson, I. M., Marcusson, J., Ross, S. B., Seckl, J. R., and Olsson, T. (2000) "Selective effects on NGFI-A, MR, GR and NGFI-B hippocampal mRNA expression after chronic treatment with different subclasses of antidepressants in the rat." *Psychopharmacology (Berl)*; 151, 7-12.

- III. Bjartmar, L., Huberman, A. D., Ullian, E. M., Renteria, R. C., Liu, X., Xu, W., Prezioso, J., Susman, M. W., Stellwagen, D., Stokes, C. C., Cho, R., Worley, P., Malenka, R. C., Ball, S., Peachey, N. S., Copenhagen, D., Chapman, B., Nakamoto, M., Barres, B. A., and Perin, M. S. (2006) "Neuronal pentraxins mediate synaptic refinement in the developing visual system." *J Neurosci*; 26, 6269-6281.

- IV. Bjartmar, L., Alkhorji, L., Ruud, J., Mohammed, A.H., Marcusson, J., Hallbeck, M. (2009) "Long-term treatment with antidepressants, but not environmental stimulation, induces expression of NP2 mRNA in hippocampus and medial habenula." Submitted to *Brain Research*

ABSTRACT

Neuronal plasticity means the ability of the brain, its cells and networks to adapt and adjust to new challenges, a process which is ongoing throughout life. The goal of this thesis was to gain better understanding of the molecular events that follow different types of stimulations of brain structures such as the hippocampus, a key region for cognitive functions with overriding control on the corticosteroid system. A better knowledge of the mechanisms involved in neuronal plasticity is fundamental in the development of strategies for improving health in patients suffering from major depression or cognitive disorders such as Alzheimer's disease.

Antidepressant drugs induce the expression of several genes involved in neuronal plasticity, a mechanism which may explain the several weeks time lag between treatment initiation and clinical effect commonly observed in patients. Besides, there are indications that disturbances in the corticosteroid system are involved in the pathogenesis of major depression. Therefore, the mRNA expression of the glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) as well as of the immediate-early genes NGFI-A and NGFI-B was analyzed using *in situ* hybridization in the hippocampus and cortex after 21 days treatment with various antidepressant drugs having different monoaminergic profiles. The mRNA expression of the transcription factors was selectively increased depending on region and also on the monoaminergic profile of the drug given. Generally, drugs with less specificity for monoamines had an overall more anatomically wide-spread inducible effect.

In a follow-up study the message expression of the synaptic protein NP2 was investigated in a similar setting where long-term (21 days) was compared with short-term (3 days) antidepressant treatment. In addition to the hippocampus, the medial habenula, a relay station within the limbic system was analyzed. Overall there was an upregulation of NP2 mRNA expression following long-term treatment irrespective of the monoaminergic profile of the drug. Simultaneously, NP2 mRNA was analyzed in rats exposed to enriched, normal or deprived environments respectively, an experimental setting known to affect neuronal plasticity. However, in contrast to the pharmacological treatment, this

environmental stimulation did not lead to alterations in NP2 mRNA expression in any of the regions studied.

Finally, the function of NP2 as well as the closely related proteins NP1 and NPR was investigated. The “knock-out mouse” technique was used to eliminate these neuronal pentraxins (NPs), both individually and in various combinations. Since previous data had suggested that the NPs are involved in synaptic development, axonal refinement in the visual system during development was analyzed in these animals. In the NP1/NP2 knock-out mice, synaptic formation, axonal development and refinement occurred at a significantly slower rate than in wild-type mice, indicating that the NPs may be necessary for activity-dependent synaptogenesis.

In conclusion, the results of the studies constituting this thesis demonstrate that long-term treatment with antidepressant drugs, possessing different monoaminergic profiles, has selective effects on the expression of NGFI-A, NGFI-B, GR and MR in the mammalian brain. In general, the least selective drugs exhibit the most profound effect suggesting that induction of neuronal plasticity is more effective with multiple neuronal inputs. The results also show that NP2 expression is induced by antidepressant drugs, in contrast to environmental stimulation, supporting the presence of different pathways for inducing neuronal plasticity depending on type of stimuli. Finally, this thesis indicates that the neuronal pentraxins play an important part in synaptic development.

ABBREVIATIONS

ACTH	adrenocorticotrophic hormone
AMPA	α -amino-3-hydroxyl-5-methyl-4-isoxazole-propionate
BDNF	brain-derived neurotrophic factor
CA	cornu ammonis
CaCl ₂	calcium chloride
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
CHO	Chinese hamster ovary
CNS	central nervous system
CREB	cAMP response element binding protein
CRH	corticotropin releasing hormone
CX	cortex
DA	dopamine
DG	dentate gyrus
dLGN	dorsal lateral geniculate nucleus
DNA	deoxyribonucleic acid
ECT	electroconvulsive therapy
EDTA	ethylene-diamine-tetra-acetic acid
EE	enriched environment
ES	embryonic stem
GABA	γ -aminobutyric acid
GR	glucocorticoid receptors
HPA	hypothalamic pituitary adrenal
5-HT	5-hydroxytryptamine (serotonin)
IEG	immediate-early gene
ISH	in situ hybridization
LHb	lateral habenula
LTP	long-term potentiation
LTD	long-term depression
MAO-A	monoamine oxidase-A
mGluR _{1/5}	metabotropic glutamate receptor 1/5
MHb	medial habenula
MR	mineralocorticoid receptors
mRNA	messenger ribonucleic acid
NA	noradrenaline

NaCl	sodium chloride
NGFI-A	nerve growth factor-induced gene A
NGFI-B	nerve growth factor-induced gene B
NMDA	N-methyl-D-aspartic acid
NP	neuronal pentraxin
PC ₁₂	pheochromocytoma cell line
POMC	pro-opiomelanocortin
PVN	paraventricular nucleus
RGC	retinal ganglion cells
RSG	retrosplenial granular cortex
SGZ	subgranular zone
SVZ	subventricular zone
SSRI	selective serotonin reuptake inhibitor
VEGF	vascular endothelial growth factor
WT	wild type

INTRODUCTION

Neuronal plasticity

Neuronal plasticity allows the CNS to reorganize neuronal networks in response to environmental stimulation in order to learn skills and remember information, and to recover from brain injuries. The neurons and their networks adjust continuously and adapt to new experiences. Plasticity can be beneficial for the organism, such as in learning processes or in compensation after injury where neighboring neuronal circuits adapt to the novel situation and resume new responsibilities and tasks. These plastic changes include reorganization of existing cortical maps [1], a mechanism which, for example, plays a role in medical rehabilitation. On the other hand, plastic events in the brain can be negative for the individual who exposes himself to toxic substances which may lead to addictive behavior [2]. Obviously neuronal plasticity is especially prominent in the developing brain, but the phenomenon continues throughout life. Due to age-related alterations in plasticity the young brain is more sensitive to addictive behavior than the adult [3] and the ability for compensation after injury does deteriorate with age [4, 5]. Basic molecular mechanisms involved in plasticity include increase or decrease in synaptic transmission, or synaptic strength; alterations in gene expression (including neurotrophic mechanisms); morphological alterations, and neurogenesis. Programmed cell death, or apoptosis, can also be considered a basic mechanism for neuronal plasticity which is especially abundant during early development, although this specific process lies beyond the scope of this thesis.

Molecular basis of neuronal plasticity

Long-term potentiation (LTP) and long-term depression (LTD) are experimental phenomena used to demonstrate the long-lasting modifications of which individual synapses are capable [6] and has been most extensively studied in the CA₁ subregion of the hippocampus. Traditionally, LTP is considered the physiological/experimental model of

long-term memory formation in the mammalian brain [7]. LTP is induced by synaptic NMDA-receptor activation leading to an influx of the trigger Ca^{2+} , and a rapid postsynaptic modification of AMPA-receptors which become increased in number due to amplified trafficking, and also increased in efficiency via direct phosphorylation [6], hence enhancing synaptic transmission. This early phase lasts 30-60 minutes and is independent of protein synthesis. However, maintenance of LTP, or late-phase LTP, requires activity-dependent changes in gene transcription and synthesis of effector proteins that allow for a stably altered neuronal function [6, 8]. The immediate-early gene (IEG) NGFI-A (*Egr1*, *zif268*) (see below) is required for the transition from early-phase to the protein synthesis-dependent late-phase LTP, in that it regulates the transcription of late-response genes [9, 10]. Morphological changes that accompany LTP include growth of new dendritic spines, enlargement of preexisting spines and their associated postsynaptic densities and also an increase in spine arborization [6]. In contrast, LTD leads to a downregulation of postsynaptic AMPA-receptors, possibly through internalization, and LTD is probably important, like LTP, for learning and memory, experience-dependent development, addiction, and neurodegenerative diseases [6].

Immediate-early genes are rapidly and transiently transcribed in response to specific stimuli as opposed to late-response genes whose transcription is dependent on new protein synthesis [11]. The *c-fos* and *c-myc* proto-oncogenes, that were among the first IEGs to be described, have important regulatory functions during cell proliferation and can be involved in the origin of malignancies [11]. In the CNS *c-fos* is induced by generalized seizures, and *fos*-deficient animals have deficits in synaptic plasticity and behavioral adaptation [8]. NGFI-A, NGFI-B and NPTX2 are other examples of IEGs that are induced by seizures [11-13], and they encode DNA-binding proteins, or transcription factors (NGFI-A, NGFI-B and NP2 respectively), controlling the transcription of the DNA allowing for specific effector function in the neuronal response [11, 14]. These transcription factors may be important in development turning on/off transcription of specific genes leading to changes in cell differentiation and morphology. Other examples of transcription factors are the cytoplasmically located corticosteroid receptors MR and GR which will be described in more detail below.

Also neurogenesis, or the formation of new neurons, remains a continuous process throughout life [15] after its most intensive period during prenatal development. Adult neurogenesis predominantly occurs in two brain regions; the subventricular zone (SVZ) which lines the brain ventricles, and the subgranular zone (SGZ) which is located adjacent to the dentate gyrus of the hippocampal formation. Neurons that develop in the SGZ are believed to contribute to hippocampal-dependent memory function [16, 17]. This neurogenesis is continuous, although certain stimuli i.e. stimulating environment promotes survival of the cells [16]. In contrast, neurogenesis can be inhibited by cytostatic drugs and stress [17, 18].

Neuronal plasticity in Alzheimer's disease and depression

Alzheimer's disease (AD) and major depression (MD) are two distinctly different disorders which significantly impact affected patients as well as the health systems worldwide. They do not share a common pathogenesis but both diseases involve the hippocampus, a brain structure well-studied in terms of different aspects of neuronal plasticity and a region with great influence in cognitive functions, especially memory.

Alzheimer's disease

Alzheimer's disease is the most common cause of dementia among people over age 65 with a rapid increase in prevalence with age. It is estimated that about 50% of people over age 85 suffer from AD. Initially, memory deficits is the principal symptom indicating an early involvement of the hippocampus, but subsequently the pathological process spreads throughout the brain causing progressive cognitive disturbance. Pathologically AD is characterized by "senile plaques" or more specifically amyloid- β ($A\beta$) deposits which may be intracellular in location; neurofibrillary tangles with tau accumulation; and neuronal loss [19]. In animal models of AD, soluble $A\beta$ oligomers have been found to disrupt LTP, delay intracellular transmission, making the cells even more vulnerable to excitotoxicity. Such changes would diminish the ability for learning and can be an explanation for mild cognitive impairment in pre-clinical AD [20]. Despite the progressive nature of the disease there are data suggesting that cognitive training can be beneficial for early stage AD

[21]. Also a higher education level may result in a greater cognitive reserve due to higher synaptic density which may help compensate for early-stage decline, but later in disease this advantage diminishes [22]. Environmental enrichment has been shown to reduce cognitive difficulties and stimulate neurogenesis in animal models of AD [23] and also lead to a reduction of A β levels and cholinergic deficits [24, 25]. Hence, given the link between symptom development and reduced neuronal function, an increased knowledge of neuronal plasticity is highly relevant in the context of influencing the pathological process of AD.

Major depression

Major depression (MD) is another great burden for involved individuals and the health-care systems. The life-time prevalence is on average 17% in the industrialized world, more specifically men 9.9% and women 24% [26], with a median age of onset in the range of 20-25 years [27]. The clinical picture is characterized by depressed mood, diminished pleasure in activities, altered appetite, sleep disturbance, loss of energy, feeling of worthlessness, diminished ability to concentrate, and recurrent thoughts of death or suicide.

One hallmark of MD is increased activity in the hypothalamic pituitary adrenal (HPA) axis. This overdrive leads to elevated levels of circulating corticosteroids [28-30] and patients with a persistent HPA disturbance after remission from a depressive episode have an increased risk for relapse [31]. Also, patients suffering from Cushing's disease, a condition where the corticosteroid levels are dramatically elevated, have a high incidence of major depression [32].

In MD as well as in Cushing's disease, there is an increased occurrence of hippocampal atrophy [32-34], due to reduced cell size instead of cell loss [35]. The cause of this atrophy is suggested to be caused by either a direct or an indirect neurotoxic effect of the corticosteroids in this highly sensitive region [36].

The HPA axis

The hypothalamic pituitary adrenal (HPA) axis is regulated via negative feedback at several levels from circulating corticosteroids, including the CNS (see Fig. 1.). Neurons located within the hypothalamic PVN release CRH into the portal vasculature, stimulating pituitary release of ACTH through enzymatic cleavage of pro-opiomelanocortin (POMC). ACTH, in turn, mediates the synthesis and release of corticosteroids from the adrenal glands. The function of the HPA axis is affected by stress. In non-stressed conditions, the corticosteroid levels show a circadian pattern, with low activity at the onset of the resting phase and high activity at the onset of the active phase, a rhythm that is coordinated by inputs from the suprachiasmatic nucleus. The regulation of corticosteroid secretion during stressful conditions is largely under the control of limbic brain regions such as the hippocampus, amygdala and prefrontal cortex [37].

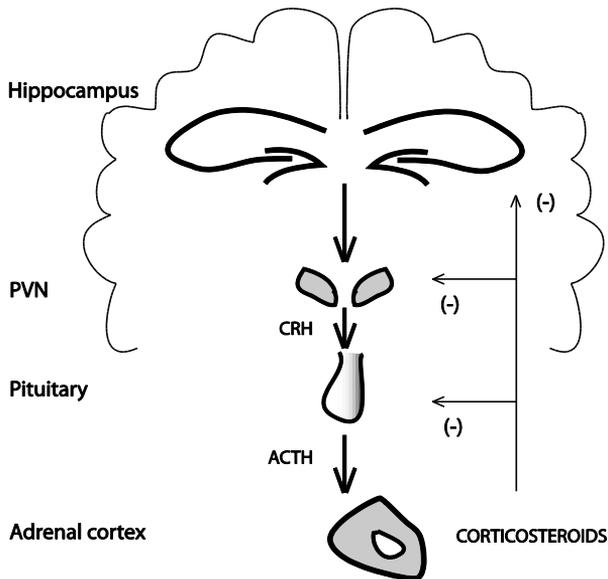


Figure 1. Schematic illustration of the hypothalamic-pituitary-adrenal (HPA) axis and the negative feedback sites for the circulating corticosteroids [38].

The hippocampus influences the HPA axis

The hippocampus contains very high levels of the corticosteroid receptors MR and GR. These receptors function as ligand-activated transcription factors that reside in the cytoplasm of the neuron, dimerize upon ligand binding, translocate into the nucleus and exert transcriptional control (positive or negative) over glucocorticoid-responsive genes [39, 40]. GR are expressed in most CNS neurons, but MR predominantly are of hippocampal location in rodents [41-43]. The GR have a relatively low affinity for corticosteroids and are only substantially occupied by their ligands at circadian peaks or during stress [44], where they act to bring the individual back to normal homeostasis, facilitating recovery, and in non-stressful conditions are important for memory storage [37, 38, 45]. The MR have a tenfold higher affinity for corticosteroids than the GR, and are highly occupied by basal corticosteroid levels, possibly enhancing inhibitory input to CRH-producing cells in the PVN [44]. Also the ratio between MR and GR appears important for control of emotional reactivity [39] and hippocampal MR are involved in cognitive functions [46, 47]. Hence, the hippocampus exerts a negative feedback control on the HPA axis via both GR and MR [37-39, 43, 48, 49] and constitutes a region important for mood and cognitive function [39, 47, 48, 50, 51], disturbance of which becomes symptomatic of depressive disorders.

Neuroanatomical structures of particular interest

The Hippocampus

The hippocampal region (hippocampus proper, dentate gyrus (DG) and the subicular complex) is part of the limbic system and constitutes anatomically related structures in the medial temporal lobe. The hippocampus proper primarily contains pyramidal neurons, and its structure is divided into four subregions CA₁-CA₄ which are located closely to the granule cells of the DG. The DG receives input from the adjacent entorhinal cortex via the perforant path. The entorhinal cortex, which is early affected in Alzheimer's disease, has prior to this connection to the DG pre-processed information from major sensory cortical areas, including the prefrontal cortex. The DG sends projections to the CA₂-CA₄, the CA₃

to the CA₁, which in turn projects to the subiculum and sequentially back to the entorhinal cortex [38]. The hippocampus is in rodents a region of major importance for spatial memory, which requires consolidation of input from several adjacent regions [52]. Also in humans the hippocampus is of major importance for most types of memory, especially the episodic type, whereas semantic and spatial memory may require the hippocampus for retrieval of memories stored mostly in cortical areas [53]. In addition to cognitive functions, the hippocampus provides input to brain regions such as the prefrontal cortex, cingulate cortex, and amygdala. It is well established that the hippocampus plays a significant role influencing altered mood and emotion in depressive disorders [54, 55].

The Habenula

The habenula which is located dorsally in the lateral walls of the third ventricle, in the epithalamus, comprises two separate nuclei on each side, the medial (MHb) and the lateral (LHb), which appear to represent distinct subcircuits within the dorsal diencephalic conduction (DDC) system [56]. The DDC is one of two major pathways that interconnect the limbic forebrain with sites in the mid- and hindbrain. It consists of afferent fibers of the stria medullaris (SM), projecting to the habenular nuclei, and the fasciculus retroflexus (FR) which projects efferent axons from the habenula towards the mid- and hindbrain [57]. The DDC regulates the activity of monoaminergic nuclei in the ventral midbrain, and involvement in cognitive processes, especially spatial learning and attention has been proposed [57, 58]. The LHb, mainly containing glutamatergic neurons, is a point of convergence for information from the basal ganglia and limbic forebrain. This structure has strong functional links with dopaminergic cells in the ventral midbrain, hence believed to be involved in modulating motor behaviors [57]. The MHb, which is more evolutionary conserved than the LHb, primarily receives inputs from the hippocampus and subiculum via the septum, and also from ascending monoaminergic nuclei [57, 59]. Most of the efferent axons from the MHb project to the interpeduncular nucleus (IPN) which contains extremely high levels of acetylcholine. The habenulo-interpeduncular pathway is one of the major cholinergic pathways in the brain and is viewed as a relay station within the limbic system [57, 60].

Pharmacological stimulation of neuronal plasticity

Antidepressants influence GR and MR

Long-term administration of various types of antidepressants results in an increase of both glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) mRNA levels in the hippocampus [41, 61-64] and is associated with improved spatial learning [65].

Mice heterozygous for the GR gene (GR +/-), with a 50% reduction of GR, manifest an increased risk compared to wild-type mice in developing depressive behavior after being exposed to stressful events. On the other hand, genetically altered mice with a two-fold increase in GR are more stress resistant [66], but manifest an impaired regulation of the HPA-axis and mild cognitive impairment suggestive of hippocampal dysfunction [67]. These “knock-in” mice also exhibit an enhanced hippocampal dynamic BDNF expression [66, 68] as opposed to a morning down-regulation of BDNF in GR +/- mice [69]. Forebrain-specific GR knock-out mice, lacking GR completely in most limbic regions from 4-6 months of age, demonstrate a robust depression-like phenotype which is reversed by chronic antidepressant treatment [48]. Together, these data support the relevance of GR in pathophysiological studies of depressive disorders and stressful conditions.

Antidepressants influence hippocampal IEGs

The genes NGFI-A and NGFI-B, IEGs which were discovered in PC12 cells exposed to NGF, may also be relevant in antidepressant-induced neuronal plasticity given their interactions with GR (see below). NGFI-A (also *zif268*; *krox24*; *egr1*) which is important for LTP [9, 70], is constitutively expressed as a transcription factor in several areas of the brain, including the hippocampus [14, 71, 72], and is induced by environmental enrichment [73] as well as by long-term antidepressant treatment [74]. NGFI-A binds the GR promoter in rat and guinea-pig suggesting that it has a crucial role in GR transcription [75, 76].

NGFI-B (*nur77*) is also constitutively expressed in the hippocampus [14, 77], and part of its sequence is highly conserved to GR and other intracellular receptors, suggesting that it may be a DNA-binding protein

[78]. There is a direct protein-protein interaction and transcriptional antagonism between NGFI-B and GR [79, 80] and NGFI-B appears to positively influence both CRH and POMC, the precursor of ACTH, and have a positive effect on the HPA-axis [49, 81]. Also, NGFI-B is closely associated with dopamine (DA) [82] and serotonin (5-HT) [83] transmission. Moreover, its expression is modulated by administration of DA receptor antagonists or psychostimulants [82]. Finally, NGFI-B knockout mice have an altered DA turn-over, further supporting that NGFI-B is involved in the regulation of DA neurotransmission [82].

Antidepressants induce hippocampal neurogenesis

Various forms of stress lead to decreased neurogenesis in the subgranular zone (SGZ) [55]. This suppression is normalized by long-term but not acute treatment with antidepressants, consistent with the “therapeutic lag” of antidepressive treatment commonly observed in clinical praxis. Such normalization is not achieved by treatment with other forms of psychoactive substances such as haloperidol and morphine [84]. The antidepressant-induced neurogenesis has been attributed to stimulation of the 5-HT_{1A}-receptor having a trophic or mitogenic effect on dentate precursor cells [85]. Alternative, perhaps more likely, mechanisms are induction of the cAMP cascade (CREB), and increased BDNF and VEGF which are consistently upregulated by chronic antidepressant treatment [86-89] as well as by ECT [90, 91]. The growth factors VEGF and BDNF increase the proliferation and survival, respectively, of newly formed neurons in the hippocampus [18]. BDNF also induces the expression of the neurotrophin VGF which in turn enhances neuronal proliferation and produces antidepressant-like behavioral effects [92]. It is possible that the mechanism behind the clinical effect of antidepressants is simultaneous stimulation of noradrenaline (NA), 5-HT, VEGF, and BDNF receptors and - via their intracellular pathways - subsequent induction of neuronal plasticity, gene transcription and cell proliferation, as well as cellular resilience and survival [18].

Environmental influence on neuronal plasticity

Environmental enrichment (EE), which may be used as a model of enhanced cognitive, sensory and motor stimulation, can induce experience-dependent plasticity at structural and functional levels in animal models of both healthy and dysfunctional brains [93]. Housing animals in an EE results in an increase of dendritic branching and length, sizes and numbers of synapses, increased hippocampal neurogenesis [16, 94-98], i.e. increased survival of progenitors [99], as well as altered expression of genes involved in synaptic function and cellular plasticity [100, 101]. Such environment also induces the expression of NGFI-A [102], synaptic strength, including LTP [103-105] and an improvement in spatial memory [73, 99]. Furthermore, increase of VEGF induced by EE and exercise is associated with improvements in spatial memory [94, 97]. Exposure to EE after ischemic stroke leads not only to increased levels of BDNF, NGFI-A, and NGFI-B, and a reversal of lesion-induced GR mRNA reduction [106, 107], but also to an improvement of deficits in learning and memory [108, 109]. Nurturing young mice in an enriched environment leads to an accelerated development of the visual system [110].

Morphological plasticity

The function of the nervous system relies on precise synaptic connections between neurons and their target cells. In the case of damage to the CNS, the neurons have the ability to adjust their morphology in order to compensate for the loss of function, for instance increased dendritic branching of non-injured axons adjacent to the lesion.

During development, morphological plasticity plays a fundamental role in the process of shaping the brain. In the juvenile CNS, there is a considerable overshoot in axonal branches, and more synapses are established than ultimately will be retained. Hence, there is an on-going elimination of synapses during maturation. The anatomical refinement of synaptic circuits occurs at the level of individual axons and dendrites through rapid elimination [111], a critical step in synaptic circuit maturation [112]. In addition, there is an initial excess in number of

neurons, which is reduced by programmed cell death, apoptosis, another important process during development. Critical phases during development are characterized by different stages of pronounced synaptic reorganization – so called “developmental windows” – during which disturbances may lead to defective synaptic wiring and functional deficits [113].

Activity-dependent refinement

It is unclear whether the formation of synapses in the immature brain is dependent on synaptic activity only. In contrast, synaptic maintenance and remodeling is completely guided by activity of the synaptic network [111-113]. The molecular mechanisms underlying such activity-dependent refinement are not completely understood.

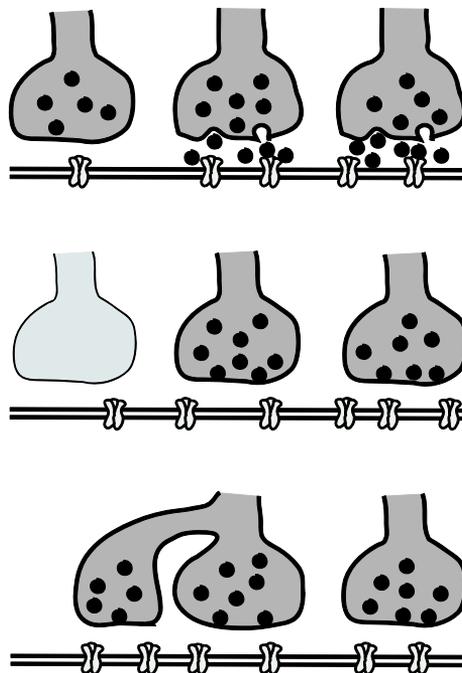


Figure 2. Illustration of activity-dependent refinement where there is axonal sprouting to compensate for withdrawal of adjacent axon that has degenerated due to non-synchronous firing. Adapted from Cohen-Cory *et al.* [112]

It is known that some terminals withdraw due to non-synchronous firing with its neighbors, whereas if there is ongoing synchronous activity in the remaining axons, sprouting will occur to establish new synapses, thereby promoting arborization [112] (see Fig. 2.). This process has been studied extensively in the context of ocular dominance in the visual system [111].

Given that elimination of synaptic components may be an important factor in the maturation of synaptic connections, the molecules involved in this process are of particular interest. The synaptic membranes are continuously undergoing remodeling. Neurotransmitters are released into the synaptic cleft via presynaptic vesicles, which fuse with the membrane, followed by reuptake of the transmitters via endocytosis and engulfment of the membrane which is subsequently recycled [114, 115]. The snake venom neurotoxin Taipoxin inhibits neurotransmission by blocking synaptic vesicle recycling at the neuromuscular junction [116, 117]. Therefore, Taipoxin has proved useful as a tool when attempting to characterize molecules crucial for synaptic membrane remodeling in the CNS.

Neuronal Pentraxins

The neuronal pentraxins (NP) were identified as proteins binding in a calcium-dependent manner to Taipoxin. They define a subfamily within the pentraxin family with whom they share 50% cDNA sequence homology. The CNS specific NP family consists of Neuronal Pentraxin 1 (NP1) [118], Neuronal Pentraxin 2 (NP2) [119, 120]; also apexin [120] and Narp [121], and Neuronal Pentraxin Receptor (NPR) [122].

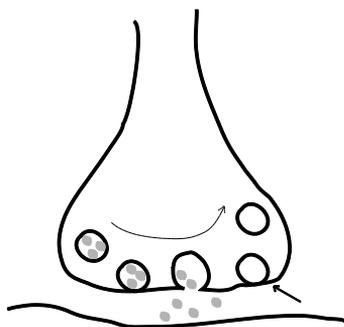


Figure 3. Synapse where vesicles containing neurotransmitters are exocytosed to release the transmitters. The vesicles are subsequently recycled via endocytosis. Taipoxin, a neurotoxin, inhibits vesicle recycling at the site of endocytosis (arrow); hence the transmission is completely inhibited.

Parallel to its characterization as a taipoxin-binding pentraxin, NP2 mRNA was demonstrated to be rapidly upregulated in the hippocampus following seizures. This revealed characteristics as an IEG with consensus binding sites for the transcription factors CREB and NGFI-A on its putative promoter region [121]. NPR contains a possible membrane-spanning hydrophobic domain, suggesting that it is presented on the cell surface [122], whereas NP1 and NP2, based on their cDNA sequence, are predicted to be secreted proteins [118, 119]. *In vitro* experiments with NP-expressing CHO cells indeed demonstrate that NPR is transmembranous and forms heteromultimeric complexes with NP1 and NP2, which thereby are presented on the cell surface [123]. Heterocomplexes of NP1 and NP2, as well as NP2 individually, play a role in synaptic formation through clustering of the excitatory glutamatergic AMPA receptor subunits [13, 124].

The neuronal pentraxins share 20-30% cDNA sequence homology with previously identified pentraxins, such as C-reactive protein (CRP) of the immune system, which is rapidly elevated in the serum during the acute phase response [125, 126]. CRP levels can increase up to 1000-fold and is believed to help mediate clearance of cellular debris during inflammation [126]. Due to this sequence homology, and structural similarities between the neuronal pentraxins with classical pentraxins such as CRP, the function of the neuronal pentraxins was initially thought to involve synaptic material uptake during synapse formation and remodeling.

AIMS

The general aim of this thesis was to gain insight into three aspects of the basis of neuronal plasticity; alterations of gene expression in response to pharmacological stimulation; the possible effect of behavioral stimulations on these genes; and the molecular concept of synaptic plasticity /remodeling during development.

The specific aim of each paper was to provide answers to the following questions:

- I. What effect does the tricyclic antidepressant drug amitriptyline, a compound with a predominant serotonergic profile, have on the transcription factors NGFI-A, NGFI-B and on the glucocorticoid and mineralocorticoid receptors? Can we find any explanation to the delayed onset of effect observed clinically after initiation of antidepressant therapy?
- II. How do various antidepressant drugs, with different mechanisms of action and monoaminergic profiles, affect the transcription factors NGFI-A, NGFI-B, and the glucocorticoid and mineralocorticoid receptors? Is there a difference in mRNA expression related to specificity or mechanism of the drugs?
- III. What role do the neuronal pentraxins play during synapse development? Are they important for synaptic plasticity?
- IV. Does environmental enrichment influence the expression of NP2? Is there any effect of short- and long-term treatment, respectively, with various antidepressant drugs on NP2 mRNA expression?

METHODOLOGICAL ISSUES

For detailed descriptions of all methods used, please refer to the original articles. This section discusses special features, advantages and limitations of selected methods.

Antidepressant treatment (papers I, II & IV)

The hypothesis that disturbance in predominantly noradrenaline (NA) and serotonin (5-HT) neurotransmission is the cause of major depression has been the main therapeutic focus for roughly 50 years. Drugs that deplete catecholamine (and to a lesser degree 5-HT) storage were shown to induce depression in many patients. The early non-selective MAO-inhibitor, iproniazid, as well as the tricyclic antidepressant, imipramine, were found to both elevate mood and NA levels. Elevation of NA levels through inhibition of the presynaptic α_2 -adrenoceptor with desipramine supported this theory. Moreover, since α_2 -adrenoceptors also are present on 5-HT nerve terminals, their inhibition elevates 5-HT neurotransmission as well. Antidepressive agents with α_2 -adrenoceptor antagonism combined with 5-HT receptor stimulation are currently available. In the mid 1970s the focus shifted to 5-HT transmission as the main pharmacological target in depression and the selective serotonin reuptake inhibitors (SSRIs) were developed. More recently, evidence for involvement of dopamine in depression has evolved which has led to development of dopaminergic drugs for antidepressant treatment. See [127] for historical review.

Even though antidepressant compounds that raise monoamine levels at the synapse are clinically successful in the treatment of depression, the reason for the delayed onset of treatment effect of several weeks [128], despite instantaneous specific binding and stimulation of the receptors, requires alternate explanations.

Antidepressants used in papers I & II were the tricyclic amitriptyline; the reversible MAO-A inhibitor moclobemide; the SSRI fluoxetine; 8-OH-

DPAT and buspirone, a full and partial 5-HT_{1A} agonist respectively (see table 1 for more details).

This panel of drugs was selected given the hypothesis that different types of pharmacological stimulations/inhibitions of the serotonergic system would differently influence intracellular events after receptor binding. In paper IV fluoxetine, which along with its active metabolite has a very long half-life and a slow development to steady-state, was replaced by the SSRI citalopram to purify the serotonerg specificity and kinetics [129]; 8-OH-DPAT and buspirone were omitted in favor of the tetracyclic maprotiline with a preferentially noradrenergic profile.

Drug	Mechanism	Specificity	Paper
Amitriptyline	TCA	DA<<5-HT>NA	I, IV
Moclobemide	MAO-A inhibitor	5-HT=NA>DA	II, IV
Fluoxetine	SSRI	DA<<<5-HT>>NA	II
8-OH-DPAT	Full 5-HT _{1A} agonist	5-HT _{1A} >>5-HT ₇ >>5-HT ₄ >>D ₂	II
Buspirone	Partial 5-HT _{1A} agonist	5-HT _{1A} =D ₂ >>α ₁ ,α ₂	II
Citalopram	SSRI	DA<<<5-HT>>>NA	IV
Maprotiline	Tetracyclic	5-HT<<NA>DA	IV

Table 1. Drugs used in papers I, II and IV, their pharmacological mechanisms and monoaminerg specificity. For 8-OH-DPAT and buspirone, specificity accounts for receptor binding [130], whereas for amitriptyline, fluoxetine, citalopram and maprotiline it accounts for the inhibition of monoamine transporters [129], and for moclobemide the specificity of the enzyme [131].

In the initial two papers all rats were treated for 21 days with subcutaneous (s.c.) injections twice daily. Since such manipulation may be associated

with pain and/or stress, there is a possibility that it could have an impact on the stress-related transcription factors analyzed. In the follow-up study (paper IV), administration of drugs was altered to an osmotic pump implanted s.c. aiming to eliminate unnecessary stressors for the animals. However, a non-specific induction was observed in the animals at the 3 day time-point, putatively due to implantation of the osmotic pumps. In order to enable a more adequate evaluation, additional time-points both prior to and after 3 days would have been of value. 21 days was elected to represent chronic treatment which for several years has been a consensus treatment length in similar studies.

Enriched Environment (paper IV)

Housing rats in an enriched environment (EE) is a standardized and well established procedure for evaluating the effect of a stimulating environment and social interactions on the brain. Pups are introduced to the different environments at weaning, hence at a very young age, and remain there for 30 days before being sacrificed. The EE cages, housing 8 animals, are larger than standard laboratory cages. These cages contain different types of items which periodically are exchanged, allowing for exploring and stimulation of physical exercise. In the standardized environment (SE) 4 rats are kept in each cage which is smaller and contains sawdust bedding only. In the poor environment (PE) group, the rats are housed individually, with minimal exposure to animal care workers. Nevertheless, despite the exposure to various stimuli with the EE cage, the type of enrichment offered still represents a deprived condition compared to environments in the wild [99].

***In situ* hybridization (papers I, II & IV)**

For the initial two papers, the radioactive method for performing *in situ* hybridization (ISH) was chosen. This method is used for quantitative analysis of mRNA in individual cells with high regional accuracy. Cells undergo a fixation procedure whereafter available mRNA hybridizes with a complementary riboprobe coupled to a radioactive ligand (³⁵S). After high

stringent washing to minimize non-specific binding, the sections are placed on autoradiographic film for a certain time period (in the present experiments 2 weeks). After this, the film is developed, allowing for rough estimations of labeling. The sections are subsequently dipped in nuclear emulsion (NTB₂) and exposed for additional 3-6 weeks where the radioactive emission converts the silver crystals of the nuclear emulsion to insoluble metallic silver. These silver grains are quantified using specific software, allowing for high accuracy.

A potential disadvantage of this method is long exposure-time to the radioactive material before development (up to 6 weeks in the experiments described here), which delays feed-back and prevents rapid methodological alterations. The need for radioactive isotopes may also render the method more hazardous. However, while using radioactive labeling, there is an additional option of co-labeling the cells for proteins using immunohistochemistry, thereby increasing the information extractable from the sections.

During the time-course of these studies the DIG-method was developed and introduced in the lab. Instead of hybridizing mRNA with radioactive ligands, the probe is labeled with the antigen digoxigenin which after hybridization is identified using immunohistochemistry. One advantage with this method is its speed, enabling results day 3, which substantially accelerates the experiments. Furthermore there is no need for radioactive isotopes and no concerns with possible decay of labeled probe, enabling storage and usage of identical probe in repeated experiments. The downside of this method is that co-staining with antibodies for proteins is non-feasible. The validity of the method regarding sufficient detail for quantitative analysis had been evaluated prior to entering study IV [132].

Generation of knock-out mice (paper III)

Development of knock-out (KO) mice is a method for studying the importance of a gene and its products. The development of this technique, which involves the use of embryonic stem (ES) cells, was awarded with the Nobel Prize in physiology or medicine in 2007. The motivation did read

that “few discoveries have had greater impact on contemporary biomedical sciences”, and “the technique is being used today in the development of new drugs for treating virtually all important human diseases” [133]. The general concept is that obliteration of a specific functional gene in the genetically engineered animal enables an estimation of its function depending on the resulting phenotype.

The neuronal pentraxins are essentially brain specific, although NP2 initially was described as a testicular protein [120] with a potential role in maturation of sperms. The concern that knocking out NP2 might make these mice sterile proved wrong however, since fertility was not affected in these KO mice.

The genes were identified and sequenced and the first (NP2) and second and third (NPR) exons respectively were replaced with a neomycin-resistance coding cassette. The constructs were electroporated into 129/SVJ-derived ES cells and neomycin-resistant clones were screened via genomic southern for the correctly sized band. Positive cells were inserted into blastocysts, derived from strains with different fur coat color than the ES cells, and implanted into pseudopregnant females serving as surrogate mothers. Newborn pups were either black (wild-type) or chimeric (mixed genotype). Chimeras are not necessarily potential breeders since the construct may have been inserted into non-germ cells, hence the altered gene will not be transferred to the next generation. Hence, chimeric animals were bred with C57Bl/6 mice to generate heterozygots, and further on homozygots. Tail tips from all pups were screened via genomic southern.

Obviously, creating and characterizing a KO mouse is a well established strategy for evaluating gene function. It is also a time-consuming technique which requires large financial and spatial resources. Even though it is a formidable method for studying specific gene function, there are limitations to the technique. Several genes are mandatory for survival and development, hence knocking out such a gene, the embryo cannot survive into adulthood, some may even die in utero. To compensate for this, conditional knock-outs have been developed. Here, additional sequences are introduced into the construct, allowing for a temporal shut-down of the gene expression.

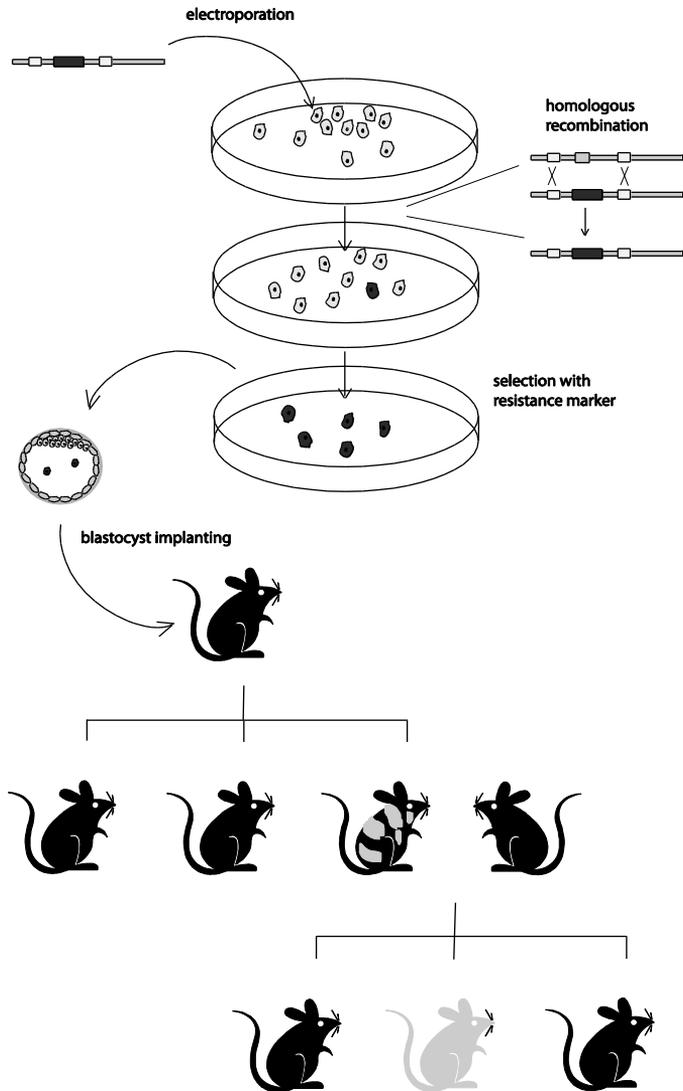


Figure 4. Illustration of “knock-out” procedure. The construct where the target gene is replaced with neomycin-resistance coding cassette is electroporated into ES cells, followed by positive selection by growth during neomycin treatment. Surviving ES cells are screened with genomic southern blots, cells containing the correct sized band, indicating the occurrence of homologous recombination, are inserted into blastocysts, implanted into surrogate mothers and sequential breeding occurs according to image above. Adapted from www.Answers.com.

Using this technique, even genes crucial for survival and development are accessible for gene silencing. It is also possible to introduce the construct specifically into desired target organs which is especially important in ubiquitously located proteins. Since the NP knock-out mice were viable and fertile there was no need for creating conditional knock-outs at this stage. However, one cannot ignore the possibility that the lack of the NPs can have been compensated by other proteins having similar functions. Such an (hypothetical) effect could have been avoided by conditional knock-outs, since compensatory mechanisms theoretically would not have sufficient time to develop. However, an involvement of the NPs in synaptic reorganization would make them especially important during development. Creating a conditional knock-out with intact NP expression during development was therefore not an obvious option for the purpose of these studies. Finally, the possibility that different mouse strains have inborn genetic variability should be taken into account, since this might limit direct generalization of the results obtained to other strains or to other species.

Affinity chromatography (paper III)

Affinity chromatography is a well established method for purification of proteins [134]. The snake venom neurotoxin Taipoxin inhibits synaptic vesicle recycling [116, 117] making it a valuable tool for studying this calcium-dependent process. Solubilized brain membranes, which presumably contain Taipoxin binding proteins, were at ambient pressure run over an agarose column coupled with immobilized toxin followed by sequential CaCl_2 -containing elution buffers with increasing NaCl concentrations. The final eluate contained EDTA in addition to 1M NaCl to remove proteins bound tightly in a calcium dependent manner. Hence, the neuronal pentraxins were 1000-fold concentrated in this final eluate [118, 122] and therefore well detectable on western blots. In paper III affinity chromatography of solubilized brain membranes from wild-type, single-, double- and triple mutants was performed on taipoxin columns in order to verify the lack of these proteins in the KO mice.

Labeling of retinogeniculate afferents (paper III)

Studying the development of eye-specific retinal ganglion cells (RGC) projections to the dorsal lateral geniculate nucleus (dLGN) of the thalamus is an established model for exploring activity-dependent axonal refinement in the mammalian CNS [111, 112]. The dLGN is a sensory nucleus in the thalamus which relays the visual information to the visual cortex. In the dLGN input from the two eyes are initially intermixed, but during maturation the axons specifically target their appropriate layer [111].

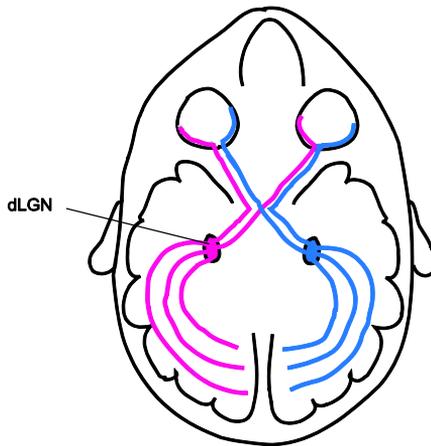


Figure 5. Schematic picture of the visual pathway demonstrating that the dLGN receives visual information from the contralateral visual field, transmitted from RGCs in both the contralateral and the ipsilateral retinas. The dLGN is highly topographic in organization with strict segregation of ipsi- and contralateral axon terminals. From the dLGN visual signals are transmitted via the optic radiation to the visual cortex where the topographic organization is maintained. Adapted from www.lea-test.fi.

Neuronal pentraxins were hypothesized to be involved in synaptic remodeling, which is the rationale behind performing labeling of retinogeniculate afferents for a more elaborate analysis of the KO mice. All the neuronal pentraxins are present in the RGC of the eye, and the NP2

immunoreactivity in the dLGN represents NP2 positive RGC axons projecting from the retina.

The eyes of the animals were bilaterally injected with fluorescent dye, green and red respectively, conjugated to cholera toxin- β unit which enables internalization and transport of the dye through the axons. Hence, the segregation of axons in the dLGN could be visualized and graded using immunofluorescence imaging. This accessibility is one of the major advantages of this method for studying axonal development and refinement.

RESULTS AND COMMENTS

A brief summary of the results of the thesis is presented in this section; please refer to the original articles for specific details.

Pharmacological studies

Papers I & II

In control animals, NGFI-A, NGFI-B, and GR mRNA were, as previously described [14, 72, 135], expressed in all of the hippocampal subregions, the DG, and the cerebral cortex at the level of the dorsal hippocampus. For MR there was, as expected, an almost exclusive expression in the hippocampus and the DG [61, 136].

Long-term (21 days) treatment with the tricyclic antidepressant amitriptyline and the reversible MAO-A inhibitor moclobemide, drugs exerting a wide-spread effect on several neurotransmitter systems, in particular the serotonin and noradrenaline systems, resulted in a similar pattern of NGFI-A gene expression in the hippocampus and the cerebral cortex. A profound induction of gene expression in all of the regions studied was observed. By using the SSRI fluoxetine, NGFI-A mRNA expression was increased in the CA₂, CA₃ and the cerebral cortex. In contrast, the effect with the full 5-HT_{1A} agonist 8-OH-DPAT was more modest with an increase in the CA₂ and the cerebral cortex. With the partial 5-HT_{1A} agonist, buspirone, there was no observed alteration in NGFI-A gene expression.

The only pharmacological effect on NGFI-B mRNA expression detected was a reduction in the CA₃ and RSG after moclobemide treatment.

Moclobemide and amitriptyline had the strongest effect on MR gene expression of the drugs investigated with an increased expression in all of the hippocampal subregions for amitriptyline and all but CA₃ for moclobemide. Treatment with 8-OH-DPAT and buspirone lead to a selective increased expression in the CA₁ and CA₂.

For GR, there was a selective increase in the CA₁ and CA₂ with amitriptyline, and in the CA₁ with moclobemide. 8-OH-DPAT induced increased in GR gene expression in the CA₁ and the DG. In contrast, treatment with fluoxetine and buspirone lead to a decreased expression in the CA₁, DG and the DG respectively.

Paper IV

In agreement with previous reports [137], NP₂ mRNA was robustly expressed in all hippocampal subregions, the dentate gyrus (DG) and the medial habenula (MHb). In addition, there was specific labeling, indicating NP₂ mRNA expression, in the cortex, the thalamus and the hypothalamus.

In rats who received long-term (21 days) antidepressant treatment, NP₂ mRNA expression was upregulated in almost all regions studied. The most pronounced increase occurred in the MHb (mean 24.5%, range 12-33%). In the DG the upregulation was less pronounced (mean 13.2%, range 10-16%). The initial (3d) message increase in the control animals, most likely due to an acute induction of gene expression due to the surgical procedure, is more profound in the MHb than in the hippocampus. This observation raises the question whether MHb could be more responsive to various types of stress than other brain regions. In contrast to drug-treated rats, animals that were kept in deprived (PE), normal (SE) or enriched environments (EE), showed no difference in NP₂ mRNA expression in any of the regions studied.

	<i>CX</i>	<i>RSG</i>	<i>CA1</i>	<i>CA2</i>	<i>CA3</i>	<i>DG</i>	<i>MHb</i>
<i>AMI</i>							
MR			↑	↑	↑	↑	n.a.
GR			↑	↑			n.a.
NGFI-A	↑	↑	↑	↑	↑	↑	n.a.
NGFI-B							n.a.
NP2	n.a.	n.a.	↑	↑	↑	↑	↑
<i>MOC</i>							
MR			↑	↑		↑	n.a.
GR			↑				n.a.
NGFI-A	↑	↑	↑	↑	↑	↑	n.a.
NGFI-B		↓			↓		n.a.
NP2	n.a.	n.a.	↑	↑	↑	↑	↑

	<i>CX</i>	<i>RSG</i>	<i>CA1</i>	<i>CA2</i>	<i>CA3</i>	<i>DG</i>	<i>MHb</i>
<u>MAP</u>							
MR	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
GR	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NGFI-A	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NGFI-B	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NP2	n.a.	n.a.	↑	↑		↑	
<u>FLUOX</u>							
MR							n.a.
GR					↓	↓	n.a.
NGFI-A	↑			↑	↑		n.a.
NGFI-B							n.a.
NP2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<u>CIT</u>							
MR	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
GR	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NGFI-A	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NGFI-B	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NP2	n.a.	n.a.	↑	↑	↑	↑	↑
<u>8-OH</u>							
MR			↑	↑			n.a.
GR			↑			↑	n.a.
NGFI-A	↑			↑			n.a.
NGFI-B							n.a.
NP2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<u>BUSP</u>							
MR			↑	↑			n.a.
GR						↓	n.a.
NGFI-A							n.a.
NGFI-B							n.a.
NP2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Table 2. Effect of drugs utilized in paper I, II and IV on target genes after 21 days treatment. Individual drugs abbreviated and underlined. The changes of NP2 expression is compared to expression after 3 days treatment as opposed to a comparison with saline-treated controls in papers I and II.

Developmental studies

Paper III

Previously, mice lacking NP1 were available [18], and for the present study, mice lacking NP2 and NPR, as well as combinations of NP1/2, NP1/R,

NP2/R and NP1/2/R knock-outs were generated. All mice were viable and fertile, with no obvious phenotype, and all crosses generated the expected mendelian numbers of each genotype. Loss of NPR caused a 50% reduction in purified NP1 and NP2 on taipoxin chromatography suggesting that NPR play an important role in the trafficking or membrane presentation of NP1 and NP2.

In wild type (WT) mice there was no detectable NP2 mRNA expression in the dLGN, but an abundance of message in the RGC layer of the retina. In the dLGN there was some NP2 immunoreactivity which most likely represents RGC axons projecting from the retina. In the KO mice, neither message nor immunoreactivity was detectable, as expected. All the NPs were present at postnatal day (P) 5 in WT retina as demonstrated by immunohistochemistry.

Delayed eye-specific refinement

Visualization of RGC-dLGN projections was used to explore activity-dependent axonal refinement [111]. Early in development, at P4, inputs from the two eyes were intermingled at the dLGN, both in WT and in mutants. It is known that in normal development the axons are very simple and sparse at this age [111], but during maturation this simplicity is followed by a loss of inappropriate synapses accompanied by a massive growth and sprouting into the correct column, a process that constitutes axonal refinement.

At P10 axons from the two eyes were segregated in WT mice, occupying complementary, non-overlapping regions in the dLGN. In contrast, the dLGN of P10 NP1/2 null mice lacked eye-specific segregation, resulting in an extensive binocular overlap. At P30, about 2 weeks after eye-opening, some normalization with a higher segregation of the axons in the NP1/2 null mice was observed, although axons from the ipsilateral eye remained distributed across an abnormally large extent of the dLGN.

These results indicate that there is a defect in the NP1/2 null mice RGC axons, hampering their ability to refine in the dLGN. The ability of the axons to refine was compared in the various combinations of NP mutants. Abnormalities were noted in all of the single null mice, although less pronounced than in the NP1/2 double mutants. Interestingly the defects in

NP1/NPR double null mice were also mild relative to the NP1/2 double null mice, whereas the alterations in the NP1/2/R triple mutants were similar to those observed in the NP1/2 double null mice. Hence, the combination of NP1 and NP2 appears most relevant for retino-dLGN remodeling.

Delayed synaptic function but normal synaptic contacts

Detailed descriptions of the methods used for acquiring the following results are available in paper III.

In order to study whether glutamatergic synaptic transmission might be altered in NP1/2 KO mice, RGC neurons were cultured in the presence or absence of WT astrocyte feeding layers and synaptic activity was recorded with patch clamping. In NP1/2 mutants, RGCs had few synaptic currents with small amplitudes, compared to the high rates of currents of various amplitudes in WT RGCs. However, leaving the NP1/2 KO RGCs in co-culture with WT glia for 4 weeks, the recordings more or less normalized, possibly reflecting a maturation event. The number of pre- and postsynaptic contacts was measured in these cultured cells, and WT and NP1/2 KO mice had similar numbers of synaptic structures. These results indicate that the development of glutamatergic synaptic function, but not formation of synaptic contacts, is significantly delayed in the absence of NP1 and NP2. Despite this delay in the maturation of synaptic function, there were no defects in basal synaptic strength, LTP or LTD in the hippocampus of P20-P40, hence adult NP1/2 KO mice. This suggests that the defects in axonal development are of a different time-frame or a separate mechanism than that of LTP and LTD.

DISCUSSION

Antidepressants induce neuronal plasticity

Long-term treatment of rats using antidepressant drugs with various pharmacological profiles influenced hippocampal and cortical expression of NGFI-A, NGFI-B, GR and MR mRNA to different extents (paper I & II). Such varied pattern was not observed for NP2 mRNA, which was consistently upregulated in the hippocampus and the MHb after long-term antidepressant treatment. In contrast, environmental stimulation or deprivation did not influence NP2 mRNA levels in this experimental model (paper IV). These results, discussed in more detail below, are interesting and indeed encourage further investigations.

Is drug selection important?

A direct comparison between papers I, II and IV is complicated by the fact that the battery of drugs used in the experiments were slightly different. Also, the study design for investigating the response on NP2 message was not completely identical to the first two studies. However, all the drugs used are antidepressants, or have a direct influence on monoamine transmission. Some have a propensity for influencing serotonin, some influence noradrenaline, whereas others increase monoamine transmission with less specificity.

In the clinical setting, SSRI is the most common first-hand choice when initiating treatment for depression. When no or in-sufficient effect is achieved, the strategy includes stimulation of noradrenaline transmission, and in some cases also enhancement of dopaminergic activity. Hence, the usage of the early, more broad-spectrum TCAs is still a valid choice, especially when the depression is severe and if the side effects can be tolerated by the patient. The results obtained in this thesis are generally in line with such clinical experience. The drugs with the least monoaminergic specificity (TCA, MAO-A-inhibitor) had an anatomically broader effect on most target genes than more specific drugs, indicating that long-term adaption could be influenced by the spectrum of neuronal input.

The 5-HT_{1A} receptor, one of the main mediators of the action of serotonin, is present at high densities in brain areas associated with memory function [138]. It functions as a postsynaptic receptor on target cells and as an inhibitory autoreceptor on serotonergic neurons of the raphe nuclei [139]. Hence, agonists for this receptor can, depending on dosage, both inhibit or enhance 5-HT_{1A} function and thus modulate various neurotransmitter systems involved in cognition [130, 138]. 5-HT_{1A}-agonists have, in clinical praxis, been used to augment the effect of 5-HT reuptake inhibitors. In paper II, long-term treatment with buspirone and 8-OH-DPAT, partial and full 5-HT_{1A} receptor agonists respectively, had a very modest effect on the target genes. This provides support for the view that stimulation of several monoamine systems is necessary for a more robust induction of neuronal plasticity.

The induction of GR and MR after antidepressant treatment

Long-term antidepressant treatment influenced the expression of MR and GR differently. MR were, as previously demonstrated [41, 61, 63], more robustly upregulated, especially by non-selective drugs. GR, on the other hand, were upregulated to a certain degree by some drugs tested, but after treatment with an SSRI and the partial 5-HT_{1A} agonist, their expression was decreased in CA₃ and DG respectively. These varying outcomes support previous data indicating that the GR and MR play different roles in stress modulation [140], which may be dependent on their widely differing affinities to corticosteroids. Pharmacological inhibition of MR suppresses LTP whereas inhibition of GR has an opposite effect, particularly under stressful conditions, which is why these receptors are suggested to maintain opposite roles in regulating synaptic plasticity [140].

What about NGFI-B?

The effect of long-term antidepressant treatment on hippocampal and cortical NGFI-B mRNA expression was merely a reduction after moclobemide treatment in the CA₃ and RSG. A number of possible factors may explain these findings. First, the selection of 21 days treatment length might not capture putative changes in NGFI-B expression; the presence of alterations at other time-points cannot be excluded. Furthermore, moclobemide, which inhibits degradation of serotonin, noradrenaline and to some extent also dopamine [131] is the least monoamine-specific drug

used in paper II. The fact that this drug selectively influenced NGFI-B expression supports previous suggestions that NGFI-B is important not only for serotonin [83] but also for dopamine [82, 83] transmission. Also, the varied results on NGFI-A and NGFI-B after plasticity-inducible stimulations in paper I and II would support recent findings that suppression of calcineurin, a negative regulator of synaptic plasticity, induces NGFI-A, but suppresses NGFI-B expression [141].

Is the CA₁ particularly responsive to antidepressant treatment?

Expression of the transcription factors studied in papers I and II, as well as NP2 in paper IV, were most consistently upregulated in the hippocampal subregion CA₁ after long-term antidepressant treatment. This may indicate a higher degree of readiness for plasticity in this particular region. Most efferent projections from the hippocampus originate in the CA₁, and LTP and LTD are predominantly studied in excitatory synapses on CA₁ pyramidal cells [6]. Moreover, this specific region has been shown to be important for spatial memory in rats [142] and has been suggested essential for episodic memory and higher cognitive functions in humans [143]. Previously, 14 day treatment with the tricyclic antidepressant imipramine has been demonstrated to enhance structural synaptic plasticity due to a significant increase in number of CA₁ excitatory synapses [144]. In addition, similar treatment had a positive effect on CA₁ LTP in a stress paradigm imitating depression [145]. The increased message of NGFI-A, MR, GR and NP2 in the CA₁ after long-term treatment with antidepressant drugs indicates that these factors are important players contributing to such plastic events.

NP2 mRNA upregulation in the medial habenula

The most pronounced increase of NP2 mRNA expression after long-term antidepressant treatment occurred in the medial habenula (MHb), which is one of the most cholinergic regions in the brain. The specific function of this region has not been extensively investigated, although the habenula has been described as a relay station for regulating monoaminergic transmission [59]. Non-selective lesions of the entire habenula (MHb + LHb) leads to impaired spatial memory in the Morris water maze [58] supporting its involvement in cognitive functions. The habenula is also identified as a structure with importance for the development of psychosis

[58, 146], and deep brain stimulation of the LHB has been suggested as a potential treatment for patients with therapy-resistant major depression [147]. Little is known about the intrinsic habenular circuitry but there are some evidence for a medial-to-lateral connection [57].

The MHB contains a high density of nicotinic receptors, and it is extremely sensitive to nicotine exposure. In fact, its nerve fibers degenerate selectively after high prolonged nicotine administration [148, 149], and axons derived from the LHB degenerate after continuous administration of drugs that stimulate dopamine signaling, such as cocaine and methamphetamine. Hence, this region is suggested to constitute the “weak link” that mediates progressive effects of drug abuse [57]. This raises the interesting question whether this specific drug sensitivity could also apply to increased sensitivity to other agents or influences.

Even though NP2 mRNA expression appears to be slightly stronger in the MHB than in the hippocampal subregions after long-term treatment, there is no significant inter-regional difference in the expression levels, nor are there any differences related to the monoaminergic profile of the drugs. However, the lack of significance may be due to the statistical power of this study. The NP2 mRNA induction occurs with long-term treatment, which supports the hypothesis that NP2 is involved in plasticity and identifies the MHB as a potential region of interest for subsequent studies on the function of NP2.

Antidepressants vs. environmental enrichment

As demonstrated in paper IV, NP2 mRNA was induced by antidepressant treatment but not affected by exposure to different environments, the latter a paradigm previously shown to induce the expression of genes involved in neuronal plasticity [73, 100, 150]. Contrary to this, NGFI-A expression is induced not only with long-term antidepressant treatment, but also by environmental stimulation [73, 74, 102]. The discrepant results of NP2 obtained here is in agreement with the suggestion that antidepressant drugs and environmental enrichment (EE) produce antidepressant and anxiolytic effects via separate pathways [151]. This hypothesis is based on findings that antidepressants appear to use a neurogenesis dependent pathway to achieve effect as opposed to EE which uses a neurogenesis independent pathway – either alone or in combination

with neurogenesis dependence. The neurogenesis independent pathway would include upregulation of growth factors and morphological changes such as more extensive dendritic branches and synaptogenesis [35, 151]. Various classes of antidepressants, as well as lithium and electroconvulsive seizures (ECS) [84], and the atypical antipsychotic drug olanzapine [152], induce neurogenesis along with a more rapid maturation of newly formed cells [153]. Classical antipsychotic drugs, such as haloperidol, or the opioid morphine, has no similar effect [84], which suggests that pharmacologically induced neurogenesis is specific for antidepressants [154], or that stimulation of the 5-HT_{1A} receptor is required [152].

The cAMP response element binding protein (CREB), brain-derived neurotrophic factor (BDNF), and vascular endothelial growth factor (VEGF) are factors that have been identified as links within the pathways of antidepressant-induced neurogenesis [18, 89, 91, 155, 156]. The possible role of NGFI-A and NP2 in this process has neither previously, nor in this thesis, been evaluated. However, such an involvement cannot be ruled out. Induction of LTP at perforant path inputs to the DG promotes proliferation and survival of progenitor cells in the subgranular zone (SGZ) [157]. The fact that NGFI-A is intimately connected with the process of LTP [10] suggests possible relationships between NGFI-A and neurogenesis. Although there are some inconsistencies between previous studies on antidepressant effect on LTP, where some show induction and others depression [158], chronic as opposed to short-term antidepressant treatment more often leads to increased LTP [159, 160].

VEGF has been suggested to play a role in antidepressant-induced neurogenesis and recently also been implicated in neurogenesis induced by environmental enrichment (EE) [94]. So far, it has not been determined whether the antidepressant/anxiolytic and behavioral effects gained by these two paradigms follow the same pathway since recent studies reveal somewhat conflicting data [94, 151, 153]. VEGF stimulates proliferation and BDNF maturation [18] of newly formed neurons, and some studies have failed to demonstrate proliferation after EE, as opposed to in antidepressant treated animals, where this occurs [99, 153]. This may support the presence of an alternate pathway for EE to generate antidepressive effects [35, 151], or, alternatively, raises the question whether a common pathway simply is yet to be described. The results presented in

this thesis support the possibility of different pathways, with an increase in NP2 mRNA expression after long-term antidepressant treatment but not after EE.

The function of the neuronal pentraxins

The results in paper III demonstrate, for the first time, that the neuronal pentraxins (NPs) play a role in synaptic refinement. This is further corroborated by more recent studies showing that complexes of NP1 and NP2 co-function in clustering AMPA receptors during synaptic plasticity [124]. NP1 and NPR participate in GluR4 synaptic recruitment *in vitro* and triple NP KO mice manifest a reduced number of hippocampal GluR4 synapses *in vivo* [161]. As demonstrated in paper III, LTP was intact in NP KO mice [161].

All of the NPs are proposed to capture glutamatergic AMPA receptors during synapse formation and the transmembrane domain of NPR appears to inhibit endocytosis. However, in synaptic depression there is an mGluR1/5-induced cleavage of NPR which allows it, together with linked NP1 and/or NP2 and an associated pool of AMPA receptors, to enter endosomes and thereby increase endocytosis [162]. These results lead the authors to propose that NPR, early in development, anchors NP1 and NP2 at sites of emerging synapses, whereas NPR forms a signaling complex required for mGluR1/5-dependent LTD in mature synapses [162]. Hence, the results from these subsequent studies emphasize an importance for all three NPs in synaptic development and remodeling.

The importance of the NPs in neuronal plasticity makes them candidates of interest for involvement in pathological processes, and the NPs have recently been in focus for investigations on certain neurodegenerative diseases.

Neuronal pentraxins and neurodegenerative diseases

NP1 has been suggested to be part of the gene expression program that leads to apoptotic cell death during a reduction in neuronal activity in cerebellar granular cells [163, 164]. Interestingly, NP1 appears to be involved in the pathology of Alzheimer's disease (AD) by playing a key role

in the synaptic loss, neurite damage, and apoptotic neurotoxicity evoked by A β oligomers, and there is an increased presence of NP1 in dystrophic neurites in brains of patients with AD [165]. Further support for an involvement of NP1 in apoptosis is the circumstance that NP1 mRNA is induced in hypoxic-ischemic brain injury and that anti-sense oligonucleotides against NP1 mRNA prevent hypoxia- and AMPA-induced neuronal cell death [166]. NPR has been proposed as a clinical marker for AD since it is significantly elevated in cerebrospinal fluid of Alzheimer but not Parkinson patients, possibly regulating above mentioned NP1 actions in AD [167].

NP2 co-localizes in hypothalamic PVN neurons with orexin [168], a protein known to stimulate glutamatergic interneurons [169] and is of major importance for regulating wakefulness [170]. Orexin neurons degenerate in Huntington's disease [171] and orexin/NP2 cells degenerate in narcolepsy [172]. A possible involvement of NP2 in sleep regulation has been discussed [172], as well as a synergistic effect with orexin on target glutamatergic neurons [168, 169], although this remains to be demonstrated. Interestingly, the efferent tracts of the NP2 dense MHB have been implicated in regulation of normal sleep patterns and duration [57], functions that are often disturbed in depressive disorders and in neurodegenerative diseases [173].

In postmortem brains from patients with Parkinson's disease (PD) there is a co-localization of NP2 with α -synuclein in Lewy bodies and Lewy neurites as well as a strong up-regulation of NP2 mRNA [174]. These findings lead to the suggestion that NP2 could be involved in uptake of synaptic material in this disease [174]. Furthermore, the hypothalamic decrease of orexin in PD, which correlates with disease progression [175], has been regarded as an explanation for the sleep disorders often observed in these patients [175, 176] and possibly in patients suffering from other synucleinopathies [177]. Whether NP2 was decreased as well was not addressed by these studies, although that specific question would be of great interest. Together, these data point to the possible involvement of all the neuronal pentraxins in neurodegenerative diseases, an attractive hypothesis which warrants further investigations.

CONCLUSIONS

From the results presented in the papers of this thesis, the following conclusions can be drawn:

- I. That long-term treatment with the antidepressant amitriptyline induces expression of NGFI-A and MR in all regions studied; that GR is selectively induced in the CA₁ and CA₂ and that effect on NGFI-B mRNA expression is lacking. NGFI-A may play a role in antidepressant-mediated neuronal plasticity.
- II. That long-term treatment with antidepressant drugs with different monoaminergic profiles has a selective effect on NGFI-A, NGFI-B, GR, and MR mRNA expression. However, moclobemide, the drug with the least monoaminergic selectivity, has the overall most profound effect on most transcription factors. NGFI-A is the transcription factor induced the most and the CA₁ of the hippocampus is the region most responsive to antidepressant treatment.
- III. That the neuronal pentraxins are important for activity-dependent synaptogenesis and synaptic refinement as demonstrated by *in vitro* and *in vivo* experiments on the visual pathway of knock-out mice.
- IV. That long-term antidepressant treatment, as opposed to short-term treatment or environmental enrichment/deprivation, has an inducible effect on NP₂ mRNA expression in the hippocampus and the medial habenula.

SVENSK SAMMANFATTNING

Den förmåga hjärnan och dess nätverk av celler har att anpassa sin funktion till den situation den befinner sig i kallas neuronal plasticitet, en viktig process som pågår kontinuerligt genom livet. Nervcellerna utvecklas tidigt enligt förprogrammerade mönster, men utvecklingen är samtidigt beroende av rätt typ av stimulering för att kopplingarna skall bli korrekta. Man känner delvis till de molekylära mekanismer som styr nervcellernas utveckling, men samtidigt är det mycket kunskap som saknas. Inte bara under den tidiga utvecklingen utan även under resten av livet sker en kontinuerlig omformning av nervcellerna. Exempelvis leder en intellektuellt stimulerande miljö till att hjärnområden som styr vårt minne får en större volym, främst genom att nervcellernas kontakter med varandra blir fler och mer effektiva, men även genom att det sker en kontinuerlig nybildning av celler. Under stress påverkas denna process, samt förgreningen av nervcellernas utskott, negativt. Detta kan leda till att personer som utsätts för långvarig stress, eller lider av depressionssjukdom vilket också påverkar stress-systemen i kroppen, drabbas av en viss förtvining av hippocampus, den hjärnregion som styr vårt minne.

För att öka förståelsen för de molekylära mekanismer som ligger bakom neuronal plasticitet behandlades råttor med olika typer av antidepressiva läkemedel. Man har tidigare visat att sådan behandling stimulerar vissa gener samt nybildning av celler i hippocampus. Vi valde olika typer av antidepressiva läkemedel med en varierande specificitet mot serotonin, noradrenalin och dopamin, signalsubstanser som man anser styra känslolivet och vars balans ofta är rubbad i en depression. De läkemedel mot depression som används kliniskt ökar tillgången till dessa signalsubstanser i hjärnan relativt omgående. Dock tar det minst 2 veckor innan man kan observera förbättring av de depressiva symtomen. Därför är alternativ förklaring till symtomlindring än enbart ökade nivåer av signalsubstanser trolig. Mycket talar för att det sker någon form av plastisk omvandling i cellerna, och att det i själva verket är denna anpassning som leder till ett kliniskt tillfrisknande.

Efter 3 veckors behandling, vilket anses vara långtidsbehandling för råttor, analyserades genuttryck i hippocampus och cortex (hjärnbarken) för 4

olika gener; GR, MR, NGFI-A och NGFI-B. GR och MR, receptorer för hormonet kortisol, är normalt rikligt förekommande i hippocampus. Man tror att kortisol (stress) systemet styrs av hippocampus och balansen i detta system är rubbad vid depressionssjukdom. NGFI-A och NGFI-B är sammankopplade med regleringen av GR och MR och anses vara s.k. "immediate-early genes" vilka är inblandade vid plasticitet. Tre veckors antidepressiv behandling ledde till varierande grad av uppreglering av samtliga dessa gener (med ett undantag). Generellt sett hade läkemedel med ett bredare tillslag över flera transmittorsystem hade en kraftigare positiv effekt på de studerade generna.

Som uppföljning av dessa resultat undersöktes genuttrycket för ett hjärn-specifikt protein, NP2. Detta protein anses vara inblandat i utvecklingen av synapser, d.v.s. kontaktytan mellan nervcellerna. Långtidsbehandling med de flesta antidepressiva läkemedel använda i studien, oavsett verkningsmekanism, hade en positiv effekt på NP2 i princip i samtliga analyserade regioner. Resultaten tyder på att det finns en gemensam mekanism i dessa läkemedel som inducerar NP2. I samma studie analyserades NP2 hos råttor som vistats i berikande, ordinär eller understimulerande miljö. Man har tidigare konstaterat att en berikande miljö har positiv påverkan på mekanismer som påverkar neuronal plasticitet. Råttor som vistats i olika miljöer uppvisade dock inte någon skillnad i NP2 uttryck. Således uppregleras NP2 av antidepressiv behandling, men inte miljöstimulering. Dessa resultat stödjer tidigare indicier att neuronal plasticitet induceras via olika system beroende på stimuli.

Avslutningsvis studerades funktionen av NP2 och dess närbesläktade proteiner NP1 och NPR, vilka tidigare antagits vara inblandade i nybildning och omformning av synapser. För studien framställdes möss som via genmanipulation saknar dessa proteiner, enskilt eller i olika kombinationer. Denna s.k. "knock-out"-teknik är välrenommerad och dess utveckling belönades med Nobelpriset i fysiologi eller medicin 2007.

Mössen som saknar NPs utvecklades utan några synbara defekter. För att ytterligare analysera möjliga effekter av brist på dessa proteiner studerades utvecklingen av synbanan. Man vet att nervcellernas axon arrangeras i ett mycket detaljrikt och förutbestämt mönster i synbanans

omkopplingsstation dLGN under utvecklingen. Det faktum att denna nervbana är relativt åtkomlig gör den anatomiskt möjlig att studera i detalj till skillnad från flertalet andra bansystem i CNS. Analyserna av synbaneutvecklingen hos "knock-out" mössen visade att möss som saknar kombinationer av NPs, ffa NP1/NP2 har en fördröjd och mindre effektiv förfining av inblandade nervceller. Dessa resultat stödjer hypotesen att NPs spelar en viktig roll i synapsutvecklingen.

Sammanfattningsvis visar denna avhandling att långtidsbehandling med antidepressiva läkemedel inducerar uttryck av specifika gener i hippocampus, vilket indikerar initiering av plastiska förändringar i dess nervceller. Dessutom hade läkemedel med relativt bredare tillslag en kraftigare effekt, vilket tyder på att parallell aktivering av flera transmittorsystem är positivt för utveckling av plasticitet. Resultaten visar också att synapsproteinet NP2 induceras av antidepressiv behandling oavsett transmittorspecificitet, vilket pekar på någon typ av gemensam mekanism hos de olika läkemedlen vid dess påverkan på NP2. Dessutom har en berikande miljö, vilket normalt påverkar plasticitet positivt, ingen effekt på NP2-uttryck, vilket tyder på att dess induktion sker via en alternativ väg. Slutligen har specifik elimination – s.k. "knock-out" - av NPs, dels individuellt men även i kombinationer, visat att dessa proteiner utvecklingen av synapser.

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