Role of IL-6 trans-signalling for sleep-wake behaviour of rats / Generation of brain-specific sgp130-Fc transgenic mice: Central blockade of IL-6 trans-signalling

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Der Dekan

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1 Introduction

1.1 The cytokine IL-6 and its multiple functions

The pleiotropic cytokine Interleukin 6 (IL-6), initially identified as a factor inducing the production of immunoglobulins in B lymphocytes [184], is produced by numerous immune and non-immune cells. They include T and B cells, macrophages, fibroblasts, endothelial cells, adipocytes and neuronal cells [274]. Expression of IL-6 occurs under physiological as well as under pathophysiological conditions, whereas its production is stronger upregulated during the latter [117, 325].

IL-6 is considered an important player in host defense as a pyrogen (causing fever) [142, 202, 236] and in regulating the acute phase response - amongst others - by inducing the release of C-reactive protein and serum amyloid A in hepatocytes [78]. Moreover, IL-6 has a pivotal role in growth and differentiation of haematopoietic precursor cells [106] and T lymphocytes [199]. For instance, the cytokine IL-6 induces together with TGFβ the differentiation of naive T cells into Th17 cells [17, 296]. Immune functions are also controlled through the autonomic nervous system by IL-6, which is provoked by neuronal activity [116]. Beyond immunological processes, IL-6 mediates many brain functions in the development and maintenance of the central nervous system (CNS) as well as in brain damage. In the CNS, IL-6 and IL-6 receptors are expressed within various regions by astrocytes, microglia and neurons [75, 76, 160, 162, 237, 243, 247, 294, 295, 321]. Together with leptin and insulin-like growth factor (IGF) IL-6 has strong implication in the regulation of metabolic balance of sugar- and fat metabolism [163]. IL-6 has further been shown to control neurotransmitter expression [49, 54], enhance differentiation and survival of primary neurons and neuronal cell lines, and to exhibit neurotrophic activities by inducing together with its soluble IL-6 receptor several neurotrophins in astrocytes [110, 160, 161, 237]. The neuroprotective potential of IL-6 has also been shown *in vivo* during cerebral ischemia in rats [147]. It is even capable of inducing the differentiation of progenitor cells into astrocytes [110, 187, 276]. Moreover, the modulation of endocrine processes within the CNS such as the activation of the hypothalamic-pituitary-adrenal (HPA) axis is a crucial effect of IL-6 action [89, 248, 262, 309]. Likewise, IL-6 is involved in memory consolidation/learning, synaptic long-term potentiation and promotes plasticity [5, 7, 16, 166, 279]. Noteworthy, IL-6 is implicated in behavioural changes after infection, called sickness behaviour, including fever, anorexia (reduced food intake) and altered sleep-wake behaviour [20, 50]. During the strong brain-immune system interactions in sleep, cytokines exert a central role [133, 135, 156, 266]. The somnogenic

properties of IL-6 are described in section 1.7. Importantly, cytokines such as IL-6 mediate the bidirectional communication between the immune system and the nervous system, that maintains homeostasis throughout the body (protective) as well as the association with pathology (degenerative).

Under various disease conditions, IL-6 levels become dysregulated or elevated and IL-6 is being produced at local tissue sites after acute infection or injury. But when chronically increased, IL-6 contributes to an overdriven immune response leading to tissue damage and thus to a prolonged or exaggerated course of illness, inflammation or autoimmunity. Altered IL-6/sIL-6R levels have been found in several human diseases such as multiple myeloma, Crohn's disease, rheumatoid arthritis, sepsis, colon cancer, cardiovascular diseases, AIDS, osteoporosis, diabetes mellitus, asthma, stroke and other neuroinflammatory/-degenerative diseases (see 1.5) [114, 117, 325].

1.2 Signal transduction of IL-6

IL-6 belongs to the gp130-signalling family of long chain four α-helix bundle cytokines containing four α-helices (termed A, B, C and D) arranged in an up-up-down-down topology [28]. These cytokines use at least one glycoprotein 130 (gp130, IL-6 receptor β-chain, CD 130) as a common signal-transducing subunit. Apart from IL-6, members of this family include interleukin 11 (IL-11), leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin 1 (CT-1), cardiotrophin-like cytokine (CLC), viral IL-6, IL-27, neuropoietin (NP) and IL-31. In addition to gp130, each cytokine uses a specific set of receptors. For example, LIF and OSM induce the formation of gp130/LIF receptor heterodimer, whereas OSM can also signal via a receptor complex of gp130 and OSM receptor. The components of the receptor complex for IL-6 include a homodimer of gp130 and the cytokine-binding glycoprotein IL-6 α-receptor (IL-6R) [93, 97] (Fig.1). The extracellular portions of both gp130 and IL-6R contain an immunglobulin G (Ig)-like domain at the N-terminus followed by one cytokine-binding module (CBM). The CBM comprises two fibronectin-type III (FN III) domains that carry a set of four conserved tyrosine residues and a tryptophan-serine-X-tryptophan-serine (WSXSW) motif. Gp130 possesses three additional FN III domains followed by a transmembrane and an intracellular cytoplasmic domain, which are involved in signal transduction. Adjacent to the CBM, the IL-6R contains a long flexible

stalk region, a transmembrane domain and a short cytoplasmatic domain that is not implicated in signalling events [27].

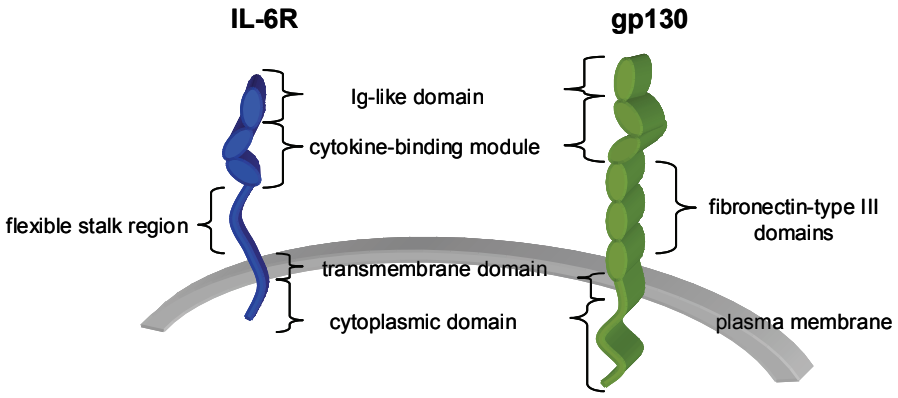

Figure 1 Introducing the IL-6R complex components: schematic assembly of domain structures for IL-6R (blue) and gp130 (green).

To initiate signal transduction, IL-6 first binds to the CBM domain of the IL-6R. Thereupon the complex of IL-6/IL-6R associates with two molecules of gp130 (Ig-like and CBM domains) and intracellular signalling events occur [93, 233, 274]. Interestingly, gp130 proteins have been shown to be present on the plasma membrane as preformed but inactive dimers, which are only activated by ligand addition [281]. The tetrameric complex of (IL-6)-(IL-6R)-$(gp130)^2$ is sufficient [83, 215] to trigger auto-phosphorylation of gp130-associated Janus-tyrosine kinases (JAK), which in turn phosphorylate several tyrosine (Y) residues of the intracellular gp130 portion, which provide binding sites for molecules with a so-called SH2 (src homology 2) domain. Such molecules are the STAT (signal transducer and activator of transcription) proteins, which are tyrosine phosphorylated/activated by JAKs and thereafter dimerise and translocate into the nucleus, where they bind as transcription factors to promoters of target genes [51, 93], e.g. C-reactive protein [313] or c-fos [96]. IL-6 mostly activates STAT3 and to a minor extent STAT1 from the seven known members of STATs [93, 326]. Additionally to the JAK/STAT pathway, the Ras-Raf-MAPK (mitogen activated protein kinase) pathway gets activated upon IL-6 stimulation. Therefore, the SHP-2 tyrosine phosphatases, which also contain SH2 domains, are recruited to the stimulated gp130 and undergo tyrosine phosphorylation by JAKs. Via their interaction with phosphorylated Grb2 (growth factor receptor bound protein) the MAPK pathway is induced [93, 274]. An illustration of the transduction pathways is given in Fig. 2.

The IL-6 transduction is tightly controlled and several mechanisms exist for downregulation of signalling [93, 315]. Upon cytokine stimulation, SOCS (suppressor of cytokine signalling)

proteins are activated which inhibit the kinase activity of JAKs and thus the tyrosine phosphorylation of gp130 and STAT1/3, representing a negative feedback loop [263]. Further, dephosphorylation of receptors and JAKs is mediated by protein-tyrosine phosphatases [90, 141]. The activity of STATs can be inhibited by PIAS (protein inhibitors of activated STATs), e.g. PIAS3 blocks STAT3 DNA binding [45]. Another possibility of IL-6 signal termination involves the degradation of the receptor-ligand complex by internalisation [59] or usage of the ubiquitin-proteasome pathway [278]. The latter is also used to degrade activated STATs.

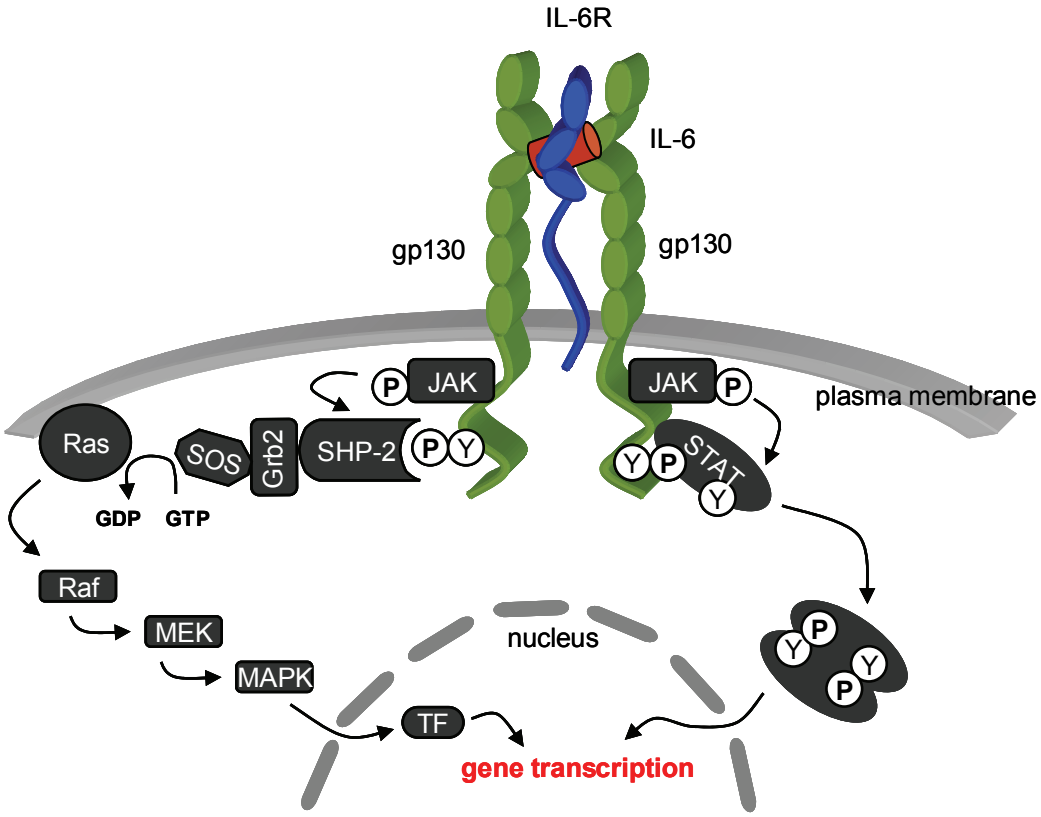

Figure 2 Major signal transduction pathways activated by IL-6: JAK/STAT pathway (right side) and MAPK pathway (left side). Cells expressing gp130 (green) and the membrane-bound IL-6R (blue) are responsive to IL-6 (red cylinder) (classic signalling).

1.3 IL-6 classic signalling versus trans-signalling

Activation of signal transduction by IL-6 (Fig. 2) via the receptor complex of membrane-bound IL-6R and gp130 is called classic signalling. The signal transducer gp130 is ubiquitously expressed on all cells of the body, whereas the membrane-bound IL-6R has only been found on hepatocytes, monocytes/macrophages, some leukocytes as well as in certain

regions of the brain (see 1.1) [162, 197, 274, 321]. Cells lacking the membrane-bound IL-6R normally would not respond to IL-6 itself. But a naturally occurring soluble form of IL-6R (sIL-6R) can bind IL-6 with a similar affinity as the transmembrane IL-6R [232]. Therefore, IL-6 complexed with the sIL-6R can stimulate cells, which only express gp130, thus virtually all cells. Contrary to other soluble receptors (e.g. IL-1R, TNFαR) that are competitive inhibitors of their ligands [58, 307], the sIL-6R acts as an agonist. Hence, IL-6-mediated responses are enhanced by presence of the sIL-6R. This alternative transduction mode (Fig. 3) has been named IL-6 trans-signalling [180, 232]. Cells only responsive to IL-6 in presence of the sIL-6R are e.g. embryonic stem cells, early hematopoietic progenitor cells, endothelial cells, smooth muscle cells and neural cells. For the latter this finding was shown for neural stem cells [110], primary rat sympathetic neurons [160], primary dorsal root ganglia sensory neurons [282], primary rat hippocampal neurons [273], human fetal astrocytes [198], newborn rat astrocytes [161] and rat pheochromocytoma cells (PC12) [159]. Likewise, IL-6 trans-signalling adopts an important role in neuronal differentiation and survival [162].

The sIL-6R has been detected in various body fluids of healthy humans (25–35 ng/ml serum [100]; 0.8–1.8 ng/ml cerebrospinal fluid [74, 158]). It is generated by two different mechanisms, alternative mRNA splicing (10%) [151] and limited proteolytic cleavage of the ectodomain (shedding; 90%) by ADAM (a desintegrin and metalloprotease) 10 and ADAM 17 [165, 180]. Stimuli leading to shedding of the IL-6R include bacterial metalloproteinases and pore-forming toxins [308], C-reactive protein [113], cholesterol depletion [165], phorbol esters [178] and apoptosis [41]. During differential splicing in humans a novel carboxy-terminal protein sequence (GSRRRGSCGL) is created, which does not change the biological properties of the sIL-6R [103].

In addition to the agonistic sIL-6R, a soluble form of gp130 (sgp130) with antagonistic properties exists at relatively high levels (100–400 ng/ml human serum) [169]. Sgp130 is generated by alternative mRNA splicing [55, 252, 277] as well as shedding [179]. The IL-6/sIL-6R complex can bind the soluble as well as the membrane-bound gp130, thus a molar excess of sgp130 is needed for antagonising IL-6/sIL-6R-triggered responses. Sgp130 is therefore seen as a natural competitive inhibitor of IL-6 trans-signalling without affecting signalling via the membrane-bound IL-6R [115, 188, 234]. This is due to the fact that sgp130 cannot bind neither to IL-6 nor to the complex of IL-6/membrane-bound IL-6R. To a much lesser extent sgp130 is also an inhibitor of LIF- and OSM-signalling [115, 245].

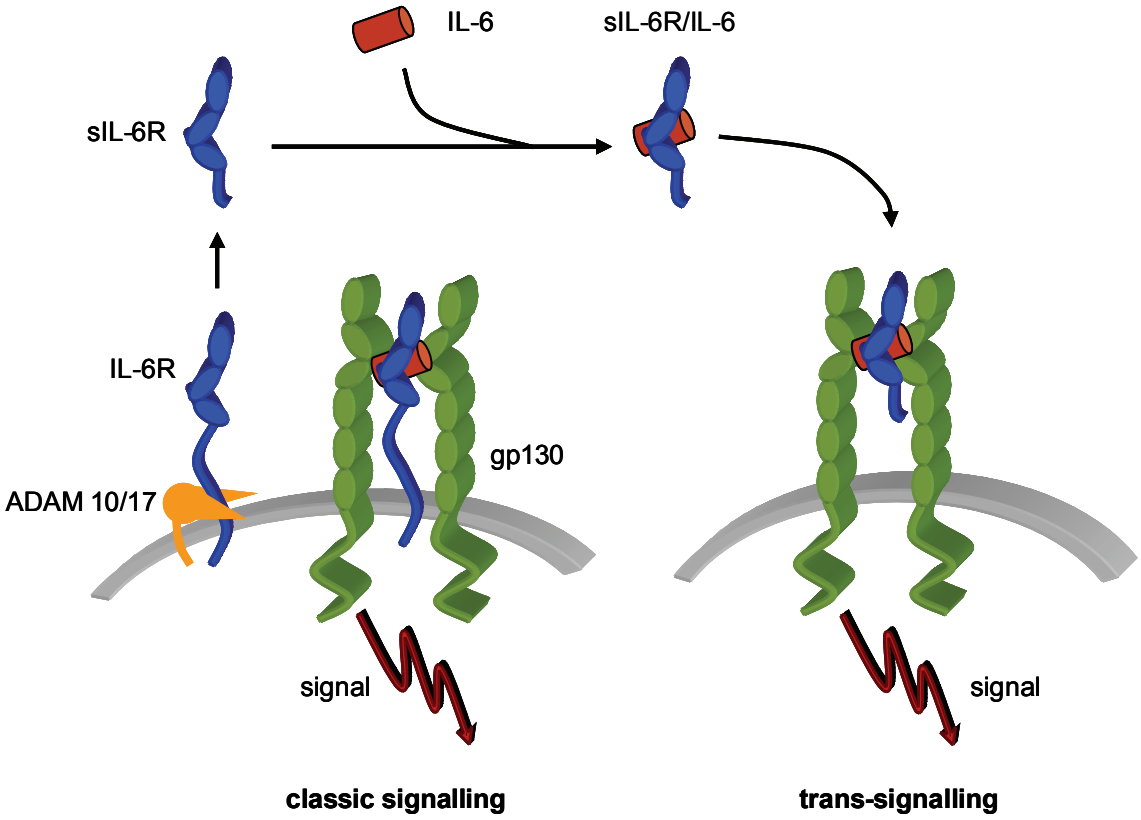

Figure 3 IL-6 classic signalling versus trans-signalling. IL-6 classic signalling is mediated by membrane-bound IL-6R and gp130 (left side). The soluble IL-6R (sIL-6R, blue) is generated via proteolytic cleavage (shedding) of proteases of the ADAM-family (yellow) or via alternative RNA splicing (not illustrated). The complex of sIL-6R/IL-6 can activate cells that only express gp130 but lack the membrane-bound IL-6R (trans-signalling, right side).

1.4 Manipulation of IL-6 signalling: molecular tools

Recombinant designer cytokines have been constructed that mimic components of IL-6 signalling. A molecular agonist of IL-6 trans-signalling is named Hyper-IL-6, which is a fusion protein of human IL-6 and the human soluble IL-6R connected by a flexible peptide linker [70]. Hyper-IL-6 has a 100- to 1,000-fold higher effectivity on gp130-expressing cells than the combination of IL-6 and sIL-6R [70, 214]. As demonstrated by several studies, this designer cytokine is highly effective on neuronal cells such as primary rat astrocytes and neural cells and can stimulate cells that would not survive with IL-6 alone [159-162, 244, 273].

Given the function of the natural soluble gp130 (see 1.3), the trans-signalling pathway can be specifically inhibited by a recombinant soluble fusion protein, which was constructed as a dimer of the extracellular part of human gp130 connected to a Fc-portion of a human IgG-

antibody, designated as soluble gp130-Fc (sgp130-Fc, Fig. 4). The antagonistic activity of the dimer of sgp130-Fc was shown to be 10-fold higher than of the monomeric sgp130 [115]. This engineered inhibitor of IL-6/sIL-6R-mediated responses offers a tool for therapeutic application. The treatment with sgp130-Fc in murine models of rheumatoid arthritis [194], colitis [4], and colitis-associated colon carcinoma [14] suppressed or ameliorated the course of the disease. In sgp130-Fc systemic transgenic mice showing sufficient levels of protein in the serum to inhibit the hepatic acute phase response after Hyper-IL-6 challenge, inflammatory processes were blocked, supporting the ample role of IL-6/sIL-6R during inflammation *in vivo* [220].

Eventually, the molecular tools Hyper-IL-6 and sgp130-Fc aid to specifically distinguish between IL-6 classic and trans-signalling at *in vitro* and *in vivo* applications.

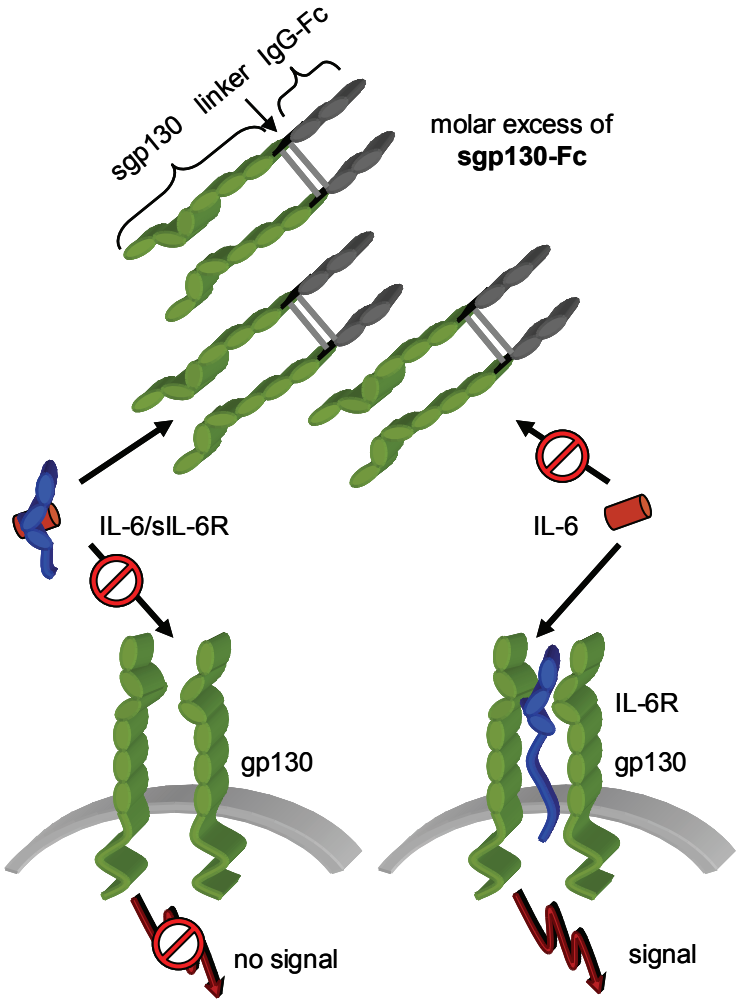

Figure 4 Competitive inhibition of IL-6 trans-signalling by sgp130-Fc. The complex of IL-6/sIL-6R binds with the same affinity to membrane-bound gp130 and to sgp130-Fc, therefore a molar excess of sgp130-Fc inhibits trans-signalling responses (left side). Sgp130-Fc does not block classic-signalling (right side), because IL-6 can associate with the membrane-bound IL-6R, but sgp130-Fc is not able to bind neither to this complex nor to IL-6 alone.

1.5 IL-6 signalling in CNS pathophysiology

Elevated levels of IL-6 and sIL-6R in the CNS are implicated in a wide range of inflammatory and degenerative disorders of the brain. Activated microglia [254] and astrocytes [230, 295], that represent the primary immunocompetent cells of the brain, are the major sources of IL-6 during CNS inflammation or injury. The initial beneficial effects of IL-6 turn then into the reverse, associated with exaggerated or chronic activation of microglia [211] and astrocytes [67], followed by overproduction of different cytokines and neurotoxins. In turn, further activation of glia cells and recruitment of inflammatory immune cells lead to persistence or progression of cell damage and disease (e.g. in multiple sclerosis, Parkinson's and Alzheimer's disease). Particularly, the sIL-6R-mediated IL-6 response and thus trans-signalling is of substantial importance for pathogenesis.

In multiple sclerosis (MS), which is a chronic inflammatory demyelinating CNS disease caused by autoreactive myelin-specific T-helper (Th) cells, including Th17 cells, that enter the CNS from the peripheral immune system through the blood-brain barrier (BBB) [98, 138, 265], human serum levels of sIL-6R and sgp130 are increased and correlate with the disease severity [205]. In contrast, the amount of sgp130 in cerebrospinal fluid of MS patients was found decreased, which might lead to a reduced inhibition of the sIL-6R/IL-6 complex [206]. Mice deficient for IL-6 (IL-6 KO) remained resistant to myelin-oligodendrocyte glycoprotein (MOG) peptide induced experimental autoimmune encephalitis (EAE), which is an animal model for MS. This finding has been explained with the absence of expression of the adhesion molecules VCAM-1 and ICAM-1 on endothelial cells of the BBB, that normally guide the entry of primed T cells into the brain [65]. Likewise, blockade of IL-6 in wildtype mice by an anti-IL-6R monoclonal antibody inhibited the development of EAE and the induction of MOG petide-specific Th17 and Th1 cells [251]. This observation indicated a marked impact of IL-6 for the differentiation of Th17 cells (see 1.1) in EAE. Remarkably, a role of IL-6 trans-signalling has been concluded for the early effector phase of rat EAE. The onset of EAE after suppression of IL-6 trans-signalling by sgp130-Fc was not prevented but less severe, mainly due to the observed reduction of adhesion molecule expression (VCAM-1) at the BBB [146].

Detection of elevated IL-6 in cerebrospinal fluid has been described for many other brain disorders, such as Parkinson's and Alzheimer's disease, stroke, traumatic brain damage, and encephalitis [116, 325]. A major role of glial cells, that produce and respond to cytokines, is discussed for the development of their neurological symptoms.

Transgenic mice strongly overexpressing IL-6 within the CNS by astrocytes developed neurological changes (e.g. tremor, ataxia), neuronal damage, astrogliosis, angiogenesis, induction of acute phase response, diffuse BBB leakage and show impairments in neuroendocrine, electrophysiological and learning functions [36, 37]. In contrast, transgenic mice that overexpressed IL-6 in neurons developed a gliosis similarly but no neuronal damage [69]. IL-6/sIL-6R double transgenic mice with systemic peripheral expression also showed strong neurological symtoms (e.g. tremor, paresis) and a massive reactive gliosis of astrocytes, which have not been observed in single transgenic mice [33].

Moreover, both IL-6 and sIL-6R plasma levels are significantly elevated in psychiatric disorders such as in schizophrenia [152, 181], during post-traumatic stress disorder [154] and major depression [1, 86, 153, 176]. These and further results for chronic fatigue, alcoholism and eating disturbances point to a psychopathological role of IL-6, especially for trans-signalling. Noteworthy, mental disorders are mostly paralleled by sleep disturbances.

Furthermore, the brain controls the allocation of glucose to the brain itself and to the musculature and adipose tissue in the periphery, and reguates the metabolic setpoints, which are consolidated during sleep [213]. Alterations of these setpoints lead to pathological glucose regulation and thus disrupted energy supply causing diseases such as obesity, type 2 diabetes mellitus, hypertension, cardiovascular diseases, anorexia nervosa or major depression [213]. Interestingly, also these mentioned disorders are connected to dysregulated circulating IL-6 and/or sIL-6R levels and altered sleep behaviour [117, 121].

1.6 The sleeping brain

1.6.1 What is sleep?

Sleep is a fundamental physiologic brain process for health and well-being. It is behaviourally defined as a state of immobility and quiescence with reduced responsiveness, which is rapidly reversible. After prevention of sleep, the body is capable of recovering the lost amount in a rebound sleep (homeostatic) [255].

In almost all mammals and birds two forms/stages of sleep can be defined electrophysiologically via specific electroencephalogram (EEG) and electromyogram (EMG) patterns: non-rapid-eye-movement sleep (non-REM sleep; in humans slow wave sleep (SWS)) and rapid-eye-movement sleep (REM sleep). Non-REM sleep is further subdivided into light and deep sleep (delta sleep). Deep non-REM sleep is characterised by high

amplitude/voltage slow delta waves (0.5–4 Hz) and a relaxed muscle tone [72, 189]. Slow waves originate from synchronised periods of neuronal depolarisation (high firing) followed by periods of hyperpolarisation (down phase) within large areas of the cortex. In addition very slow cortical oscillations (<1Hz) are present [269]. The delta wave power (=slow wave activity) reflects the intensity of sleep, e.g. it increases during rebound deep sleep after a sleep loss [23]. REM sleep characteristics are the low voltage EEG (low EEG amplitude, as in wakefulness), high values of theta waves (4–9 Hz) from hippocampal origin, and muscle atonia [72, 189].

Sleep architecture is organised in cycles of alterations between non-REM sleep followed by REM sleep. Physiological noctural sleep in humans consists of 4 to 5 cycles of about 90–100 min each. The length of sleep stages varies in the course of sleep, in which the first half of sleep is dominated by non-REM sleep and the second half by REM sleep. There are differences in sleep architecture between species. In rats, whose main activity period is during the dark, the sleep is polyphasic. They sleep about 70% during the light phase and 30% during the dark phase. The periodicity of the non-REM/REM cycle with 7–13 min is much shorter than in humans [156, 255].

1.6.2 Regulation of sleep

For a better understanding of sleep, it is useful to get a brief insight into the mechanisms underlying its regulation in the CNS. Homeostatic, circadian, neuronal and humoral (endocrine, metabolic, immunologic) processes are linked in a very complex network leading altogether to regulation of sleep-wake behaviour. Even though there are still many open questions, various hypotheses have been established on different levels of organisation of sleep, which are explained here briefly.

The circadian control by the master clock located in the suprachiasmatic nucleus of the hypothalamus receives retinal input, and the time of day information (light/dark) is relayed to several brain regions. It coordinates the timing of the sleep-wake cycle by its circadian clock genes maintaining a 24-h rhythm [170, 256]. Borbely [22, 24] combined the interaction of circadian control with the homeostatic regulation of sleep in the “two process model”. In accordance, the homeostatic need to sleep accumulates during prolonged wakefulness and is discharged during sleep. It includes the process of synaptic homeostasis. The circadian component controls the thresholds at which sleep and wake occur [22, 24].

On an anatomical view, critical brain areas, neuronal circuits and neurotransmitters for sleep-wake regulation have been delineated. Networks of cell groups of the posterior hypothalamus,

the basal forebrain and brainstem are crucial for wake maintenance, that activate the thalamus and cerebral cortex. Their neurons are monoaminergic (contain e.g. noradrenaline, dopamine, serotonin, histamine), cholinergic (e.g. actelycholine) and orexinergic (orexin=hypocretin). Cell groups for arousing are inhibited during sleep by basal forebrain, median and ventrolateral preoptic (VLPO) hypothalamic regions, which contain the inhibitory neurotransmitters gamma-aminobutyric acid (GABA) and galanin. These non-REM sleep active neurons are inhibited during wakefulness by the monoaminergic system. This direct mutual inhibition between the non-REM sleep-promoting neurons (e.g. VLPO) and the monoaminergic arousal groups forms the first switch of the "flip-flop switch model". Only one side is active avoiding mixed states. This switch is stabilised by the wake active orexin neurons of the posterior lateral hypothalamus [52, 167, 240]. A second switch lies between non-REM and REM sleep, where "REM-on" neurons in pons and adjacent midbrain get activated, that also trigger REM muscle atonia [52, 150, 167].

A more recent view on brain organisation of sleep revealed a mechanistic hypothesis. It describes sleep as a fundamental, local and use-dependent property of neuronal assemblies [135-137]. Neuronal assemblies are anatomically defined neuronal networks, e.g. cortical columns, where the high amplitude EEG delta waves of non-REM sleep is a local property. It has been found that cortical columns alterate between a sleep-like and a wake-like state by changing their input-output relationships. When an animal is awake, the wake-like state is present in most of the cortical columns. During sleep, most of the columns are synchronised in a functional sleep-like state. These states are also regulated homeostatically, thus the longer a column stays in a wake-like state, the higher the probability that it will change into the sleep-like state [223]. Importantly, these state oscillations are dependent on the past activity of the column state. For example, EEG delta power was enhanced during subsequent non-REM sleep after disproportionally local hand stimulation in wakefulness [123]. Column state alterations are not only dependent on the amount of afferent activity but also on the production of sleep regulatory substances (SRSs) [135-137]. SRSs that build the humoral part of sleep regulation include substances like adenosine, nitric oxide (NO), NF-κB, peptides such as growth hormone (GH), orexins, prostaglandins, serotonin, and other proteins such as cytokines. Cytokines present a humoral link in the interaction between immune and central nervous system and interact with other cytokines, neurotransmitters and hormones involved in sleep regulation [52, 82, 107, 133, 136, 156, 195, 203, 264]. Effects of cytokines are always dependent on the dose and circadian phase. During wakefulness in response to neuronal activity, SRS production by neurons and glia is enhanced locally. In a network of biochemical

cascades ("non-REM Sleep homeostat"), the downstream actions of SRSs on neurotransmitter and cytokine release, gene transcription and translation, including feedback loops, act on neuronal assemblies to alter the functional sleep/wake-like state (ATP-contributed) and communicate to the sub-cortical sleep regulatory circuits [120, 135-137].

Well-characterised SRSs are the cytokines TNFα and IL-1β, whose administration (central and systemic) enhance duration and intensity of non-REM sleep in laboratory animals and humans [135, 136, 203]. The interaction of IL-1β and serotonin appears to have a central role in the regulation of sleep [107]. There is also strong evidence that IL-6 affects sleep modulation (see section 1.7). Many other cytokines have somnogenic properties for non-REM sleep like IL-8, IL-18, Epo, NGF; are non-REM sleep inhibitors such as IL-4, IL-10, sTNF-R; or inhibit REM sleep like IL-1β, IL-2, IL-15, IL-18, TNFα [107, 120, 133-136, 156, 195, 203].

Hormones of the hypothalamic-pituitary-adrenal (HPA) axis such as adrenocorticotropin (ACTH), corticotropin releasing hormone (CRH) as well as cortisol build a component of the interaction between sleep and stress system. The circadian secretory pattern of these HPA hormones in humans indicate lowest levels during early noctural sleep and a maximum at the morning (arousal). Furthermore, several research studies observed that CRH, ACTH and cortisol induced increased arousal [26, 156, 264, 299]. Cytokines including IL-1, IL-6 and TNFα have been shown to stimulate the HPA-axis (see 1.1) [262]. Hormones of the somatotropic system as growth hormone-releasing hormone (GHRH) and GH belong to the sleep-promoting SRSs. Taken together, the complex network of cytokines and the neuroendocrine system is part of the sleep regulatory/modulatory machinery (sketched in Fig. 5). In turn, sleep itself affects cytokine expression [107].

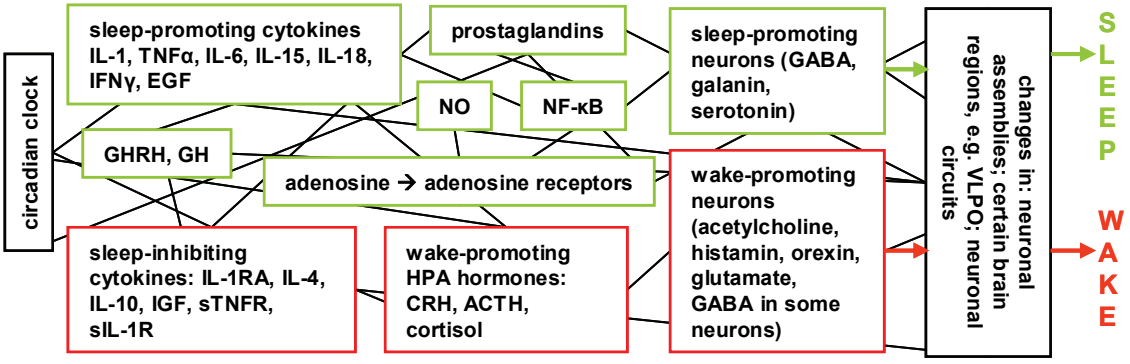

Figure 5 Schematic overall model of humoral regulation of sleep organised in a large network. Sleep-promoting components (green) are inhibited during wakefulness (red) and vice versa. For further information see 1.6.2.

1.6.3 Functions of sleep

Sleep is a fundamental brain process [137, 283] but currently it is not possible to determine a specific function. Nevertheless, there is no question that sleep serves multiple functions to ensure physical and mental health. Sleep has recovery and restorative functions to the brain and body that include cellular, endocrine, immune, metabolic, thermoregulatory and behavioural processes like learning and synaptic plasticity [167].

The detrimental effects of sleep loss corroborate the importance of sleep. Rats that were not allowed to sleep died after 2–3 weeks [222]. Sleep deprived humans showed impaired cognitive performance, tiredness, metabolic, endocrine and immune dysregulation [8, 128, 290, 291]. Prevention of sleep altered the numbers of NK-cells, lymphocytes, monocytes and enhance levels of HPA hormones and proinflammatory markers such as CRP and the cytokines TNFα, IL-1β, IL-6 and IL-17. These sleep-restricted changes of immune functions contribute to many disorders such as higher susceptibility to infections, diabetes, impaired glucose tolerance, cardiovascular diseases, hypertension, obesity and depression [25, 108, 109, 121, 292].

The other way around, bacterial or viral infections, inflammation processes, stress situations, or other pathologies that alter cytokine and hormone secretion patterns often lead to dysregulated sleep-wake behaviour, e.g. in influenza infection, HIV infection, in cardiovascular diseases, diabetes and rheumatoid arthritis [107, 121, 134]. Beneficially, sleep is a component of host defense against microbial pathogens, where cytokines play a key role, including acute phase response and sickness behaviour. Sleep architecture is therefore altered in a manner that supports the development of fever to promote recovery. Thus, non-REM sleep is enhanced and fragmented during infection, so that fever can be generated and heat loss is reduced. REM sleep is strongly suppressed, since shivering that is needed for generation of fever does not occur during REM sleep [107]. The role of sleep for the immune response has additionally been illustrated for vaccination effectiveness. Sleep at the night after Hepatitis A vaccination strongly enhanced the antibody titer in comparison to a wakeful night [140]. Otherwise, the functions of T-helper and regulatory T cells, which are coupled to the sleep-dependent circadian rhythm, are modulated by sleep [21].

On the mechanistic level many researchers have proposed the theory that synaptic scaling is the primordal function of sleep [124, 132, 137, 284], which means the up- or downregulation of the strength of synapses to stabilise the activity-dependent changes in neurons and synaptic networks. Tononi and Cirelli [284] described in the “synaptic homeostatic hypothesis” the function of synaptic downscaling for the benefits for learning/memory consolidation, which

belongs to the essential sleep function [166, 270]. Briefly, learning during wakefulness leads to long-term potentiation, the total synaptic strength increases, whereas it decreases (downscaling) during sleep (SWS). Then only the most robust connections stay intact, with benefits for energy and space requirements and memory consolidation. Krueger and Obal [132, 137] concluded that SRSs are involved in synaptic scaling, as it was shown for TNFα [119, 267].

1.7 IL-6 and sleep

Multiple research in humans and animals pointed to a role for the cytokine IL-6 as a sleep modulatory factor. IL-6 and sIL-6R are present in periphery and brain. Although their concentrations under normal conditions are very low, they are sufficient to influence brain functions [216]. Plasma IL-6 is secreted in a circadian rhythm that correlates with the sleep-wake cycle. IL-6 peaks in healthy humans during sleep and has low values during the day [13, 85, 108, 259, 298, 301, 303]. Similarly, IL-6 release increased in rats during their major sleeping period (light period) in brain (cortex, hippocampus, hypothalamus), adipose tissue and blood [84]. Thus, sleep onset is associated with elevated levels of circulating IL-6. Mainly monocytes, adipocytes and muscle cells contribute to the production of IL-6 in blood [224]. For IL-6-producing monocytes (humans) a noctural rise has been found [56]. During human sleep, IL-6 plasma levels differ between sleep stages. Higher IL-6 levels were measured in REM and light sleep but not in deep sleep (SWS) [224, 298]. A negative correlation between SWS and IL-6 release became evident. Likewise, daytime IL-6 concentrations are negatively correlated to the amount and efficiency (depth) of noctural sleep (SWS) [168, 298]. Sleep deprivation in healthy humans led to a change in the circadian pattern of IL-6 plasma levels, thus to an undersecretion of IL-6 during the night of sleep loss and to an oversecretion at the following day [253, 298, 303]. The recovery night (rebound sleep), with increased amount of SWS and delta power, was associated with reduced IL-6 levels [298, 303]. However, when sleep restriction was stopped [224] or interrupted by a nap [253], or after a 2-h midafternoon nap [304], IL-6 levels again decreased. Even mild sleep restriction from 8 to 6 h/night for 1 week in healthy humans resulted in elevated daytime IL-6 plasma levels, sleepiness and impaired psychomotor performance [302]. Also in rodents, increased levels of IL-6 were observed after sleep deprivation in serum as well as in the brain [84, 104]. Taken together,

these results indicate that increased IL-6 levels during wakefulness in consequence of sleep loss might contribute to the homeostatic sleep pressure.

In a number of previous studies sleep-wake behaviour after exogenous manipulation of the IL-6 system in humans and animals was investigated. In healthy humans a subcutaneous (s.c.) injection of recombinant human IL-6 in the evening before sleep, leading to blood concentrations comparable to values during bacterial infection, increased the feeling of tiredness. Furthermore, IL-6 reduced the amounts of REM sleep and SWS in the first half of the night followed by an enhanced SWS during the second half [262]. Recently, an intranasal application of human IL-6 to humans has increased slow wave activity of SWS (delta wave power) during the late part of the night together with an improvement of sleep-related declarative memory. Here, REM sleep was not affected [16]. The outcome from several animal studies remain conflicting with regard to the effect of IL-6. Central (i.c.v.) and peripheral (i.p.) injection of human IL-6 into rabbits did not alter non-REM sleep and REM sleep [202]. However, i.c.v. administration of rat IL-6 to rats before the dark phase caused an initial increase followed by subsequent suppression of non-REM sleep, and sleep fragmentation in a dose-dependent manner without changes in REM sleep amount. But antagonising IL-6 via anti-rat antibodies did not affect sleep-wake behaviour [99]. Remarkably, mice deficient for IL-6 (IL-6 KO) displayed normal non-REM sleep but 30% more REM sleep. After sleep deprivation, IL-6 KO mice required more time to obtain the same non-REM sleep amount as wildtype mice [174]. To bacterial LPS challenge, normal wildtype mice responded with enhanced non-REM sleep, that was slighter in IL-6 KO mice [175]. This suggests that IL-6 may be a modulator of non-REM sleep during sickness/pathologies, in which IL-6 is elevated.

Besides, IL-6 is a modulator of the neuroendocrine system, such as the HPA axis, that influences sleep (see 1.1.) [262, 286]. In clinical human studies an interesting constellation between peripheral IL-6 and cortisol levels has been observed. A hypersecretion of IL-6 accompanied by relatively high cortisol levels (activation of the HPA) led to poor sleep and fatigue, as it is the case e.g. in chronic insomnia or in elderly people (aging). On the other hand, elevated IL-6 and low cortisol levels were associated with sleep and sleepiness [182, 300, 301, 303]. IL-6 levels are elevated in disorders and pathologies associated with excessive daytime sleepiness (EDS) such as insomnia, narcolepsy, sleep apnea, obesity, aging [34, 39, 121, 200, 297, 298, 300]. Examples for chronic insomnia and elevated IL-6 levels include several psychological disorders such as depression, alcoholism and schizophrenia [1, 86, 108, 149, 176, 225].

Only a few studies elucidating the effect of IL-6 on sleep addressed the different receptors of the IL-6 signalling pathways. Cirelli *et al.* [46] found a downregulation of the rat IL-6R in the cerebral cortex during wakefulness via a gene array study. Dimitrov *et al.* [56] discriminated between components of IL-6 classic and trans-signalling during normal human sleep behaviour. This publication revealed rising plasma concentrations of the shed variant of the sIL-6R during sleep with a maximum after awakening in the morning, which is paralleld by elevated noctural IL-6 levels. These findings implicate that sleep enhances IL-6 trans-signalling. The normally high amount of REM sleep during late sleep correlated with this high sIL-6R amount, suggesting a putative connection of IL-6 trans-signalling and REM sleep.

1.8 Aim of this work

Numerous previous studies implicated the cytokine IL-6 in sleep modulation. Since the peripheral human sIL-6R is upregulated during sleep, which is regulated by the brain, it is the aim of this study to determine the role of IL-6 trans-signalling within the CNS on sleep-wake behaviour. Therefore the effect of IL-6/sIL-6R on sleep was investigated *in vivo* by intracerebroventricularly (i.c.v.) injection of the designer cytokine Hyper-IL-6 (agonist of trans-signalling) into rats and subsequent EEG/EMG recoding. Additionally, transgenic mice were generated, that overexpress the inhibitor of IL-6 trans-signalling sgp130-Fc within the brain under an astroycte-specific promoter. In the future, these mice will be investigated in terms of sleep and learning behaviour.

IL-6 mostly exerts a pathophysiological role in the periphery and the CNS via trans-signalling. Thus, the constructed transgenic brain-specific sgp130-Fc mice could be subjected to reasearch models of peripheral and central inflammation.

In summary, these experiments will help to define the relevance of IL-6 trans-signalling in the regulation/modulation of sleep and in the coordination of neuroinflammatory processes.

2 Material and Methods

2.1 Material

2.1.1 Chemicals

General buffer chemicals were purchased by the companies Carl Roth (Karlsruhe), Sigma-Aldrich (Deisenhofen), Merck (Darmstadt) and Serva (Heidelberg). The used water (Aqua bidest) was deionised and if needed autoclaved or sterile filtered.

2.1.2 Radiochemicals

Deoxyadenosin-5´-triphosphate, [α-^{32}P], 3000 Ci/mmol (MP Biomedicals GmbH, Eschwege).

2.1.3 Plasmids and vectors

pmax–GFP (Amaxa GmbH, Köln);

pGfa2–LacZ [29];

p409–sgp130-Fc (Jürgen Scheller);

pCR-script–sgp130-Fc_optimised (GENEART, Regensburg);

pTZ–PEPCK–βglob.intron–opt_sgp130Fc [220];

pGfa2–sgp130-Fc_optimised–mP1 (this work);

pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-blobin (this work).

2.1.4 Primers

polyA rabbit β-globin forward (for cloning):

5’-GAC TGA ATT CGA TCT TTT CCC TCT GCC-3’

polyA rabbit β-globin reverse (for cloning):

5’-GAC TGC GGC CGC GTC GAG GGA TCT CCA TAA G-3’

2nd intron rabbit β-globin forward (for cloning) :

5’-GTC GTC GAC AGA TCT CGA TCC TGA GAA CTT CAG-3’

2nd intron rabbit β-globin reverse (for cloning):

5’-GATCAAGCTT GATTCTTGCC AAATGATG-3’

sgp130-Fc-screen forward: 5’-GAG TTC AGA TCC TGC GAC-3’

sgp130-Fc-screen reverse: 5’-TCA CTT GCC AGG AGA CAG-3’

β-globin (mouse) forward:

5'-CCA ATC TGC TCA CAC AGG ATA GAG AGG GCA GG-3'

β-globin (mouse) reverse:

5'-CCT TGA GGC TGT CCA AGT GAT TCA GGC CAT CG-3'

sgp130-Fc-screen-new forward: 5'-CTG TCC TCC TTG TCC TGC TC-3'

sgp130-Fc-screen-new reverse: 5'-ATG TCA CCT CAG GGG TTC TG-3'

Oligo d(T): 5'-TTT TTT TTT TTT TTT TTT-3'

Primers were synthetised by Metabion GmbH (Martinsried).

2.1.5 Recombinant Cytokines

human IL-6: prepared and purified as described in [289];

Hyper-IL-6: fusion protein of human soluble IL-6 receptor connected by a flexible peptide linker; prepared and purified as described in [70];

sgp130-Fc : dimer of soluble gp130 fused to the Fc-portion of a human IgG antibody; prepared and purified as described in [115]; kindly provided by Steffi Schnell (Institut of Biochemistry, Christian-Albrechts-University Kiel).

2.1.6 Antibodies

2.1.6.1 Primary antibodies

mouse anti-human gp130 B-P4 (Diaclone, Besancon, France): diluted 1:1,000 in 5% low fat milk powder/TBS for Western blot analysis;

mouse anti-human IL-6 receptor clone 14-18 [41]: diluted 1:5,000 in 5% low fat milk powder/TBS for Western blot analysis;

rabbit anti-phospho-STAT3 (Cell Signaling Technology, New England Biolabs GmbH, Frankfurt/Main): diluted 1:2,000 in 5% BSA/TBS for Western blot analysis;

mouse anti-STAT3 (Cell Signaling Technology, New England Biolabs GmbH, Frankfurt/Main): diluted 1:2,000 in 5% low fat milk powder/TBS for Western blot analysis.

2.1.6.2 Secondary antibodies

Goat anti-mouse IgG peroxidase (POD) conjugate (Pierce, Fisher Scientific GmbH, Schwerte): diluted 1:10,000 in 1% low fat milk powder/TBS for Western blot analysis;

Goat anti-rabbit IgG peroxidase (POD) conjugate (Pierce, Fisher Scientific GmbH, Schwerte): diluted 1:10,000 in 1% low fat milk powder/TBS for Western blot analysis.

2.1.7 Cell lines

U251: human astroglioma cell line (ATCC, Rockville, MD, USA), adherent cells;

Ba/F3-gp130: murine peripheral blood pre-B-cell line, stably transfected with cDNA encoding human gp130 [207], suspension cells;

Ba/F3-gp130-IL-6 receptor: murine peripheral blood pre-B-cell line, stably transfected with cDNAs encoding human gp130 and murine IL-6 receptor [79], suspension cells.

2.1.8 Mouse strains

C57BL/6N (Charles River Laboratories, Germany, Bad Sulzfeld);

pepck–sgp130-Fc (systemic expression of sgp130-Fc, [220]);

gfa2–sgp130-Fc (astrocyte-specific expression of sgp130-Fc, this work).

2.1.9 Standard buffers

TBS: 50 mM Tris-HCl, pH 7.6, 200 mM NaCl;

TBS-T: 50 mM Tris-HCl, pH 7.6, 200 mM NaCl, 0.05% Tween-20;

PBS: 250 mM NaCl, 20 mM KCl, 48 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4;

PBS-T: 250 mM NaCl, 20 mM KCl, 48 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4, 0.05% Tween-20.

2.2 Molecular biological methods

2.2.1 Agarose gel electrophoresis

DNA was separated on an agarose gel in 0.5 x TBE buffer (45 mM Tris, 45 mM boric acid, 1 mM EDTA, pH 8) at 70–100 V (gel chambers by BioRad Laboratories GmbH, München). Depending on the size of the DNA fragment 0.7–2% of agarose was used. Before loading the DNA sample onto the gel, 1/6 volume of 6 x loading dye (10 mM Tris-HCl, pH 7.6, 0.03% bromphenol blue, 0.03% xylene cyanol, 60% glycerol, 60 mM EDTA) was added. The DNA

intercalating substance ethidiumbromide (0.5 µg/ml) was applied to the gel, to visualise the DNA under UV light (GelDoc 2000, BioRad Laboratories GmbH, München). For estimating the band size of DNA fragments, a 1 kb DNA marker was used (Fermentas, St. Leon-Rot).

2.2.2 Determination of nucleic acid concentrations

To measure the concentration of DNA or RNA, UV spectrophotometry (Ultrospec 3000, Amersham Pharmacia Biotech Europe GmbH, Nümbrecht) was used. Concentrations and purity were determined by measuring absorbances at 260 nm, 280 nm and 320 nm.

2.2.3 Cloning

Steps involved in cloning of DNA constructs and generation of new vectors/plasmids, such as isolation of plasmids, restriction of DNA, dephosphorylation of vector DNA, purification of DNA fragments via agarose gel extraction, PCR, ligation, transformation of *E.coli*, are described below.

2.2.4 Plasmid DNA Isolation

Plasmid DNA was isolated from 100-ml liquid bacteria cultures (in LB medium including an antibiotic) using the NucleoBond® Midi AX 100 Kit (Macherey-Nagel, Düren) according to the manufacturer's protocol.

2.2.5 Enzymatic restriction of DNA

The DNA was incubated with appropriate amounts of restriction enzymes (Fermentas, St. Leon-Rot) in the recommended buffer for 2 h or overnight at 37°C. Restriction was terminated by addition of 6 x DNA loading dye (10 mM Tris-HCl, pH 7.6, 0.03% bromphenol blue, 0.03% xylene cyanol, 60% glycerol, 60 mM EDTA) and loaded on an agarose gel. For further preparation, the resulted digested DNA fragment was eluted from the agarose gel (2.2.8).

2.2.6 Vector dephosphorylation

To avoid self-ligation of restricted vector/plasmid DNA, the 5'-phosphate groups were dephosphorylated enzymatically. The restriction reaction mixture was incubated with 1 U

Calf Intestinal Alkaline Phosphatase (CIAP, 1U/µl, Fermentas, St. Leon-Rot) for 60 min at 37°C.

2.2.7 Polymerase Chain Reaction (PCR) [183]

During PCR, a target DNA sequence between two oligonucleotid primers is amplified exponentially. The recombinant Taq DNA-Polymerase (Fermentas, St. Leon-Rot) was utilised in a screening PCR for identification of sgp130-Fc-positive transgenic mice. DNA amplification for a cloning strategy was achieved using the Pfu DNA-Polymerase (Fermentas, St. Leon).

PCR reaction mix:

10 x Taq-Polymerase buffer	5 µl
dNTP mix (10 mM)	1 µl
$MgCl_2$ (25 mM)	4 µl
forward primer (100 pmol)	0.5 µl
reverse primer (100 pmol)	0.5 µl
Taq-Polymerase (5 U/µl)	1 µl
DNA template	10–200 ng
water	ad 50 µl

Standard PCR temperature programme (equipment: Robo Cycler Gradient; Stratagene, La Jolla, USA):

initial denaturation	94°C	2 min	1 cycle
denaturation	94°C	1 min	30 cycles
primer annealing	55–57°C	1 min	
elongation	72°C	1 min	
final elongation	72°C	5 min	1 cycle

The annealing temperature depends on the melting temperature of the primer pair.

2.2.8 Purification of DNA fragments from agarose gels

DNA fragments were cut out with a scalpel and purified from agarose gels using the PureLink™ Quick Gel Extraction Kit by Invitrogen Corporation (Karlsruhe) following the manufacturer's protocol.

2.2.9 Precipitation of DNA

After purification of DNA from an agarose gel (2.2.8) it is possible to further purify, concentrate or change the buffer of the DNA solution. An ammoniumacetat/ethanol precipitation of the transgene-containing DNA cassette was applied, needed for the pronucleus microinjection to generate transgenic mice (2.5.2). For precipitation, 1/10 volume of 7 M ammomium acetate and 2 volumes of 96% ethanol were added to the DNA solution and mixed. After centrifugation (20 min, 16,000 x g, 4°C) the DNA pellet was washed with 1 ml 70% ethanol and centrifuged again (10 min, 16,000 x g, 4°C). The DNA pellet was dried at 50°C or during vacuum centrifugation before it was dissolved in an appropriate solvent like TE buffer (5 mM Tris-HCl pH 7.4, 0.1 mM EDTA) as requested for DNA microinjection (2.5.2) to a DNA concentration of 30 µg/ml. The DNA solution was additionally cleared by centrifugation (10 min, 16,000 x g, 4°C).

2.2.10 Ligation

For ligation of the DNA fragment of interest into a vector, 50 ng vector DNA and a 3–5 fold molar excess of insert DNA were incubated with 1 U T4 Ligase (Fermentas, St. Leon-Rot) in ligation buffer (supplemented, Fermentas, St. Leon-Rot) for 1 h at room temperature. The reaction mixture was used directly for transformation of *E. coli* (2.2.12).

2.2.11 Generation of chemo-competent *E. coli*

To prepare chemo-competent *E. coli* XL1-Blue (Stratagene, La Jolla, USA) the calcium chloride method was used. Therefore the bacteria were grown to an OD_{595nm} of 0.6 and subsequently chilled on ice at 4°C. The cells were centrifuged (3,000 x g, 4°C) and resuspended in 50 mM $CaCl_2$. After a second centrifugation step and resuspension in 50 mM $CaCl_2$ supplemented with 10% glycerin, the cells were aliquoted and snap frozen in liquid nitrogen. The competent bacteria were stored at –80°C.

2.2.12 DNA Transformation of *E. coli*

Chemo-competent *E. coli* XL1-Blue bacteria were transformed by the heat-shock method. Bacteria were thawn on ice. Fifty µl of competent *E. coli* XL1-Blue and 10 ng plasmid or 5 µl of ligation mixture were incubated on ice for 5 min. After a heat-shock of 90 seconds at 42°C the bacteria were chilled on ice for 5 min. Thereafter 1 ml LB medium without antibiotics was added and the bacteria were incubated for 1 h at 37°C under continous shaking. The transformed bacteria (100–400 µl) were plated onto LB agar plates containing the appropriate antibiotic and incubated overnight at 37°C. For isolation of a transformed plasmid DNA via the NucleoBond Kit (Macherey-Nagel, Düren, 2.2.4), a single *E. coli* colony was picked from the plate and grown in liquid cultures at 37°C overnight.

"Luria Bertani" (LB) medium: 10 g/l NaCl, 5g/l yeast extract, 10 g/l tryptone

LB agar plates: 10 g/l NaCl, 5g/l yeast extract, 10 g/l tryptone, 15 g/l agar agar

The autoclaved medium was supplemented, if required, with antibiotics: ampicillin (50 µg/ml) or kanamycin (30 µg/ml, both from Carl Roth, Karlsruhe).

2.2.13 DNA Sequencing

Sequencing of DNA was performed by the company Seq-Lab (Göttingen). The DNA (0.7 µg) and 20 pmol sequencing primer were diluted in 7 µl water.

2.2.14 Isolation of genomic mouse DNA

Genomic DNA was isolated from a 0.5-cm-long tail of a 4-week-old mouse, which was cut with sterile scissors. The tail was incubated overnight at 55°C, slightly shaked in 600 µl tail-buffer (50 mM Tris-HCl, 1 mM EDTA, 20 mM NaCl) containing 30 µl 10% SDS and 20 µl Proteinase K (20 mg/ml; Fermentas, St. Leon-Rot). Undissolved material was removed by centrifugation (5 min, 16,000 x g, 4°C) and DNA was purified by adding 600 ml phenol/chloroform/isoamylalcohol (25:24:1; Carl Roth, Karlsruhe) and shaken for 10 min at 4°C. After a centrifugation step (10 min, 16,000 x g, room temperature) the upper aqueous phase was collected and again treated with phenol/chloroform/isoamylalcohol followed by mixing and centrifugation according to the same procedure, which was repeated 2 times if necessary. After the following extraction step with only chloroform, the DNA of the upper

aqueous phase was precipitated with 100 µl 5 M NaCl and 800 µl 96% ethanol. The mixture was inverted 20 times, incubated for 20 min at -20°C and centrifuged (20 min, 16,000 x g, 4°C). The DNA pellet was washed once with 70% ethanol and thereafter dried for 20 min at 37°C. The DNA was dissolved in 100 µl water, incubated for 10 min at 65°C and overnight at 4°C. The concentration of DNA was determined by UV spectrophotometry (2.2.2). Genomic DNA was used for Southern blot analysis (2.2.17) and PCR (2.2.7).
As an alternative method a phenol-free kit was used (Gentra Puregene Tissue Core Kit; Qiagen, Hilden) according to the manufacturer's instruction, and the genomic DNA was used for PCR (2.2.7).

2.2.15 Isolation of total RNA

Total RNA from tissue or cells was isolated with the NucleoSpin® RNA II Kit (Macherey-Nagel GmbH & Co. KG, Düren). Frozen mouse tissue was grinded with pestle and mortar in liquid nitrogen. During this procedure, thawing of organs was avoided. The pulverised organs were processed immediately or kept at –80°C. The total RNA amount was measured by UV spectrophotometry (2.2.2).

2.2.16 Reverse Transcriptase PCR (RT-PCR)

During the reverse transcription (RT) reaction, mRNA is translated into a single stranded cDNA followed by a conventional PCR using gene-specific primers. RevertAid M-MuLV Reverse Transcriptase (Fermentas, St. Leon-Rot) was employed according to the manufacturer's protocol. Oligo(dT) primers were used, which target the 3' poly(A) mRNA tail. For RT reaction 1.5 µg RNA and for further PCR reaction (2.2.7) 1 µl cDNA were used.

2.2.17 Southern blot

2.2.17.1 Electrophoretic separation of digested genomic DNA

Ten micrograms of genomic mouse DNA were digested with the restriction endonuclease *Eco*RV overnight at 37°C. Afterwards the generated DNA fragments were separated on a 0.7% agarose gel at 90 V.

2.2.17.2 Preparation of the Agarose gel and blotting procedure

After electrophoresis the gel was treated with 0.25 M HCl for 15 min (depurination), 2 x 20 min with a denaturation solution (0.5 M NaOH, 1.5 M NaCl) and finally with a neutralisation solution (1 M Tris-HCl, pH 8, 1.5 M NaCl) for 10 min. After washing the gel in 10 x SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7) for 5 min, the capillary blot was assembled according to Sambrook *et al.* [239]. The transfer buffer 10 x SSC was used. During blotting, the DNA fragments were transferred and immobilised onto a positive charged nylon membrane (Hybond-N+, Amersham Biosciences Europe GmbH, Freiburg). After the transfer process the membrane was washed in 5 x SSC for 15 min and dried at 65°C. The DNA was crosslinked to the membrane by radiation with 120 mJ UV light.

2.2.17.3 Hybridisation of the membrane

The α-^{32}P-dATP-labelled probes, which contain the DNA sequence of interest, were generated using the Prime-It II Random Primer Labeling Kit (Stratagene, La Jolla, USA) according to the manufacturer's instruction in a reaction volume of 50 µl. To remove unbound nucleotides the probes were purified via ProbeQuant^{TM} G-50 Micro Columns (Amersham Biosciences Europe GmbH, Freiburg) according to the manufacturer's instructions.

To avoid unspecific binding of the probe, the membrane was prehybridised for 1 h at 65°C in prewarmed hybridisation solution (500 mM NaP_i, pH 7.2, 7% SDS, 1% BSA, 1 mM EDTA) under constant rolling. The radioactive-labelled probe was incubated for 5 min at 95°C to obtain single-stranded probes and then put on ice for 5 min. For hybridisation, the membrane was incubated with the probe (50 µl) in 20 ml fresh prewarmed hybridisation solution over-night at 65°C under constant rolling.

To remove unspecifically bound probes, the membrane was washed with stringent washing buffers: 2 times for 15 min with 2 x SSC, 0.1% SDS (high stringency); 2 times for 10 min with 0.1 x SSC, 0.1% SDS (moderate stringency) at 65°C under rolling conditions. Binding of the probe to the desired target sequence was visualised by exposing the membrane on a Kodak BioMax MS X-ray film (Amersham Biosciences Europe GmbH, Freiburg) for several days at –70°C. The film was developed until bands were seen visually and fixed in solutions by Tenenal (Norderstedt).

2.2.18 Northern blot

2.2.18.1 Electrophoresis of RNA

To avoid RNA degradation all solutions and water were treated with 0.05% DEPC (diethylpyrocarbonate; Carl Roth, Karlsruhe) overnight at 37°C. DEPC inactivates RNAses by derivatisation of histidine residues. Subsequently the treated solutions were autoclaved, whereupon DEPC breaks down to CO_2 and ethanol.

Total RNA was separated by electrophoresis under denaturating conditions on a 1% agarose gel containing 7% formaldehyde. The gel was prepared as follows. One gram of agarose was dissolved in 62 ml DEPC treated water by boiling, then cooled down to 60°C. Thereafter 20 ml 5 x MOPS buffer (0.1 M MOPS, pH 7.0, 40 mM sodium actetate, 5 mM EDTA, pH 8.0), 18 ml formaldehyde (37%) and 1 µl ethidiumbromide were added. Five micrograms of RNA in a volume of 5 µl were mixed with 15 µl RNA sample buffer, heated to 65°C for 15 min and cooled on ice. Before loading the RNA onto the gel, 2 µl of RNA loading buffer was added. The gel was running in 1 x MOPS buffer at 75 V.

2.2.18.2 Preparation of the Agarose gel and blotting procedure

After electrophoresis the gel was washed overnight in DEPC-treated water under gentle shaking to remove the formaldehyde. Under UV light (GelDoc 2000, BioRad Laboratories GmbH, München) the 2 bands of 28S and 18S rRNA should be visible as an indicator of intact RNA. Before the transfer onto the nylon membrane (Hybond-N+, Amersham Biosciences Europe GmbH, Freiburg), the gel was washed in DEPC-treated 50 mM NaOH for 30 min and finally in 20 x SSC (3 M NaCl, 0.3 M sodium citrate, pH 7) for 45 min under gentle shaking.

The following steps of Northern blotting up to exposition on a X-ray film were the same as for Southern blotting (2.2.17.2–3.), but for transfer 20 x SSC was used.

2.3 Protein biochemical methods

2.3.1 SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) [139]

Proteins were separated on 10% SDS polyacrylamide gels at 120–160 V using the Mini-Protean III System (BioRad Laboratories GmbH, München). The gel casting frames, stands and running chambers were assembled according to the manufacturer's protocol. Before loading the samples onto the gel, they were mixed with 4 x SDS gel loading buffer and heated

to 95°C for 5 min. Estimation of protein molecular weight occurred via the Prestained Molecular Weight Marker (Fermentas, St. Leon-Rot). Gel run was stopped when the blue bromphenol blue line reached the end of the gel and Western blot analysis (2.3.2) proceeded.

10% running gel:
3.870 ml water, 2.55 ml 1.5 M Tris-HCl, pH 8.8, 0.1 ml 10% SDS, 3.33 ml 30% acrylamid/bisacrylamid 29:1, 0.1 ml 10% ammoniumpersulfat, 0.01 ml TEMED

4% stacking gel:
3.725 ml water, 0.625 ml 0.5 M Tris-HCl, pH 6.8, 0.05 ml 10% SDS, 0.67 ml 30% acrylamid/bisacrylamid 29:1, 0.025 ml 10% ammoniumpersulfat, 0.005 ml TEMED

SDS running buffer: 192 mM glycine, 25 mM Tris-HCl, pH 8.3, 0.1% SDS

4 x SDS gel loading buffer:
240 mM Tris-HCl, pH 6.8, 20% β-mercaptoethanol, 8.0% SDS, 40% glycerol, 0.2% bromphenol blue

2.3.2 Western blot analysis and immunochemical detection of proteins

Proteins from a SDS gel were transferred onto a PVDF Hybond-P membrane (Amersham Biosciences Europe GmbH, Freiburg) by blotting via a "Trans-Blot SD Semi-Dry Transfer Cell" (BioRad Laboratories GmbH, München) at 20 V for 60 min. The membrane was activated before the blotting procedure by incubation steps in methanol, water and transfer buffer (25 mM Tris-HCl pH 8.3, 192 mM glycine, 20% methanol) for 5 min each. The blotting sandwich was assembled according to the manufacturer's instructions.
The membrane was then blocked in 5% low fat milk powder dissolved in TBS for 1 h at room temperature. After 3 washing steps for 10 min each with TBS-T, the membrane was incubated overnight at 4°C with the primary antibody (titer and dilution buffers: 2.1.6.1). Afterwards unbound antibodies were removed by repeated washing in TBS-T (3 times, 10 min, room temperature) before the secondary POD conjugated antibody (see 2.1.6.2) was applied and incubated for 1 h at room temperature. Subsequently the membrane was washed again in TBS-T (3 times, 10 min) and once in TBS. Immunoreactive bands onto the membrane were detected using the "ECL Plus Western Blotting Detection Reagents" (Amersham Biosciences Europe GmbH, Freiburg). Therefore the membrane was soaked for 2 min in the POD

substrate solution and exposed to a chemiluminescent sensitive “Hyper ECL” film (Amersham Biosciences Europe GmbH, Freiburg). Alternatively, chemiluminescence was detected by the Luminescent Image Analyzer LAS-1000 CH (FUJIFILM Europe GmbH, Düsseldorf).

For reprobing the membrane additionally with a different antibody, the blot was stripped in solution I (0.5 M NaCl, 0.5 M acetic acid) for 40 min and in solution II (100 mM Tris-HCl, pH 8.0) for 40 min at room temperature. After a short washing step in TBS, the membrane was blocked again and proceeded as described above.

2.3.3 Immunoprecipitation (IP) with Protein A sepharose

This method was used to precipitate sgp130-Fc from cell culture supernatants and from sgp130-Fc transgenic mouse sera. Protein A is able to bind to Fc-portions of antibodies, in this case to the Fc-portion of sgp130-Fc [115]. To precipitate sgp130-Fc from transfected U251 cell supernatant, 500 µl of cell-free conditioned medium was incubated with 50 µl Protein A sepharose (50% slurry in PBS, GE Healthcare, München) overnight under continous rolling at 4°C. The next day the mixture was centrifuged (5 min, 16,000 x g, 4°C) and the Protein A sepharose pellet was washed 3 times with IP washing buffer A (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.2% NP-40, 2 mM EDTA) and twice with IP washing buffer B (10 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.2% NP-40, 2 mM EDTA). After the last centrifugation step, the Protein A sepharose pellet was heated at 95°C in 50 µl of 4 x SDS gel loading buffer for 5 min. After SDS-PAGE (2.3.1) and Western blotting (2.3.2) of 20-µl samples, precipitated sgp130-Fc was detected using a monoclonal antibody against human gp130 (B-P4, Diaclone, Besancon, France). Via the same procedure, sgp130-Fc was precipitated from mouse serum (40 µl) and from supernatant of primary transgenic murine astrocytes (1 ml) using 50 µl Protein A sepharose solution including 10 x IP buffer (0.25 M Tris-HCl, pH 7.5, 5% Triton X-100, 5% NP-40) filled up with PBS to 1.5 ml.

For coimmunoprecipitation of Hyper-IL-6 with sgp130-Fc of transgenic mouse brain homogenates, 40 µl of brain homogenate was incubated with 100 µl 10 x IP buffer (0.25 M Tris-HCl, pH 7.5, 5% Triton X-100, 5% NP-40), 4 µl Hyper-IL-6 (100 ng/µl) and 856 µl PBS overnight under continous rolling at 4°C. For pulldown of sgp130-Fc, 50 µl Protein A sepharose was added the next day and again incubated for 4 h or overnight under rolling conditions at 4°C. Subsequently the Protein A sepharose was washed and treated as described above. Coprecipitated Hyper-IL-6 was detected by Western blotting by a monoclonal antibody against the human IL-6 receptor (clone 14-18, [41]).

2.3.4 Cell lysis

Eukaryotic cells (rat primary astrocytes and microglia, mouse primary astrocytes; all adherent) were washed in ice-cold PBS, removed by a cell scraper and transferred to a 1.5 ml plastic tube. After centrifugation (30 s, 16,000 x g, 4°C) the cell pellet was snap frozen in liquid nitrogen. Subsequently the cell pellet was lysed in lysis buffer (100 µl/ 0.5 x 10^6 cells) for 2 h at 4°C. Cell debris was removed by centrifugation (15 min, 16,000 x g, 4°C) and the cell lysate (supernatant) was stored at –20°C.
Alternatively, when cell lysates were only subjected to SDS-PAGE and Western blot analysis, the cultured cells were lysed directly in hot SDS gel loading buffer, transferred after scraping in a 1.5 ml plastic tube, vortexed vigorously and heated to 95°C for 10 min.

cell lysis buffer:
50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% NP-40, 1% Triton X-100, 1 mM NaF, 1 mM sodium-ortho-vanadate, 1 tablet/50 ml protease inhibitor complete (Roche Diagnostics, Mannheim)

2.3.5 Enzyme-linked immunosorbent assay (ELISA)

The ELISA for human sgp130 (DuoSet ELISA Development Kit; R&D Systems Europe, Abingdon, UK) was performed following the manufacturer's instructions. Recombinant sgp130-Fc [115] in 2-fold serial dilutions starting from 10 ng/ml to 0.3125 ng/ml served as standard. Mouse serum was diluted 1:2,000, and brain homogenates were diluted 1:750 and 1:1,000.
To perform the murine sIL-6R sandwich ELISA, microtitre plates (Greiner Microlon, Solingen, Germany) were coated with goat anti-mouse IL-6R polyclonal antibody AF1830 (R&D Systems Europe, Abingdon, UK) diluted to 0.8 µg/ml in PBS buffer and incubated overnight. After blocking with 5% BSA/PBS for 1–2 h, 100-µl aliquots of brain homogenates (1:50; 1:100; 1:500) and standard (mouse IL-6R 1830-SR; R&D Systems) diluted in 1% BSA/PBS were added and incubated for 2 h. Bound IL-6R was detected by biotinylated goat anti-mouse IL-6R polyclonal antibody BAF1830 (R&D Systems Europe, Abingdon, UK) diluted to 0.2 µg/ml (2-h incubation) followed by incubation in the dark for 30 min with streptavidin horseradish peroxidase (R&D Systems Europe, Abingdon, UK) diluted 1:200 in 1% BSA/PBS. After each incubation step the plate was washed 3 times in PBS/0.05% Tween-20. The soluble peroxidase substrate (BM blue POD; Roche Diagnostics, Mannheim,

Germany) for the enzymatic reaction was added and incubated in the dark until colour development. The absorbance was read at 450 nm on an SLT Rainbow plate reader (Tecan, Maennedorf, Switzerland) after the reaction was stopped by addition of 1.8 M H_2SO_4.

2.4 Cell Culture

2.4.1 Cell culture media and cultivation

Cells were usually grown in 10-cm dishes (Cell Tissue Dishes, Sarstedt, Nümbrecht) in DMEM High Glucose (4.5 g/l) Medium with L-Glutamine (PAA Laboratories GmbH, Cölbe) supplemented with 10% FCS and 1% penicillin/streptomycin at 37°C with 5% CO_2 in a 90% humified atmosphere (Hera Cell CO_2-Incubator, Kendro Laboratory Products GmbH, Langenselbold). For maintenance, the culture was split 1:10. Cells were passaged at 80–90% confluency. Adherent cell lines were therefore detached by 2 ml trypsin/EDTA (PAA Laboratories GmbH, Cölbe) for 5 min at 37°C. After centrifugation (5 min, 1,000 x g) the cell pellet was gently resuspended in fresh medium.

2.4.2 Transient transfection of U251 cells with Lipofectamine-2000

Cells grown to confluency of 80–90% were transiently transfected in a 24-well format using Lipofectamine-2000 (Invitrogen Corporation, Karlsruhe) according to the manufacturer's instructions. In contrast to the original protocol, only 0.5 µg DNA and 1.5 µl Lipofectamine-2000 were used for one transfection.

2.4.3 Proliferation assay

The cell viability test was performed with Ba/F3-gp130 and Ba/F3-gp130-IL-6 receptor cells (=Ba/F3-gp130-IL-6R cells). Cells were washed 3 times with sterile PBS and resuspended in DMEM Medium (PAA Laboratories GmbH, Cölbe) containing 10% FCS and seeded at 5,000 cells/well in a 96-well plate in a volume of 100 µl/well. The cells were cultured in presence of the cytokines to test for the proliferation capability in different concentrations. After 3 days, the cell viability was determined by using CellTiter-Blue Reagent (Promega GmbH, Mannheim) according to the manufacturer's instructions. For measurment, the Lambda Fluoro

320 fluorimeter (excitation filter: 560/25 nm; emission filter: 590/35 nm; sensitivity 75; Software KC4) was used.

2.4.4 Isolation of primary astrocytes and microglia

Primary astrocytes and microglia were isolated from 2-days-old rats by Dr. Claudia Röhl and Monika Grell at the Institute of Anatomy as described in [229]. Primary murine astrocytes, derived from cerebral cortices, were preparated using the same method. For experiments rat astrocytes were seeded in 6-well plates in DMEM medium complemented with 10% FCS (inactivated at 56°C) in a cell density of 30,000 cells/cm²; murine astrocytes in 75 cm² flasks. Microglial cells were seeded in astrocyte-conditioned medium at a density of 100,000 cells/cm² in a 6-well format.

2.5 Generation of transgenic mice

2.5.1 Animal treatment

Procedures involving animals and their care were conducted in conformity with national and international laws according to home office approved project license 66-6/06 from 9/15/2006. Mice were housed in a 12/12 h light/dark rhythm with *ad libitum* access to standard chow and tap water. All mice were maintained under barrier conditions and were pathogen free as assessed by regular microbiological screening. The animals were kept at 21°C ± 2°C and 60% ± 5% humidity in individually ventilated cages (IVC) in the animal facility Viktor-Hensen-Haus of Medical Faculty of the Christian-Albrechts-Universität zu Kiel.

2.5.2 Pronucleus microinjection of DNA

A pronucleus microinjection of DNA is a method to produce transgenic animals. The transgene DNA was prepared as follows. The vector containing the transgene DNA was transformed into *E. coli* (2.2.12), the DNA vector was isolated (2.2.4) and cutted with appropriate restriction enzymes (2.2.5). The DNA fragment comprising the transgene DNA was purified from a 1% agarose gel via gel extraction (2.2.8) and subsequent precipitation with ammonium acetate and ethanol (2.2.9).

The transgene DNA was injected into pronuclei of fertilised oocytes of the hybrid strain B6D2/F1. Viable injected oocytes were then transferred into oviducts of pseudopregnant foster mothers. The hybrid strain B6D2/F1 was created by a cross of a C57BL/6J (B6) female and a DBA/2 (D2) male mouse. B6D2/F1 mice are heterozygous for B6 and D2 alleles at all loci in their genome. Advantages of F1 hybrid strains over inbred strains are a larger litter size, thus increased fertility, higher resistance to disease, better survival under stress, longer life (information by The Jackson Laboratory, Bar Harbor, USA). Therefore the B6D2/F1 hybrid strain is often used for the generation of transgenic mice.
The DNA microinjection and implantation into foster mice was accomplished at the Biotechnologielabor (BTL) of the University of Heidelberg.

2.5.3 Animal breeding

The offspring of the foster mothers were analysed for integration of the transgene DNA into the chromosomal murine DNA. DNA isolated from tail biopsies (2.2.14) of 3-weeks-old animals was screened via PCR (2.2.7) and Southern blotting (2.2.17). Identified transgenic founder animals (B6D2/F1) were intercrossed with C57BL/6N mice to establish different lines, with regard to transgenic protein expression and activity. After 10 generations of backcrossing the genome will be >99% of C57BL/6N-genetic homogenous background and include the transgene allele. Thereupon the heterozygous mice can be crossed with each other to generate homozygous animals.

2.5.4 Preparation of mouse brain homogenates

After cervical dislocation, the mouse brain was dissected and snap frozen in liquid nitrogen and stored at –80°C. The frozen brain was homogenised in 2 ml TNE buffer (25 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA supplemented with 1 tablet protease inhibitor complete/50 ml TNE (Roche Diagnostics, Mannheim)) by use of a potter. After centrifugation (15 min, 1,000 x g, 4°C), the soluble substances contained in the supernatant of the brain homogenate were collected and stored at –20°C.

2.5.5 Preparation of mouse blood serum

Whole blood was collected from a tail vein of living mice or shortly after sacrifice by cardiac puncture. After self-coagulation of the blood for 2 h at room temperature or overnight at 4°C,

the serum was separated by centrifugation (10 min, 600 x g, room temperature) and stored at –20°C.

2.6 Sleep Studies on rats

Recording sleep-wake behaviour of rats, including surgeries and i.c.v. injections, were performed at the Washington State University, Department of VCAPP, College of Veterinary Medicine, Pullman (WA, USA), in the group of Prof. James M. Krueger, in the animal facility (Bustad building).

2.6.1 Animals

Male Sprague-Dawley rats weighing 300–380 g at the time of surgery were used. Rats were kept individually in Plexiglas cages in sound-attenuated, temperature-regulated environmental chambers at 23 ± 1°C on a 12-h light: 12-h dark cycle (light on at 5:00 a.m.). Water and food were available *ad libitum*. Institutional guidelines for the care and use of research animals were followed and approved by the Institutional Animal Care and Use Committee.

2.6.2 Surgery

All materials and tools used during surgery and injections were sterilised and autoclaved.

2.6.2.1 Anaesthesia

For surgery, the rats were anaesthetised by intraperitoneal (i.p.) injection of ketamine (87 mg/kg body weight) and xylazine mixture (13 mg/kg body weight). Breathing was controlled over the time of surgery. The rats were wrapped in a cloth to avoid hypothermia.

2.6.2.2 Implantation of EEG, EMG electrodes and i.c.v. cannula

After shaving the head, the rat was placed in a stereotaxic frame (David Kopf instruments) and fixed with earbars, toothbar and nosebar. The head skin was desinfected with 10% povidone-iodine topical solution followed by skin incision and extirpation of the skull membrane with scissors and skull scraper. For hemostasis, the tissue site of bleeding was burned with high temperature by a surgerical cautery and treated with the desinfectant hydrogen peroxide. These steps were repeated several times until bleeding had stopped.

Thereafter a 37% phosphoric Etchant gel was added to remove all traces of tissue and to make the skull bone rough. After 5 min the skull was cleaned with water. Stainless steel screw electrodes for electroencephalographic (EEG) recordings were implanted over the frontal and parietal cortices into holes of the skull burred with a dentist's drill (0.9 mm diameter drill tip) and by hand (1.4 mm diameter drill tip). See Fig. 6 for position of EEG electrodes. The EEG electrodes were screwed into the scalp in a depth of 1 mm from the skull surface. To insert electromyographic (EMG) electrodes, the skin was separated from the dorsal neck muscle and the EMG electrode wire wrapped around the muscle.

An intracerebroventricular (i.c.v.) cannula (PlasticsOne inc., 22 gauge) was inserted into the right lateral ventricle by using stereotaxic equipment. The guide cannula was implanted into a drilled hole at the coordinates 0.80 mm posterior, 1.8 mm lateral to the bregma, and 4.5 mm ventral from the surface of the skull, according to the rat brain atlas by Paxinos and Watson [210]. The size of the internal cannula (for injection procedure) was 30 gauge and extended 0.5 mm beyond the tip of the guide cannula. The guide cannula and the screws of the EEG electrodes were fixed to the skull with dental glue and dental cement. The EEG/EMG wires were connected to a pedestal pin (PlasticsOne inc.) and a round headpiece was built with dental cement covering all wires, fixing the i.c.v. guide cannula and the pedestal pin. The guide cannula was closed with a dummy cap to avoid contamination.

After surgery the rats were allowed to recover for at least 7–10 days.

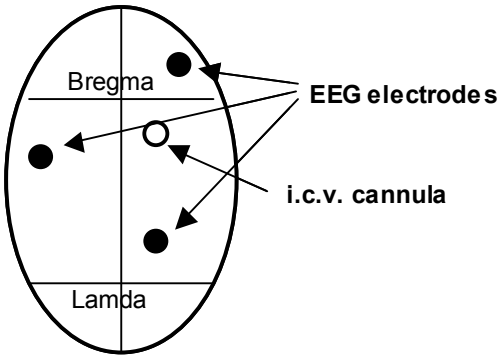

Figure 6 Schematic illustration of the positions of EEG electrodes and i.c.v. cannula onto the skull.

2.6.2.3 Verification of correct i.c.v. cannula placement

The correct i.c.v. cannula placement was determined by the gravity method (sudden drop in pressure) during implantation. Additionally 3–4 days after surgery the drinking response to i.c.v. injection of 200 ng angiotensin II [64] was tested. Only rats responding to angiotensin, verifying the correct position of the cannula, were used for experiments.

2.6.3 Injection procedure

The dummy cap was removed from the rat's head and the guide cannula was cleaned carefully with a 30-gauge needle. An internal cannula was connected to a 10-µl Hamilton syringe by a silicon tube. This injection tool was filled with sterile water followed by an air bubble before the injection substance solution was drawn up into the tube. The air bubble created a contact barrier between the injection substance and the water (Fig. 7). Thus, the syringe was only filled with sterile water without contaminations.

The internal cannula of this injection system was inserted carefully into the implanted guide cannula when holding the rat gently but firmly. A volume of 2–4 µl were i.c.v. injected within a 1-min period and left in place for an additional minute to allow the solution to flow completely into the ventricle. After removing the injection tool and replacing the dummy cap, the rat was connected to the EEG/EMG wires for recording.

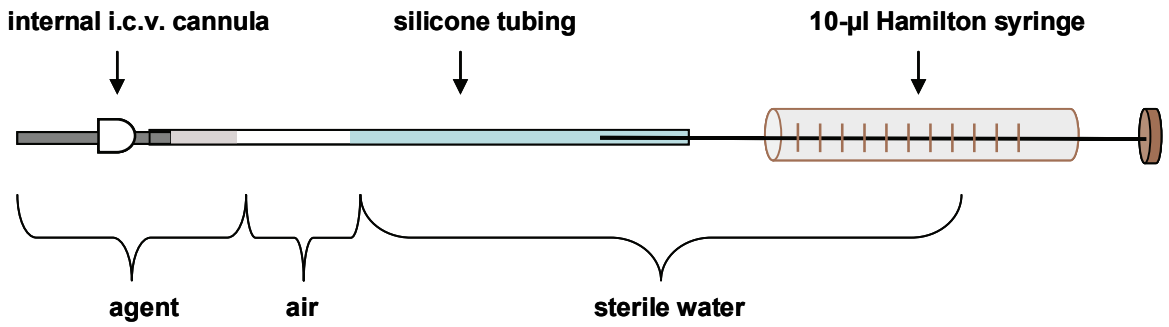

Figure 7 Scheme of the i.c.v. injection tool. For further information see 2.6.3.

2.6.4 Setup for sleep-wake recording

At least 3 days before the experiments of sleep and wake recording, the rats were connected to the recording cables for adaptation to the experimental conditions. The recording cables, plugged to communicators, were connected to amplifiers. The signals of EEG and EMG were converted from analog to digital at a sampling rate of 128 Hz and collected by computers. The EEG was filtered below 0.5 Hz and above 30 Hz. EMG activity aided to determine the vigilance state.

2.6.5 Experimental procedure of sleep-wake recording

Rats were injected i.c.v. with 2 µl pyrogen-free isotonic NaCl (vehicle) at dark onset (prior to the dark phase) following 23-h sleep-wake recording as baseline. The next experimental day the rats received an i.c.v. injection at dark onset of either 100 ng human Hyper-IL-6 (n=4) or 500 ng Hyper-IL-6 (n=4), in a volume of 2 µl. EEG and EMG signal recording started immediately after injections.

2.6.6 Body core temperature recording

For temperature measurement, an e-mitter without batteries (Vital View®) was surgically implanted into the peritoneal cavity. This was realised before implanting the EEG electrodes and i.c.v. cannula during the same surgery. After shaving the stomach and desinfection of the skin with 10% povidone-iodine topical solution, the skin, muscle and peritoneal membrane was cutted with a blade at a length of 1 cm. Then the sterile e-mitter was placed into the peritoneum and could freely moved e.g. within the intestinal loops. Thereafter the muscle and the skin was sutured (with Chromic Gut 12``), and the closed wounds were desinfected.
For temperature recording, the animal cage was placed onto a receiver plate (Vital View®) and the signal was routed to a computer.

2.6.7 Analysis of recorded EEG/EMG data

2.6.7.1 Sleep state scoring

The states of vigilance were visually scored off-line in 10-s epochs and classified as non-REM sleep (high voltage EEG waves, predominant EEG in delta range of 0.5–4.0 Hz, no body movement), REM sleep (low voltage EEG, dominance in fast theta power of 4.0–9.0 Hz, no body movement) or wakefulness (Wake; less regular low voltage fast EEG, frequent body movements) according to the criteria of Neckelman and Ursin [189]. The amount of time spent in each vigilance state was calculated for 2-h time blocks. The number of transitions from one vigilance to another served as indicator of sleep fragmentation. The programme “Sleep Sign” was used for analysis.

2.6.7.2 Spectral analysis

For spectral analysis (frequency analysis), EEG of each artefact-free 10-s epoch scored as non-REM or REM sleep was subjected to a Fast Fourier Transformation (FFT) analysis, yielding power spectra between 0.5 and 20 Hz with a 0.5 Hz resolution (“Sleep Sign”). EEG

power in REM and non-REM sleep was expressed as percentage of individual maximum EEG power in non-REM sleep to correct for individual differences in the absolute power [73].

2.6.7.3 Statistical analysis

Values are means ± S.E.M. For comparison of duration of each vigilance state and numbers of transitions between the baseline and experimental day, two-way analyses of variance (ANOVA) for repeated measures (factors: treatment and time effect) were calculated. A α-level of $P<0.05$ was considered to be significantly different. As post-hoc analysis the Tukey test was used. The same statistical analysis method was used for comparison of EEG power spectra (factors: treatment and frequency effect).

3 Results

3.1 Role of Hyper-IL-6 for sleep in rats

This chapter describes the experiments and results investigating the effect of intracerebroventricularly (i.c.v.)-injected Hyper-IL-6 on sleep-wake behaviour in rats. By means of Hyper-IL-6 (see 1.4), which activates almost all cells of the brain in contrast to IL-6 alone, the IL-6 trans-signalling pathway could be investigated exclusively.

3.1.1 Activity test of used cytokine samples

Since construction of the designer cytokine Hyper-IL-6 by Fischer *et al.* [70], several studies have indicated the specific binding to the receptor gp130, with no report showing cellular activation independent of gp130. In order to avoid non-specific side effects by the cytokine Hyper-IL-6 itself within the rat brain, its specificity was tested again before i.c.v. injection into rats with subsequent sleep recording. The biological activity of relevant Hyper-IL-6 samples was verified in a primary rat brain-derived cell-based analysis. Hyper-IL-6 is known to be a potent activator of STAT3-dependent gene transcription [221]. Therefore primary rat astrocytes and microglia cells (2.4.4) were treated with 50 ng/ml Hyper-IL-6 and IL-6 for 20 minutes after overnight starvation of confluent cells. Equal amounts of cell lysates (2.3.4) were applied to SDS-PAGE (2.3.1) and Western blot detection (2.3.2) of Y705-phosphorylated STAT3 proteins. The Western blot analysis (Fig. 8) revealed a specific strong reaction of primary rat astrocytes and microglia to Hyper-IL-6, presented as activation of transcription factor STAT3 by phosphorylation via gp130 stimulation. Unstimulated cells showed very week or not any phosphorylated STAT3 (negative control). IL-6, which only can signal through the membrane-bound IL-6 receptor, induced a very week signal intensity of phosphorylated STAT3 in the range of the unstimulated samples. This might point to a very low presence of the IL-6R on the membrane of rat astrocytes and microglia, if at all. As a loading control, STAT3 levels were determined showing comparable amounts of STAT protein in all samples.

In addition, after use of Hyper-IL-6 for the i.c.v. injection into rats, residual samples were retested for their biological activity in cell-based proliferation (viability) assays (2.4.3).

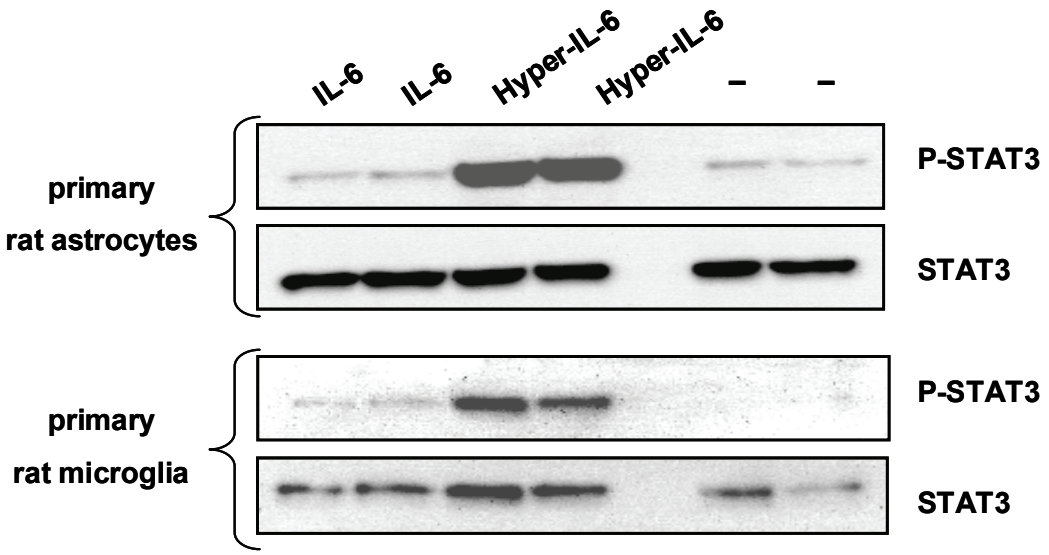

Figure 8 Detection of phosphorylated STAT3 (P-STAT3) after stimulation of primary rat astrocytes and microglia with Hyper-IL-6 and IL-6 (50 ng/ml each) via Western blotting. For comparison, STAT3 was detected. Unstimulated cells served as negative control.

The ability of Hyper-IL-6 to elicit proliferation of different Ba/F3 cell lines was utilised. The original Ba/F3 cell line, which is a murine peripheral blood pre-B cell line, grows IL-3-dependent. After stable transfection with human gp130 (Ba/F3-gp130 cells), cell growth has become dependent on IL-3 or Hyper-IL-6 [207]. Ba/F3 cells stably transfected with both gp130 and IL-6R are IL-3- or IL-6-dependent [79].

Ba/F3 cells that express gp130 on its membrane but not the IL-6R (Ba/F3-gp130 cells) and cells that express both gp130 and the membrane-bound IL-6R (Ba/F3-gp130-IL-6R cells) were cultured in presence of Hyper-IL-6 and IL-6 in different concentrations to monitor the cell viability after 3 days. Ba/F3-gp130 cells cannot respond to IL-6 alone and therefore they did not grow (Fig. 9A), because the lack of the membrane-bound IL-6 receptor excludes classic signalling. Hyper-IL-6, which only needs gp130 to bind to and to signal via IL-6 trans-signalling, maintained cell proliferation and thus viability of Ba/F3-gp130 cells. Ba/F3-gp130-IL-6R cells could respond to Hyper-IL-6 as well as to IL-6 (Fig. 9B). This proliferation assay revealed that the cytokine Hyper-IL-6 used for the rat sleep study was biologically active.

Taken together, these data again confirmed nicely the high specificity and wide reactivity of Hyper-IL-6.

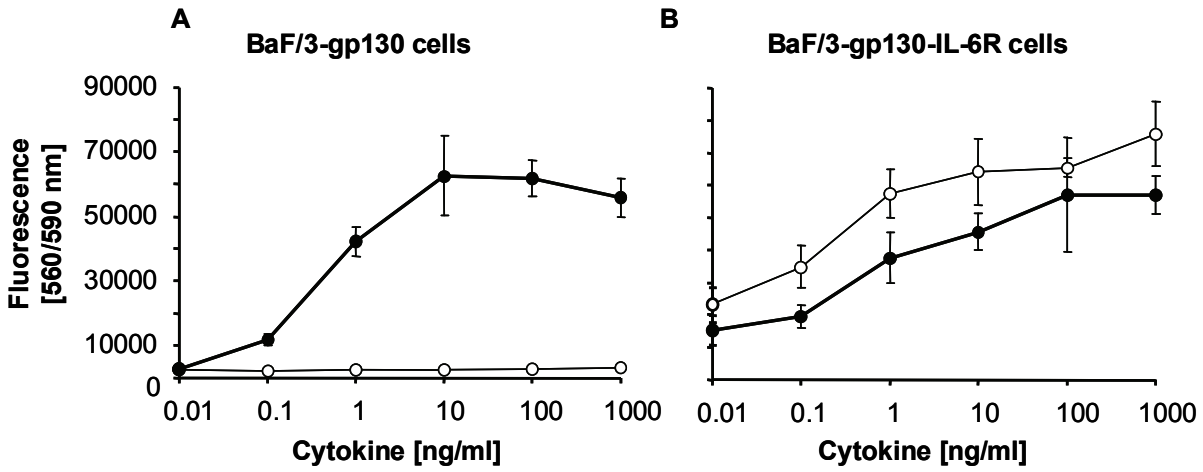

Figure 9 Proliferation assay measuring cell viability (as fluorescense) after stimulation of Ba/F3-gp130 cells (A) and Ba/F3-gp130-IL-6R cells (B) with Hyper-IL-6 (closed circles, thick line) and IL-6 (open circles, thin line). Samples were retested after use for i.c.v. injection into rats to ensure biological activity.

3.1.2 Effect of i.c.v. injection of Hyper-IL-6 on sleep-wake behaviour

In order to inject the cytokine Hyper-IL-6 into the brain of a rat, an i.c.v. cannula (22 gauge) was surgically inserted into the right lateral ventricle. EEG electrodes were implanted over the cortices and EMG electrodes in the dorsal neck muscle for sleep recording (2.6.2).

All operated animals reacted to i.c.v. administration of angiotensin II with a drinking response, thus proving the correct placement of the i.c.v. guide cannula, so that these rats could be used for experiments. The functionality of the implanted EEG/EMG electrodes to record brain waves and muscle activity respectively was tested successfully.

The study of Hogan *et al.* [99] (see 1.7) was used as a guideline, where IL-6 was injected before the dark period (dark onset) with subsequent EEG/EMG recording for 23–24 h. To investigate the effect of Hyper-IL-6, a comparable experimental set-up was chosen, which is illustrated in Fig. 10. After surgery, recovery and adaptation to conditions of connection with EEG/EMG recording wires in an environmental chamber, the rats were i.c.v. injected with 2 µl of saline shortly before the dark period (dark onset), followed by recording of EEG/EMG signals for 24 h (2.6.3; 2.6.4; 2.6.5). These signals represented the baseline sleep (for each individual rat). After 24 h of i.c.v. application of saline, Hyper-IL-6 was administered (i.c.v.), again at dark onset, and EEG/EMG recording succeeded for more than 24 h. Two different dosages of Hyper-IL-6 were analysed for their effect on sleep architecture. One group of 4

rats were treated with a low dose of 100 ng Hyper-IL-6 per rat and another group of 4 animals with a high dose of 500 ng/rat. In parallel the body core temperature was recorded, which is described in section 3.1.4.

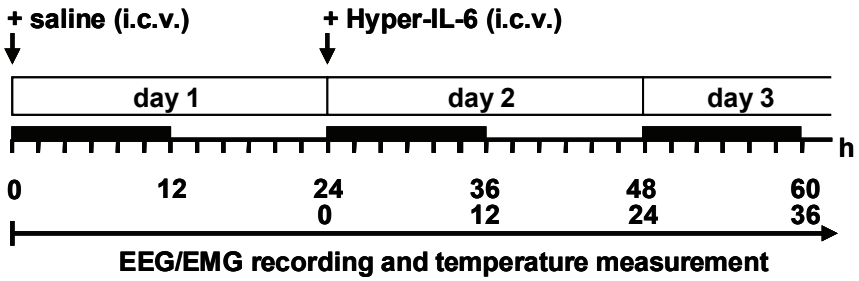

Figure 10 Scheme of experimental sleep study set-up. Black bars indicate dark period.

After i.c.v. exposure to the cytokine Hyper-IL-6, a modified or abnormal behaviour of the rats was not observed visually during the sleep study. Examples of recorded EEG/EMG waves are shown in Fig. 11.

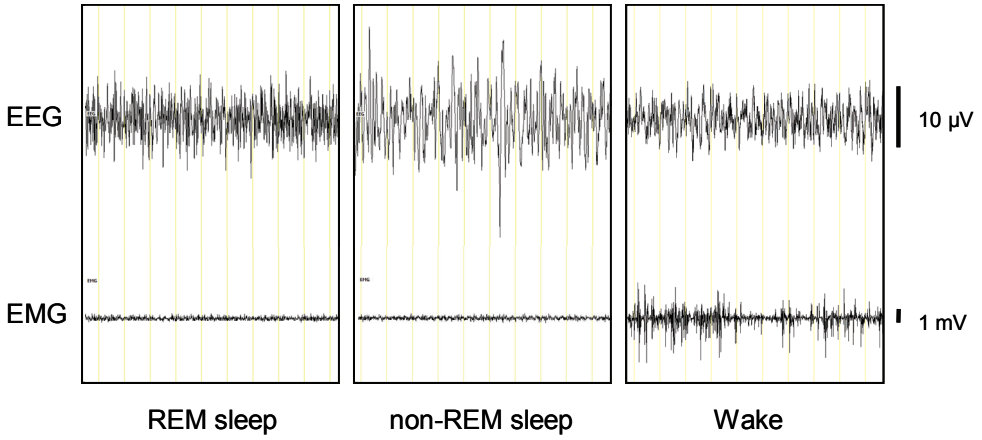

Figure 11 Example of a 10-s EEG/EMG record for each sleep-wake state. These signals were taken from an animal after i.c.v. injection of 100 ng Hyper-IL-6.

The EEG/EMG signals were scored in 10-s epochs to identify the amount of time the rats spent in each of the sleep states and the number of changes between different stages (transitions), calculated for 2-h time blocks, according to the specific patterns of electric brain signals (2.6.7.1). The resulting diagrams for a time period of 24 h after i.c.v. injection of saline and Hyper-IL-6 into rats (Fig. 12) revealed a clear circadian rhythm of the sleep-wake

behaviour. Thus, the dark period was dominated by physical activity, the light period by sleep. This is reflected in the highly significant ANOVA time effects (P<0.0001) within the recording time.

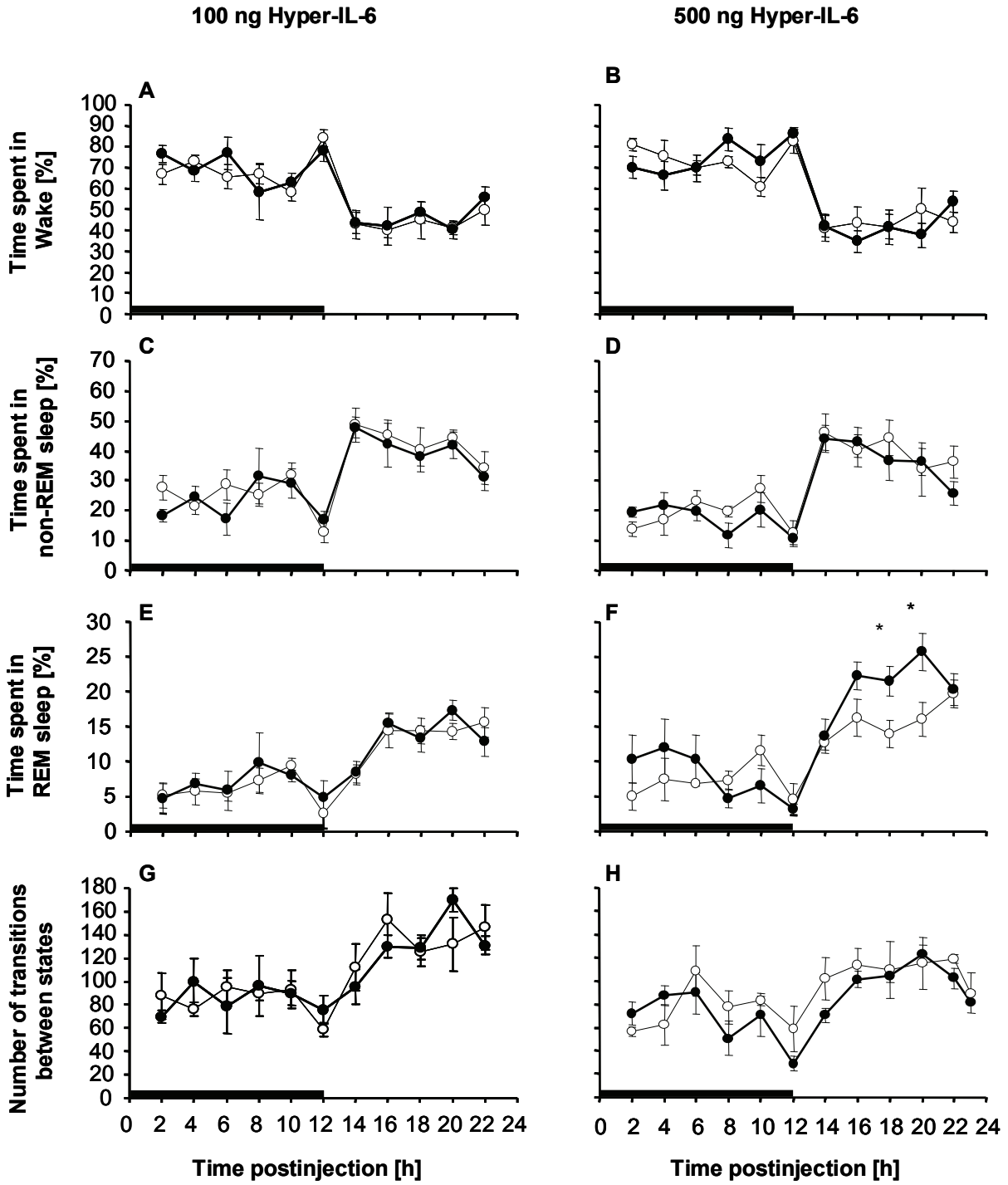

Figure 12 Effects of i.c.v. injection of 100 ng and 500 ng recombinant Hyper-IL-6 (closed circles, thick lines) and pyrogen-free isotonic saline (open circles, thin lines) into rats on Wake (A, B), non-REM sleep (C, D), REM sleep (E, F) and transition of states (G, H). Values are means ± S.E.M. (n=4). Asterisks denote significant treatment effect. Horizontal black bars indicate the dark period.

The i.c.v. administration of the low dose of Hyper-IL-6 (100 ng) did not alter sleep in terms of Wake (Fig. 12A), non-REM sleep (Fig. 12C), REM sleep (Fig. 12E) and transitions between the states (Fig. 12G). Treatment of 500 ng Hyper-IL-6 did not change the amounts of Wake (Fig. 12B) and non-REM sleep (Fig. 12D), but significantly increased the amount spent in REM-sleep (Fig. 12F; ANOVA results: treatment effect, $F(1,33)=7.559$, $P=0.0096$; interaction "treatment x time", $F(10,33)=2.015$, not significant). For the time points 18 and 20 h during the light period, the post-hoc performed Tukey test (multiple pairwise comparisons of treatments) for each 2-h time blocks revealed statistical increase. The transitions between sleep states after the application of 500 ng Hyper-IL-6 did not statistically increase (Fig. 12H; ANOVA results: treatment effect, $F(1,36)=2.907$, $P=0.0968$; interaction "treatment x time", $F(11,36)=0.8848$, not significant).

The question arose whether the observed REM sleep-promoting effect of 500 ng Hyper-IL-6 continued in the second dark period of the following day (24–34 h postinjection). Therefore the contributing EEG data were scored (Fig. 13) and these 12 h of the second dark were compared with the first 12 h after saline injection, since EEG after saline treatment was recorded only for one day. The amount of REM sleep of the second dark period after 500 ng Hyper-IL-6 i.c.v. injection into rats did not differ statistically from saline baseline (ANOVA results: treatment effect: $F(1,30)=0.163$, not significant; interaction "treatment x time": $F(5,30)=0.83$, not significant). The same finding applied to non-REM sleep and transitions between the states (data not shown).

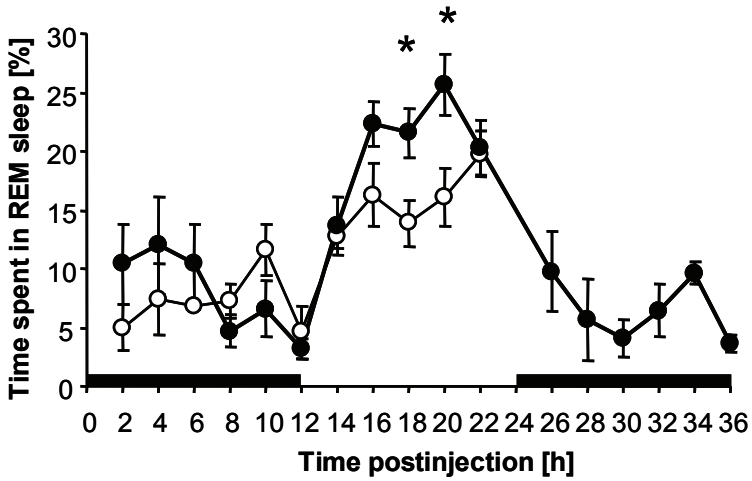

Figure 13 Effects of i.c.v. injection of 500 ng Hyper-IL-6 (closed circles, thick line) and saline (open circles, thin line) into rats on REM sleep including the second dark period. Values are means ± S.E.M. (n=4). Asterisks denote significant treatment effect. Horizontal black bars indicate the dark period.

3.1.3 Effect of Hyper-IL-6 on EEG power spectrum

EEG signals consist of many different frequencies in various amounts. A power spectral analysis shows how the power of an EEG wave (power density) is distributed with frequency. Power density differs among sleep states and varies over a wide range of frequencies [73]. EEG signal epochs defined as non-REM sleep show a higher power density of the delta frequency band (0.5–4 Hz); REM sleep waves of the theta frequency band (5–9 Hz). A spectral analysis (2.6.7.2) of 24-h EEG signals derived from rats receiving an i.c.v. injection of 500 ng Hyper-IL-6 and saline respectively is depicted in Fig. 14, determined for light and dark period separately. It displayed no effect of Hyper-IL-6 on EEG spectra for non-REM sleep (Fig. 14A). For REM sleep no shift in theta peak frequency was present but a significantly reduced relative power density (Fig. 14B; ANOVA results: treatment effect, $F(3,126)=17.09$, $P<0.0001$; frequency effect, $F(20,126)=6.173$, $P<0.0001$, interaction "treatment x frequency", $F(60,126)=0.7095$, not significant).

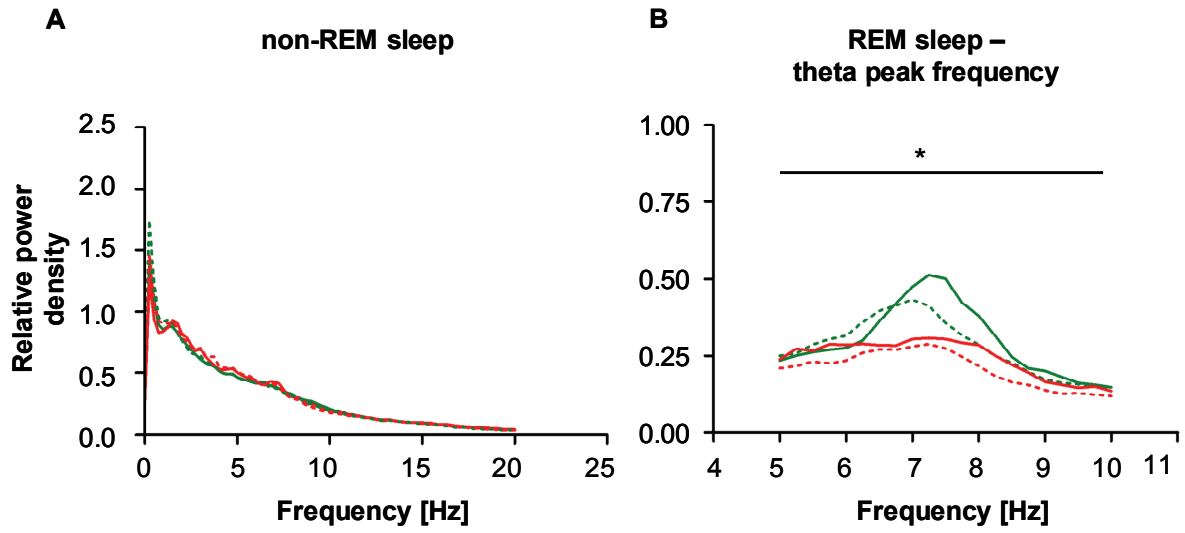

Figure 14 Effect of i.c.v. injection of 500 ng Hyper-IL-6 (red line) and pyrogen-free isotonic saline (green line) into rats on EEG power spectra obtained from non-REM (A) and REM epochs (theta peak frequency, B) shown for light (dashed line) and dark period (solid line) respectively. Mean values are shown. The black bar with the asterisk indicates a significant treatment effect (ANOVA).

3.1.4 Effect of Hyper-IL-6 on body core temperature

As the cytokine IL-6 has been shown to be pyrogenic [99, 142, 202, 236], it was tested if Hyper-IL-6 also was able to induce fever. The body core temperature was measured by a so-called e-mitter that was surgically placed into the peritoneal cavity (2.6.6). After i.c.v. injection of 500 ng Hyper-IL-6, the body temperature did not significantly differ from saline treatment (Fig. 15).

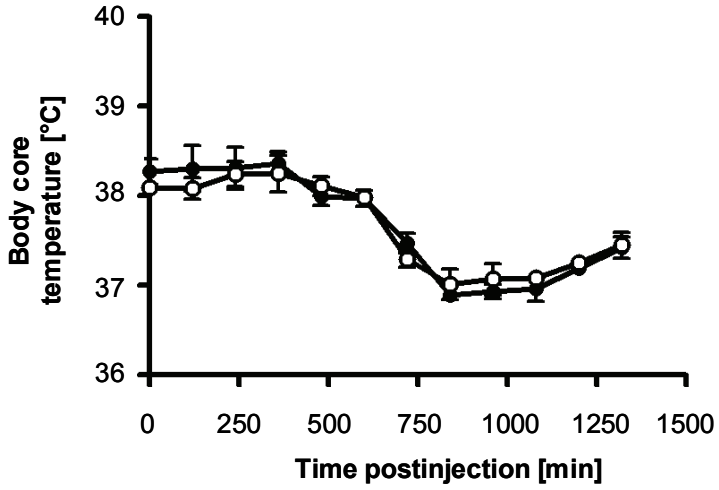

Figure 15 Body core temperature after i.c.v. injection of saline (open circles) and 500 ng Hyper-IL-6 (closed circles) into rats (n=4). Values are means ± S.E.M.

3.2 Central blockade of IL-6 trans-signalling in gfa2–sgp130-Fc transgenic mice

This chapter involves the generation and first characterisation of mice, transgenic for the antagonistic protein of the IL-6 trans-signalling pathway sgp130-Fc (see 1.4) under the brain-specific promoter gfa2.

3.2.1 Cloning of the transgene vector for gfa2–sgp130-Fc mice

To generate transgenic mice expressing sgp130-Fc within the brain, sgp130-Fc was set under transcriptional control of the human gfa2 promoter. Gfa2 is a 2.2 kb promoter fragment of the glial fibrillary acidic protein (GFAP) that is specifically active in astrocytes. It is activated from embryonic day 12.5 [29]. Genes under control of the human gfa2 promoter were successfully used in the past to generate various different transgenic mouse strains [30], including TGFβ [77] and tau [71].

The cDNA for sgp130-Fc was codon-optimised by the company GENEART (Regensburg) to achieve high and stable expression rates [220]. Thus, e.g. only the most frequent codons of the mammalian genome appeared in the cDNA.

For construction of an appropriate sgp130-Fc DNA vector, the expression vector pGfa2–LacZ–mP1 [29] served in a first approach as a backbone (Fig. 16). The lacZ cDNA was removed by restriction with the enzyme *Bam*HI and replaced with the sgp130-Fc cDNA, which was excised before with *Bgl*II from the vector p409–sgp130-Fc (Jürgen Scheller), resulting in the expression vector pGfa2–sgp130-Fc_optimised–mP1. This was possible since *Bgl*II and *Bam*HI produce compatible sticky ends (5'-GATC-3'), but these endonuclease recognition sites were destroyed after ligation. A segment of murine protamine 1 (mP1) including an intron for efficient expression [32] and a polyadenylation site (PolyA) that enhance mRNA stability [238], was located downstream of the sgp130-Fc cDNA.

In a second cloning approach, the sgp130-Fc-containing vector pCR-script–sgp130-Fc_optimised (GENEART, Regensburg) served as backbone plasmid (Fig. 17). The cDNAs for the polyadenylation site and the second intron of rabbit β-globin were amplified via PCR from the vector pTZ–PEPCK–βglob.intron–opt_sgp130-Fc [220] with addition of the appropriate endonuclease restriction sites for insertion into the plasmid pCR-script–sgp130-Fc_optimised as shown detailed in Fig. 17. The *Bgl*II/*Bam*HI fragment derived from the vector pGfa2–LacZ–mP1 [29] comprising the gfa2 promoter was ligated into the *Bgl*II opened vector pCR-script–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin, with loss of the

*Bam*HI/*Bgl*II restriction sites between promoter and intron. In the resulting expression plasmid pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin, the intron was located upstream of the sgp130-Fc-encoded sequence. This location of an intron should yield to a more potent increased expression of the gene [209]. Also this type of composition of a transgene expression cassette provided an optimal expression efficiency for the vector pTZ–PEPCK–βglob.intron–opt_sgp130-Fc used for generation of sgp130-Fc transgenic mice with systemic protein expression [220].

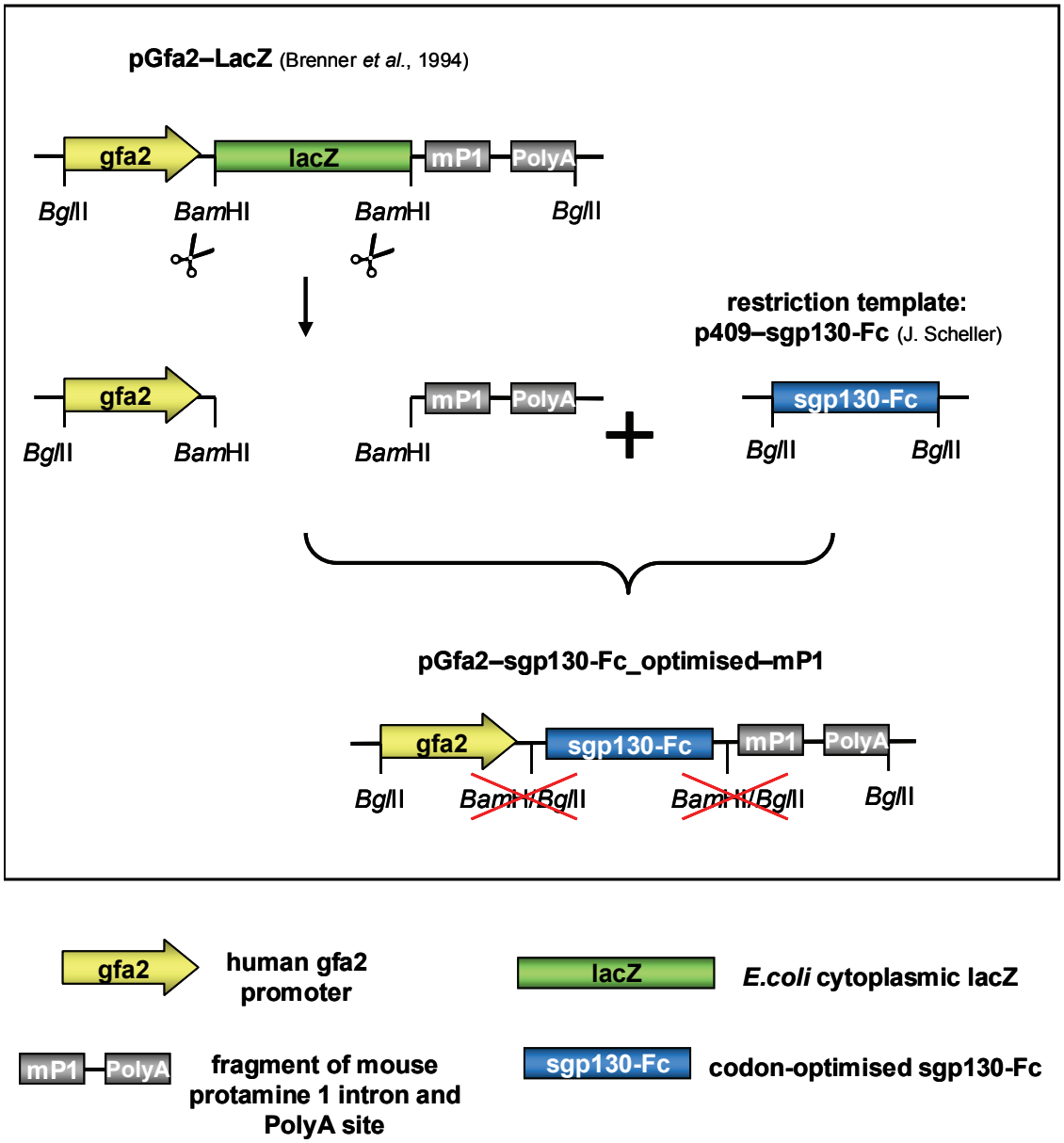

Figure 16 Cloning scheme of the vector pGfa2–sgp130-Fc_optimised–mP1. The intron is located downstream of the sgp130-Fc cDNA.

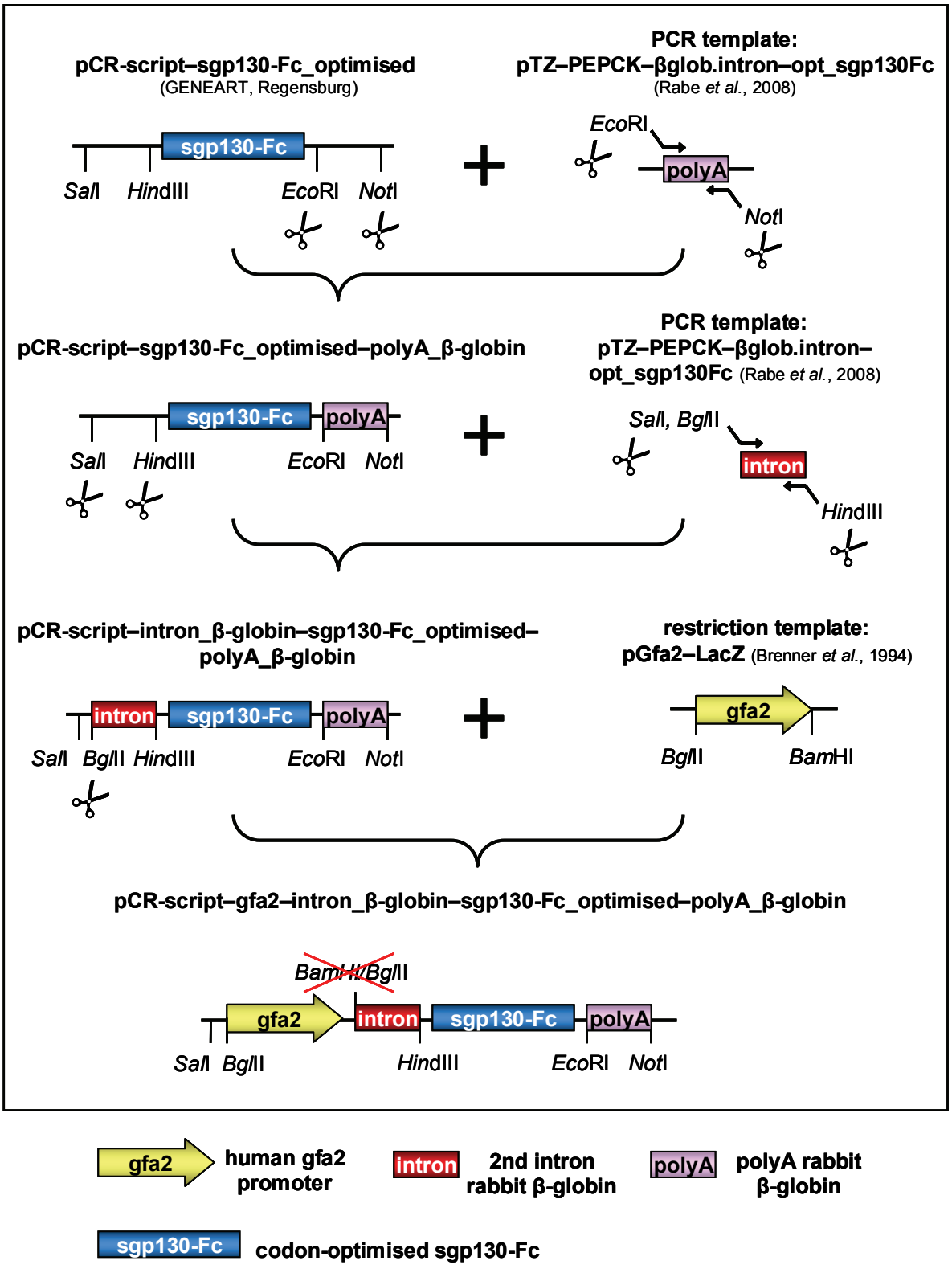

Figure 17 Cloning scheme of the vector pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin. The intron is located upstream of the sgp130-Fc cDNA.

3.2.2 Protein expression of sgp130-Fc in cell culture

Both expression vectors, pGfa2–sgp130-Fc_optimised–mP1 and pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin, were transiently transfected (2.4.2) into U251 cells to test the strength of sgp130-Fc expression *in vitro*. U251 is a human astroglioma cell line that provided an ideal model for the astrocyte-specific GFAP promoter gfa2-driven sgp130-Fc protein expression. As a control the plasmid pmax-GFP (for green fluorescent protein) was transfected with about 50% transfection efficiency. Given that sgp130-Fc is a soluble, secreted protein, the cell supernatants 3 days after transfection were subjected to immunoprecipitation with protein A sepharose (2.3.3), SDS-PAGE (2.3.1) and Western blot analysis (2.3.2). Protein A sepharose has the ability to bind Fc-portions. Sgp130-Fc was detected by the antibody B-P4 at a size of 130 kDa in the Western blot (Fig. 18), where recombinant purified sgp130-Fc served as a positive control. The vector that located the intron upstream of sgp130-Fc (pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin) showed stronger expression rates than the vector with the intron cloned downstream of sgp130-Fc (pGfa2–sgp130-Fc_optimised–mP1). Hence, the expression vector pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin was chosen for the generation of gfa2–sgp130-Fc transgenic mice.

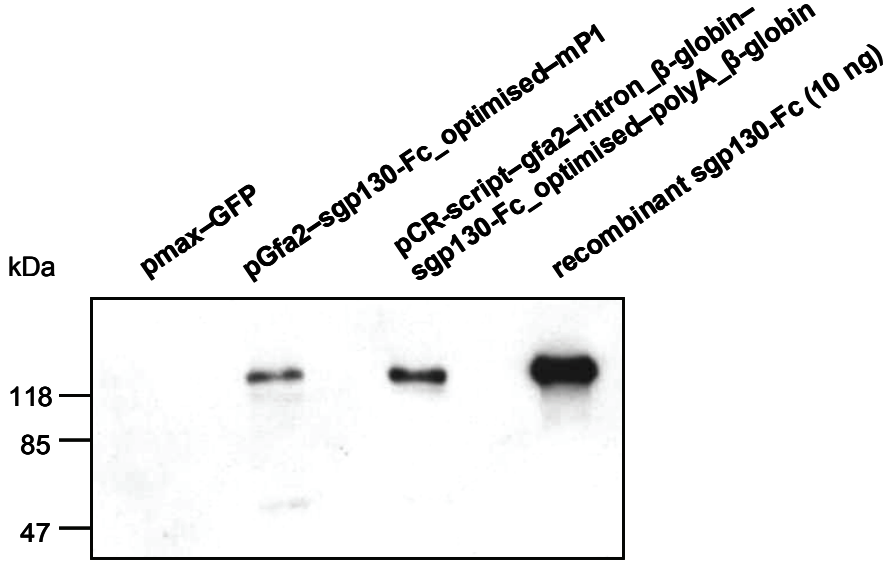

Figure 18 Protein expression of sgp130-Fc in U251 astroglioma cells. Transfected cells with different vector constructs were detected in medium supernatants after immunoprecipitation with protein A sepharose via Western blotting using the anti-gp130 antibody B-P4.

3.2.3 Generation of astrocyte-specific gfa2–sgp130-Fc transgenic mice

3.2.3.1 DNA microinjection into pronuclei

The gfa2–sgp130-Fc expression cassette, illustrated in Fig. 19A and B, was restricted by *Bgl*II and *Not*I from the vector pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin. This transgene-coding DNA fragment was purified and injected into pronuclei of fertilised mouse oocytes as described in 2.5.2. A total of 328 oocytes were transferred into 12 foster mothers, which gave birth to 151 alive animals. The offspring were then analysed for integration of the transgene cDNA cassette into the genome to identify founder animals.

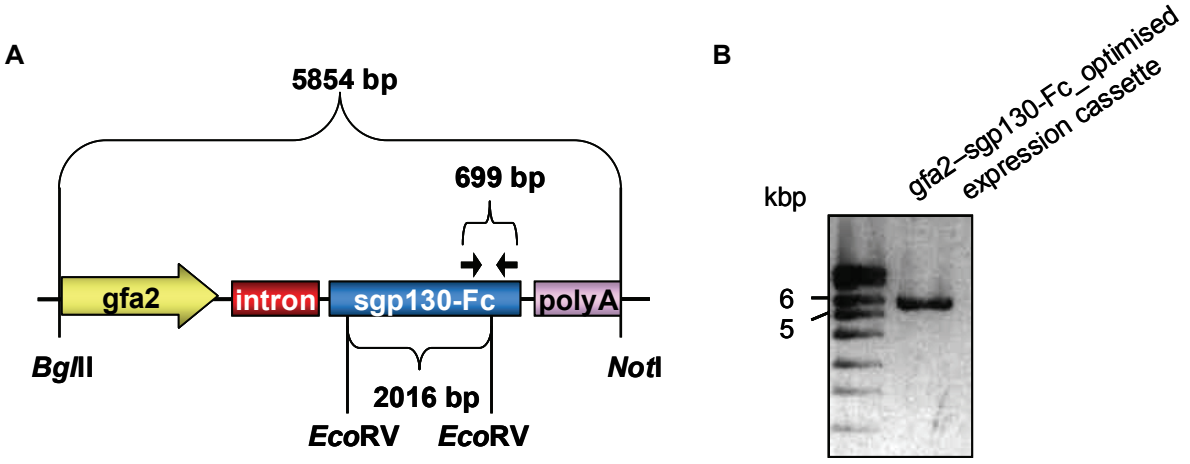

Figure 19 Organisation of the gfa2–sgp130-Fc_optimised cDNA expression cassette with all relevant regions, restriction and primer binding sites (A). The *Bgl*II/*Not*I restricted fragment of the expression vector (pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin) shown on a 1% agarose gel contained the gfa2–sgp130-Fc expression cassette for generation of transgenic mice (B).

3.2.3.2 Identification of founder animals transgenic for sgp130-Fc

A transgene integrates randomly into the mouse genome mostly at a single site with numerous copies as concatemers (up to 50 or more) [31, 48, 208]. The genomic mouse DNA (2.2.14), isolated from tail biopsies of the offspring of the foster mothers, was genotyped via PCR (2.2.7) and Southern blotting (2.2.17). The PCR screening, using sgp130-Fc-screen primers (2.1.4) that span 699 bp between sgp130 and the Fc-part of the transgenic DNA construct, identified 18 transgenic founder animals (Fig. 20). Founder mice were heterozygous for sgp130-Fc under transcriptional control of the brain-specific gfa2 promoter. As a positive control DNA of a transgenic sgp130-Fc mouse under control of the PEPCK promoter (systemic expression) [220] was used.

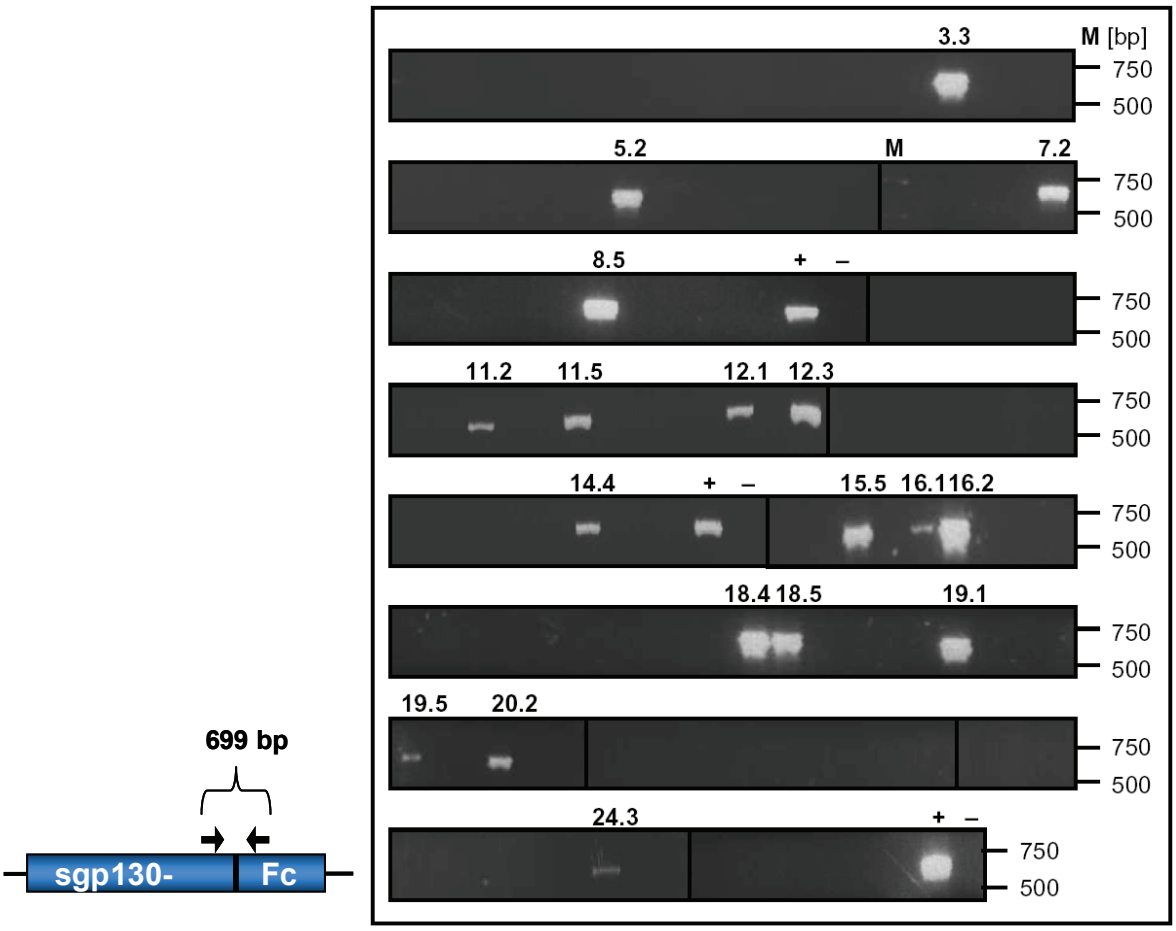

Figure 20 Identification of gfa2–sgp130-Fc founder animals via PCR analyses and separation on a 1.5% agarose gel. The offspring of 151 mice by 12 foster mothers resulting from DNA pronucleus microinjection were tested. Sgp130-Fc-specific primers amplified a 699 bp product (scheme at the bottom, left). Numbers designate founder names. M indicate the DNA marker (1 kb Generuler). The symbol + denotes the positive control, – the negative water control.

The PCR-based data of identified founder animals were additionally verified by Southern blot analysis, as presented in Fig. 21. The α-^{32}P-dATP-labelled probe contained a sgp130-Fc cDNA fragment of 2,016 bp, resulting from the restriction of the gfa2–sgp130-Fc_optimised expression cassette with the endonuclease *Eco*RV (see Fig. 19A). For 14 out of 18 founder mice the integration of the sgp130-Fc transgene could be confirmed. The differences in signal intensity generally reflect the different copy numbers of the transgene integrated into the genome.

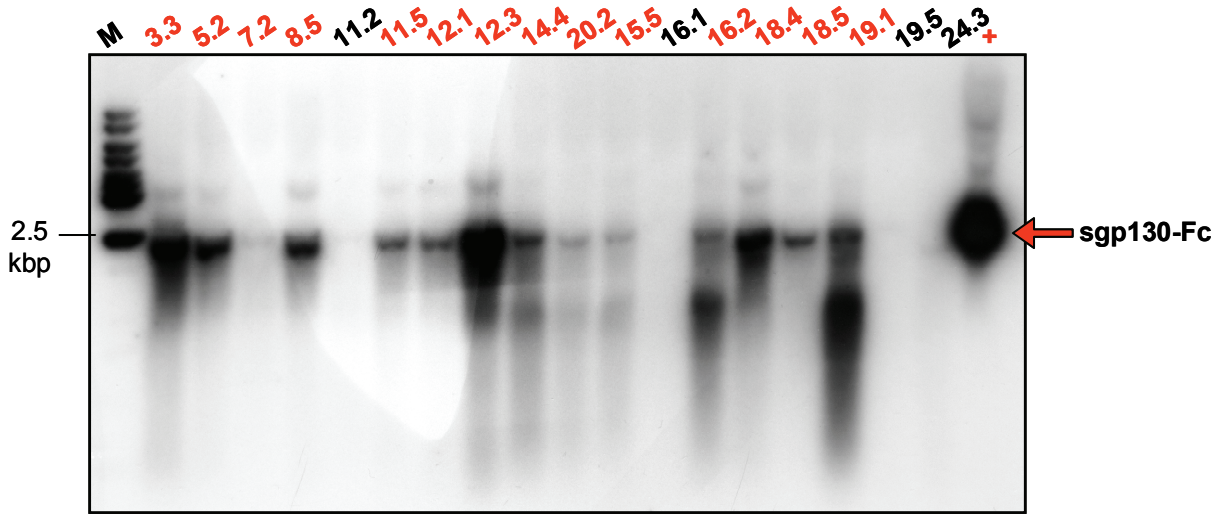

Figure 21 Verification of gfa2–sgp130-Fc founder animals via Southern blot analysis. Ten micrograms of genomic DNA were digested with *Eco*RV, loaded on an agarose gel, transferred onto a nylon membrane and hybridised with a radioactive-labelled probe consisting of sgp130-Fc cDNA (2,016 bp). Founder mice are designated as numbers. Positive signals are marked in red. M indicate the DNA Marker (1 kb Generuler), + the positive control, – the negative water control.

3.2.3.3 Backcrossing and general genotyping

Founder mice were crossbred onto a C57BL/6N background (2.5.3) to establish different transgenic lines. Backcrossing is mandatory to produce animals at a genetic homogenous background, since the offspring after pronucleus microinjection (founder mice) belonged to the hybrid strain B6D2/F1, which is a cross of C57BL/6 and DBA/2 (2.5.2). Foremost after 10 generations the genome will have reached a >99% C57BL/6N-specific background.

Because at least 14 animals had been identified as founder mice (3.2.3.2), the number of lines was reduced. Lines were excluded that produced no or a low litter size after the first backcross and/or did not express the sgp130-Fc protein within the brain in relatively high amounts (see 3.2.4.1). Finally, the transgenic lines designated as 5.2, 15.5 and 19.1 were continued.

The offspring was genotyped to identify transgenic animals via the following scheme in Fig. 22. First the quality of the isolated genomic mouse DNA (2.2.14) was checked in a PCR (2.2.7) for the housekeeping gene β-globin (494 bp). The transgene-specific PCR was performed using the "sgp130-Fc-screen" primers that produced a 699 bp product or, from backcross 5 on, with the "sgp130-Fc-screen-new" primers resulting in a 176 bp product. Both primer pairs span the boarder of sgp130 and the Fc-part of the cDNA. Untill backcross 3 the PCR results were additionally verified via Southern blotting (2.2.17) using α-^{32}P-dATP-

radioactive-labelled sgp130-Fc-specific probe of the *Eco*RV restricted gfa2–sgp130-Fc_optimised cDNA fragment (2,016 bp; see Fig. 19A). While writing this thesis the backcross was progressed to number 8.

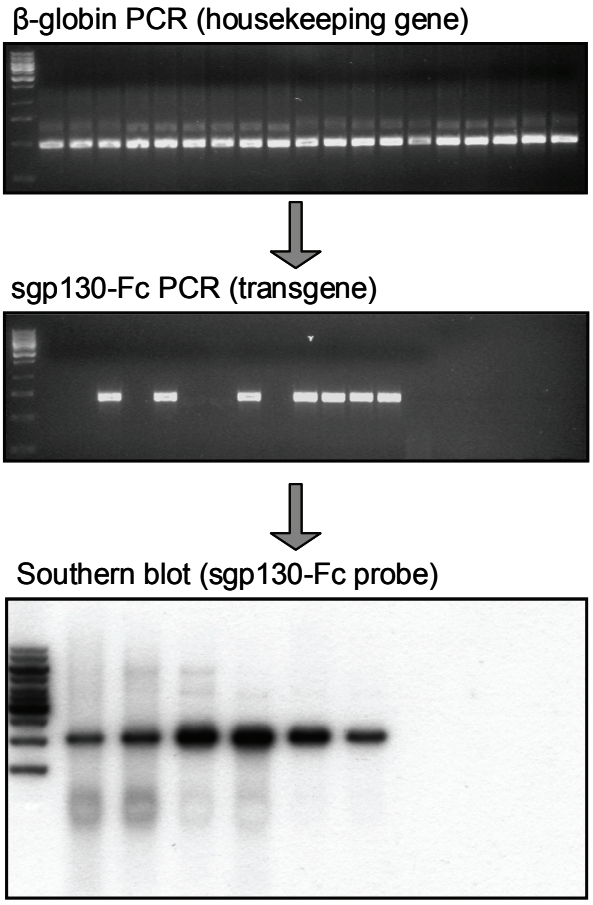

Figure 22 General genotyping scheme for the identification of gfa2–sgp130-Fc transgenic mice. See 3.2.3.3 for details.

Identification of homozygous mice, which e.g. could be used for the preparation of primary astrocytes, was determined by Southern blot analysis comparing the relative copy number of the integrated transgene between transgenic littermates. While heterozygous animals carry the transgene on one allele, homozygous mice contain the transgene on both alleles. This fact is reflected in signal intensity twice as strong for a homozygous littermate as for a heterozygous on the Southern blot, as far as equally amounts of genomic DNA have been loaded onto the agarose gel. The identification of homozygous sgp130-Fc mice is represented in Fig. 23.

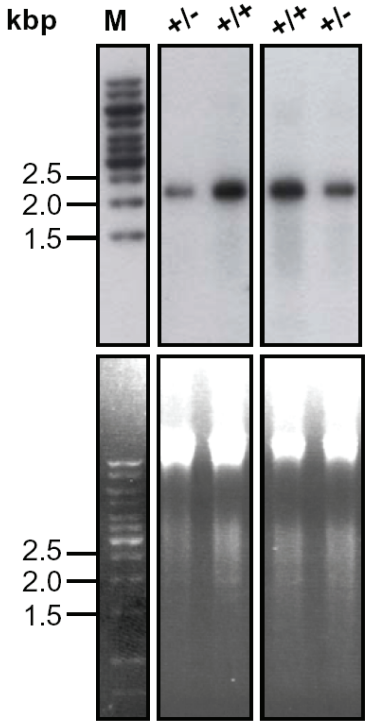

Figure 23 Identification of homozygous transgenic gfa2–sgp130-Fc mice via Southern blotting (upper panels) shown for 2 animals of 2 different litters. The correspondent agarose gel (lower panels) displayed that equal genomic DNA amounts were loaded. Symbol M indicates the 1kb Generuler, +/- indicates heterozygous, +/+ indicates homozygous animals.

3.2.4 Expression analyses of sgp130-Fc in transgenic gfa2–sgp130-Fc mice

3.2.4.1 Brain-specific expression of transgenic sgp130-Fc on protein level

Sgp130-Fc expression is targeted specifically to astrocytes by use of the gfa2 promoter. Detection of a transgene on DNA level does not necessarily lead to strong protein expression. Therefore the protein expression of sgp130-Fc within the brain of founder animals was examined. For this purpose it was ensured that the founder animals produced sufficient transgenic offspring, since the founder mice had to be sacrificed for production of whole brain homogenates. The brain was homogenised in a volume of 2 ml TNE buffer (2.5.4), and 10 µl of the soluble fraction was applied to SDS-PAGE (2.3.1) followed by detection of sgp130-Fc protein (130 kDa) in Western blot analysis (2.3.2) with the monoclonal antibody B-P4 raised against the human gp130. The highest protein amount was detected in founder animals 19.1, 12.3 and 5.2, a moderate sgp130-Fc content in founders 5.2, 8.5 and 14.4, and a lower concentration in founder 18.5 (Fig. 24). All those protein bands correlated with the DNA band intensities of the transgene seen by Southern blotting, thus with their copy numbers (see

Fig. 21). Sgp130-Fc protein expression within the brain was absent in the founder mice 11.5 and 18.4, but by contrast they showed moderate and high copy numbers of the transgene in Southern blot analysis. Interestingly, founder 15.5 that displayed an obvious low copy number, expressed rather a high protein amount of sgp130-Fc.

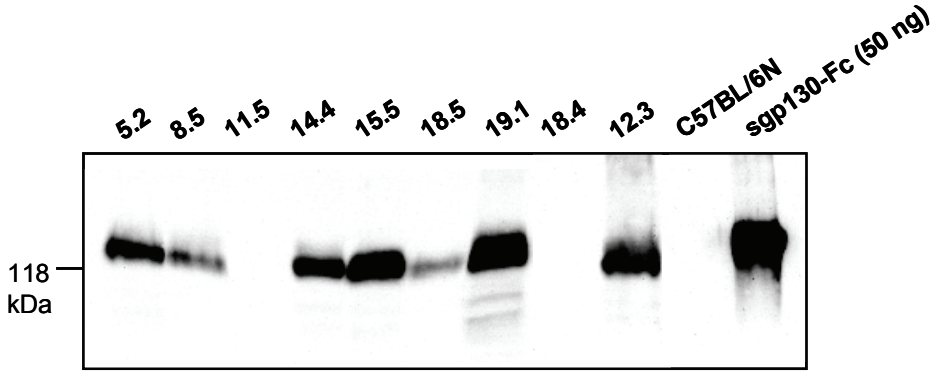

Figure 24 Detection of sgp130-Fc proteins in brain homogenates of different founder mice (designated as numbers) via Western blot analysis using the monoclonal anti-gp130 antibody B-P4. The wildtype C57BL/6N mouse did not express sgp130-Fc (negative control). Recombinant sgp130-Fc was included as positive control.

In addition, the amount of sgp130-Fc protein in brain homogenates was quantified by gp130 sandwich ELISA (2.3.5, Fig. 25), which confirmed the Western blot results. The highest protein levels of sgp130-Fc over 1 µg/ml of brain homogenate were measured in the founder mice 19.1 and 12.3. The founder mice 5.2 and 15.5, which expressed sgp130-Fc at about 750 ng/ml of brain homogenate, were chosen additionally to founder 19.1 to establish different mouse lines. During the backcross breedings, the presence of sgp130-Fc was continuously analysed to avoid production of subsequent generations without transgenic protein production. Expression of transgenic proteins may vary through generations up to cessation of activity [208].

The levels of the endogenous murine sIL-6R (msIL-6R), the crucial component of IL-6 trans-signalling that should be inhibited by sgp130-Fc, were determined by msIL-6R sandwich ELISA (2.3.5) of brain homogenates of some founder animals. Founder mice displayed amounts of endgenous msIL-6R in the range of 10–15 ng/ml brain homogenate (Fig. 26).

Blockade of IL-6 trans-signalling strictly relies on a molar excess (at least 10-fold) of sgp130-Fc [115]. Therefore the molar ratios of sgp130-Fc versus msIL-6R were calculated from their protein levels measured by ELISA (Fig. 25, Fig. 26). The molar values are shown in Table 1.

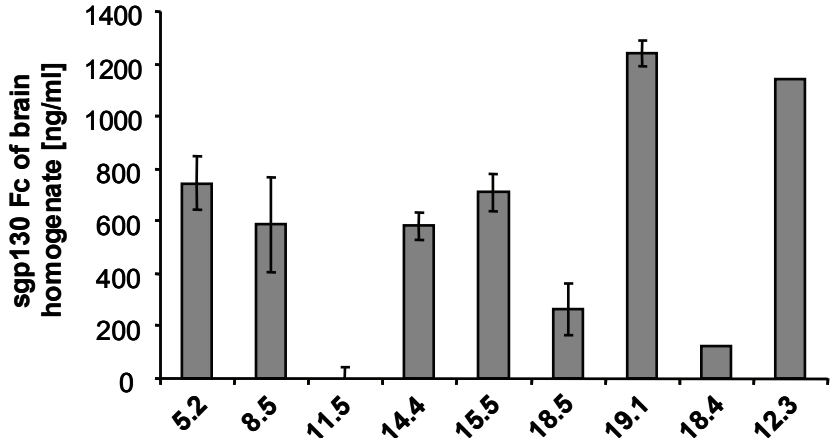

Figure 25 Sgp130-Fc protein levels in brain homogenates of different founder mice (designated as numbers on x-axis) measured via gp130 ELISA. Data represent mean values ± S.D. Values were measured in quadruplets; but 18.4 and 12.3 in duplets.

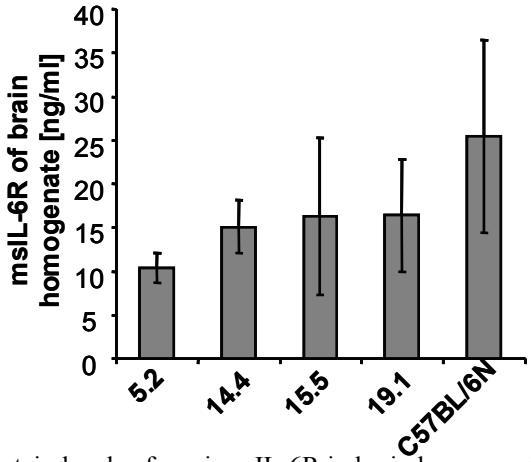

Figure 26 Endogenous protein levels of murine sIL-6R in brain homogenates of founder mice (designated as numbers on x-axis) and wildtype C57BL/6N measured via msIL-6R sandwich ELISA. Data represent mean values ± S.D. Values were calculated of 3 different sample dilutions.

Table 1 Protein levels of sgp130-Fc in brain homogenates of gfa2–sgp130-Fc founder mice measured via gp130 ELISA and calculated molarities. Shown are mean values. Protein levels were measured in quadruplets; but 18.4 and 12.3 in duplets. Molar relations of sgp130-Fc to msIL-6R were calculated for 15 ng/ml msIL-6R (1 M sgp130-Fc=130,000 g/l; 1 M sIL-6R=80,000 g/l). See 3.2.4.1 for further details.

founder	**5.2**	**8.5**	**11.5**	**14.4**	**15.5**	**18.5**	**19.1**	**18.4**	**12.3**
sgp130-Fc [ng/ml]	745.71	588.91	0	581.49	710.62	263.47	1241.98	121.72	1145.08
sgp130-Fc [mol/l]	5.736×10^{-9}	4.530×10^{-9}	0	4.473×10^{-9}	5.466×10^{-9}	2.027×10^{-9}	9.554×10^{-9}	9.363×10^{-10}	8.808×10^{-9}
molar factor sgp130-Fc : msIL-6R	30.59	24.16	0	23.86	29.15	10.81	50.95	4.99	46.98

For calculation of the molar relation of sgp130-Fc versus msIL-6R in each founder animal, an endogenous level of msIL-6R within brain homogenates at 15 ng/ml (1.875×10^{-10} M) measured by ELISA (Fig. 26) was considered to be present in all transgenic brain samples. Thus, theoretically this amount of msIL-6R could be bound by IL-6, which has to be inhibited by sgp130-Fc. Table 1 revealed a 10- to 50-fold molar excess of sgp130-Fc within brain homogenates of the various founder mice, with exception of the founders 11.5 and 18.4 that expressed only low or no levels of transgenic protein.

Because sgp130-Fc is a soluble protein, its secretion by transgenic astrocytes derived from heterozygous mice was examined. For isolation of primary astrocyte cultures, a newborn transgenic mouse pub was needed. Therefore heterozygous mice were bred to homozygousity (see Fig. 23). As the identified homozygous animals were both male, one was mated with a female wildtype C57BL/6N mice resulting in a sgp130-Fc heterozygous uniform litter (line 14.4, 2nd backcross). Two-days-old (P2) animals were dissected to isolate cortical astrocytes for primary cultures (2.4.4). The primary transgenic astrocytes grew 1 week in 75 cm²-flasks in medium either with or without FCS, before the supernatant/medium was used for SDS-PAGE (2.3.1) followed by Western blot detection (2.3.2) of secreted sgp130-Fc (130 kDa) via the anti-gp130 antibody B-P4. Supernatant was used directly (30 µl) or after immunoprecipitation (IP) with protein A sepharose that binds to the Fc-part of sgp130-Fc (1 ml medium for IP, 50% loaded onto SDS gel, 2.3.3). Additionally, as positive controls, cell lysate (2.3.4) of transgenic astrocytes (approximately 500,000 cells) and recombinant purified sgp130-Fc was used. Medium of primary wildtype rat astrocytes after IP served as negative control. The Western blot analysis (Fig. 27) clearly showed the protein expression of sgp130-Fc and secretion into the medium by primary cortical astrocytes of transgenic gfa2–sgp130-Fc mice.

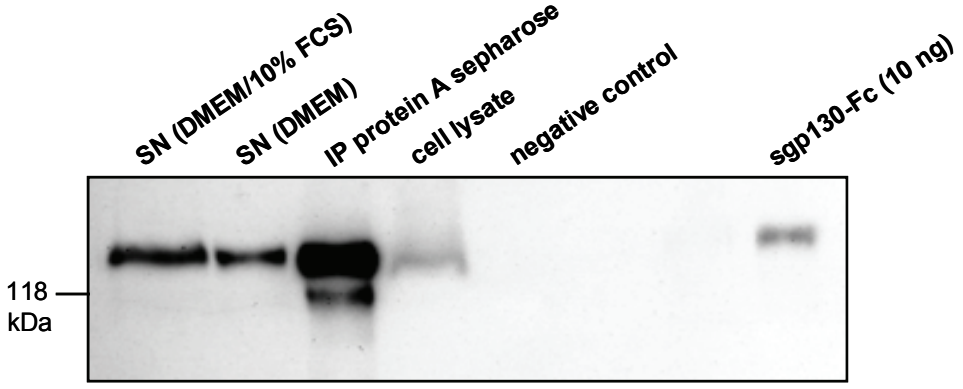

Figure 27 Secretion of sgp130-Fc by transgenic murine primary astrocytes (line 14.4, 2nd backcoss) detected via Western blotting using the monoclonal anti-gp130 antibody B-P4. Medium supernatants (SN) were used directly or after IP with protein A sepharose. As negative control served medium of primary wildtype rat astrocytes after IP . Recombinant sgp130-Fc was included as positive control.

3.2.4.2 Biological activity of transgenic sgp130-Fc

The specific ability of Hyper-IL-6, which is the fusion protein of human IL-6 and human soluble IL-6 receptor (sIL-6R) connected by a flexible peptide linker [70], to bind gp130 was employed to prove the functionality of the transgenic sgp130-Fc protein. Therefore, transgenic and wildtype brain homogenates (40 µl) were coimmunoprecipitated with Hyper-IL-6 (400 ng) and protein A sepharose for pulldown by centrifugation (2.3.3). Binding of precipitated proteins was monitored by SDS-PAGE (2.3.1) followed by Western blotting (2.3.2) via an monoclonal antibody recognising the human sIL-6R part of Hyper-IL-6 (80 kDa), named clone 14-18 [41], and the sgp130-Fc-detecting antibody B-P4 (130 kDa). The principle of this binding test including the subsequent Western blot analysis of the coimmunoprecipitated samples, achieved with recombinant purified sgp130-Fc (400 ng) and Hyper-IL-6 (400 ng), are illustrated in Fig. 28A and B.

The Western blot analyses of coimmunoprecipitated brain homogenates from astrocyte-specific sgp130-Fc-expressing mice (lines 5.2, 15.5, 19.1; 1st backcross) indicated that the transgenically expressed sgp130-Fc protein was able to bind and precipitate its ligand IL-6/sIL-6R (Hyper-IL-6) revealing its biological activity (Fig. 29). Wildtype brain homogenate (of non-transgenic littermate) did not show protein signals after coimmunoprecipitation for sgp130-Fc (negative control), as well as for Hyper-IL-6 that did not bind unspecifically to the protein A sepharose.

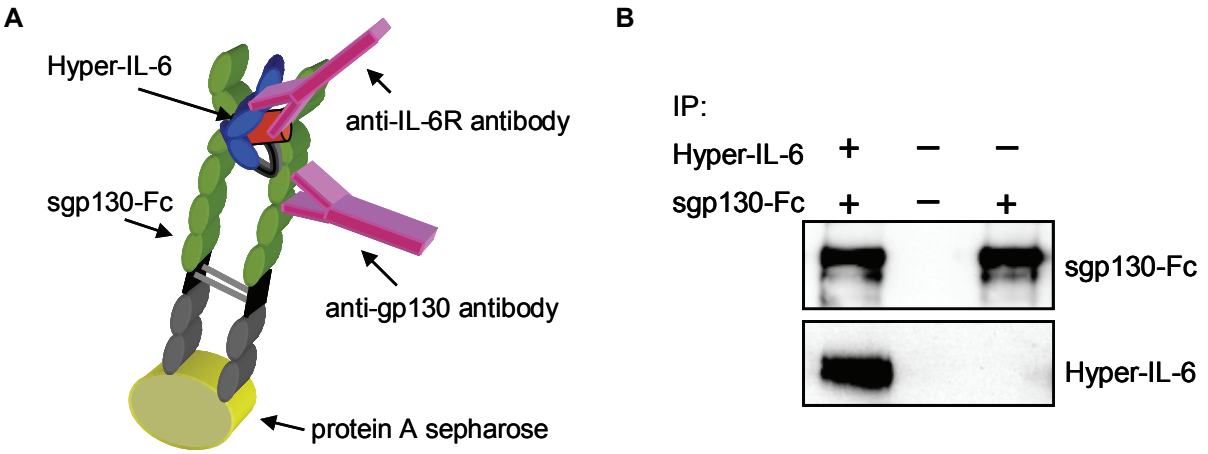

Figure 28 Principle of coimmunoprecipitation of Hyper-IL-6 with sgp130-Fc. Schematic principle of coimmunoprecipitation (A), principle of Western blot analysis of a coimmunoprecipitation using recombinant proteins of sgp130Fc and Hyper-IL-6 (B). Sgp130-Fc was pulled down after incubation of Hyper-IL-6 with protein A sepharose that binds to the Fc-portion. Bound Hyper-IL-6 was detected by a monoclonal antibody raised against human sIL-6R (clone 14-18), and sgp130-Fc via the monoclonal anti-gp130 antibody B-P4.

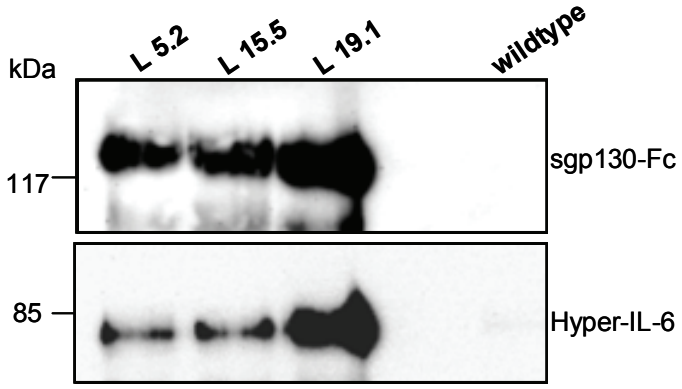

Figure 29 Western blot analysis after coimmunoprecipitation of Hyper-IL-6 with brain-derived sgp130-Fc of gfa2–sgp130-Fc mice. Sgp130-Fc in brain homogenates of transgenic mice (3 different lines after 1st backcross; designated as numbers) and wildtype (non-transgenic) were incubated with Hyper-IL-6 and for pulldown with protein A sepharose that binds to the Fc-portion of sgp130-Fc. Bound Hyper-IL-6 was detected by a monoclonal antibody raised against human sIL-6R (clone 14-18), and sgp130-Fc via the monoclonal anti-gp130 antibody B-P4.

3.2.4.3 Differential tissue expression of transgenic sgp130-Fc on RNA level

The GFAP promoter gfa2 directs expression almost exclusively to astrocytes of the CNS. However, outside of the brain expression of transgenes driven by this promoter has been reported to a weak extent [30, 271]. For the generated gfa2–sgp130-Fc mice, the tissue-specific expression of sgp130-Fc mRNA was explored in the organs brain, liver, kidney, spleen and lung by Northern blot analysis using a radioactive-labelled sgp130-Fc-specific probe (2.2.18). Beside the gfa2–sgp130-Fc mice (line 12.3, 1st backcross), transgenic sgp130-Fc mice under control of the PEPCK promoter (pepck–sgp130-Fc mice [220]) and wildtype C57BL/6N were examined. The Northern blot analysis (Fig. 30) indicated that the sgp130-Fc expression profile did not overlap between gfa2– and pepck–sgp130-Fc transgenic mice. The expression of sgp130-Fc mRNA was clearly restricted to the brain in gfa2–sgp130-Fc mice, but very faint amounts also appeared in spleen and lung. The pepck–sgp130-Fc mice transcribed sgp130-Fc mRNA mainly in liver and kidney with some amounts in lung, consistent with the finding of Rabe *et al*. [220]. The wildtype mouse strain did not display any presence of human sgp130-Fc mRNA. The total RNA was proven to be of optimal quality, indicated by the distinct RNA bands for 28S and 18S rRNA of the RNA agarose gel carried out before the blotting procedure.

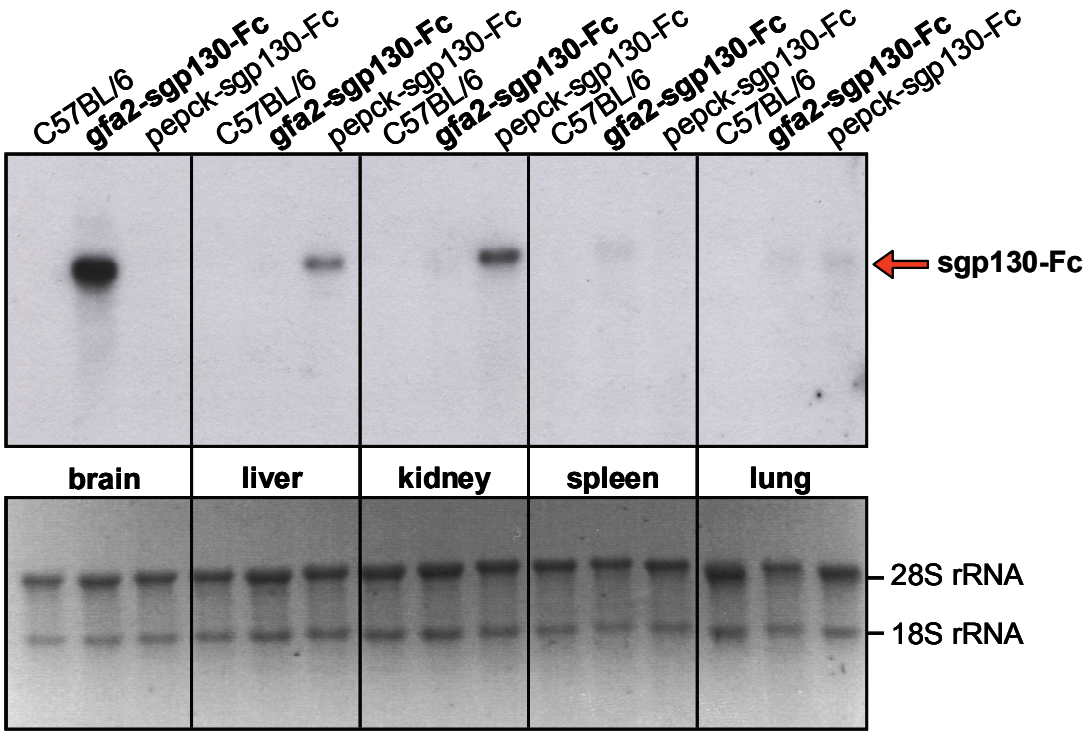

Figure 30 Analysis of sgp130-Fc mRNA expression via Northern blotting. Five micrograms of total RNA of different organs of heterozygous gfa2–sgp130-Fc mouse line 12.3 (astrocyte-specific, 1st backcross), homozygous pepck–sgp130-Fc mouse line opt3 (liver-specific) and wildtype C57BL/6N strain (negative control) were separated on a 1% agarose/formaldehyde gel (lower panel), transferred onto a nylon membrane and hybridised with α-^{32}P-dATP-radioactive-labelled sgp130-Fc-specific probe (upper panel).

In addition, the mRNA expression of sgp130-Fc was monitored via RT-PCR (2.2.16) using the same RNA samples as for Northern blotting (see above). Even very small amounts of mRNA can be amplified via this sensitive method. The results of the RT-PCR (Fig. 31) with sgp130-Fc-specific primers ("sgp130-Fc-screen") led to a 699 bp product and confirmed the findings obtained by Northern blot analysis. However, the generated gfa2–sgp130-Fc mouse also showed very low expression of sgp130-Fc in lung and spleen, and additionally in liver and kidney. Nevertheless, the most intense sgp130-Fc mRNA signal for this mouse strain remained within the brain.

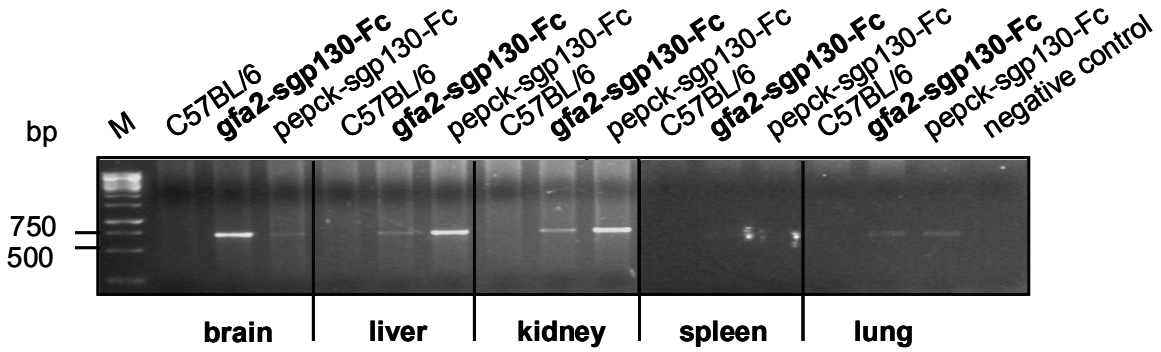

Figure 31 Analysis of sgp130-Fc mRNA expression via RT-PCR. Total RNA (1,5 µg) of different organs of heterozygous gfa2–sgp130-Fc mouse line 12.3 (astrocyte-specific, 1st backcross), homozygous pepck–sgp130-Fc line opt3 (liver-specific) and wildtype C57BL/6N strain (negative control) was transcribed to cDNA (RT), 1 µl cDNA assessed to PCR (25 cycles) and 10 µl loaded onto a 1% agarose gel.

3.2.4.4 Transgenic sgp130-Fc protein in the serum

Although expression of sgp130-Fc, under specific transcriptional control of the astrocyte-specific promoter gfa2, indeed could be assigned mainly to astrocytes of the transgenic murine brain, small amounts of sgp130-Fc mRNA were detected in organs outside of the brain (see 3.2.4.3, 3.2.4.1). Therefore, transgenic protein expression in sera of gfa2–sgp130-Fc founder animals were investigated. Forty µl of serum (2.5.5) of different founder mice were tested after IP with protein A sepharose (2.3.3) in Western blot analysis detecting sgp130-Fc by the antibody B-P4 (2.3.2), after loading 50% of IP-precipitate onto the SDS gel (2.3.1). The strain C57BL/6N served as negative control. A serum sample of pepck–sgp130-Fc line 4 strain, which expressed low sgp130-Fc levels in serum (0.55 µg/ml) [219], was included as a positive control. Varying amounts of precipitated sgp130-Fc were detected within the serum of several gfa2–sgp130-Fc founder mice (Fig. 32).

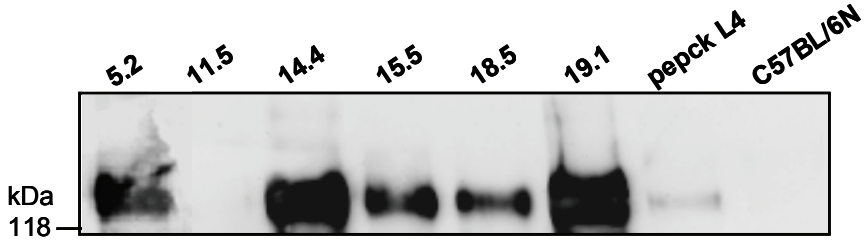

Figure 32 Detection of transgenic sgp130-Fc protein in the serum of several gfa2–sgp130-Fc founder mice after IP with protein A sepharose by Western blotting using the monoclonal anti-gp130 antibody B-P4. Serum of C57BL/6N served as negative control, serum of pepck–sgp130-Fc L4 as positive control.

Additionally, the sgp130-Fc protein concentrations in serum were quantified in gp130 sandwich ELISA (2.3.5) for several heterozygous mouse lines after the first backcross. Here, the positive control consisted of serum from the homozygous pepck–sgp130-Fc mouse line opt3 that has been shown to express concentrations between 29–36 µg sgp130-Fc/ml serum [220]. In accordance with that outcome, the ELISA (Fig. 33) revealed a concentration of approximately 29 µg sgp130-Fc/ml serum for a mouse of this opt3 line. As negative control a non-transgenic littermate (wildtype) was utilised.

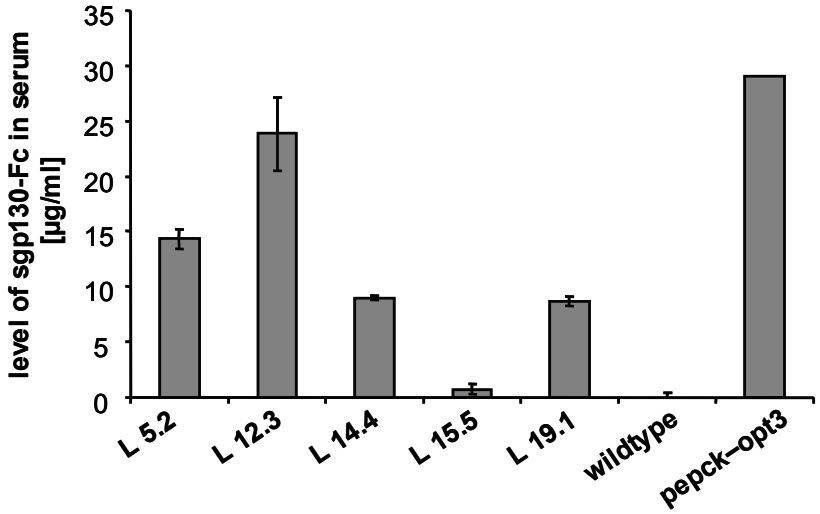

Figure 33 Quantification of sgp130-Fc protein concentrations in the serum of several heterozygous transgenic gfa2–sgp130-Fc mice by gp130 ELISA. Several lines after first backcross are designated as numbers on the x-axis. A non-transgenic wildtype serum was included as negative control and pepck–sgp130-Fc opt3 serum as positive control. Data represent mean values ± S.D. (quadruplets).

Measurement of sera of gfa2–sgp130-Fc animals indicated that the line 12.3 contained the highest sgp130-Fc protein level of about 24 µg/ml serum. Line 5.2 showed a high sgp130-Fc concentration of approximately 14 µg/ml serum. The lines 14.4 and 19.1 displayed moderate amounts of the transgenic protein with about 9 µg/ml serum, and in line 15.5 a low sgp130-Fc content of approximately 700 ng/ ml was present in the serum.

The sgp130-Fc protein expression of the currently three established gfa2–sgp130-Fc mouse lines 5.2, 15.5 and 19.1 within brain and serum was compared. This is exemplified by gp130 ELISA analyses for a mouse per line (Fig. 34). Blood from the brain (as in capillaries, meninges) were removed/replaced by perfusion with 30 ml of PBS buffer. The animals were crossed onto a C57BL/6N background to number 5 (line 19.1), 6 (line 15.5) and 7 (line 5.2).

The outcome is summarised in Table 2. The data indicate that the sgp130-Fc protein levels in serum of gfa2–sgp130-Fc transgenic mice did not correlate with the amount found in the brain. The line 19.1 that displayed the highest sgp130-Fc level in brain homogenate (about 1,900 ng/ml) revealed a moderate concentration in serum (about 6,400 ng/ml). The lines 5.2 and 15.5 that express comparable sgp130-Fc amounts within the brain homogenates (about 340 and 360 ng/ml respectively) differed in serum levels of sgp130-Fc. In the serum, line 15.5 showed a low sgp130-Fc amount (1,100 ng/ml), whereas line 5.2 contained more than a fivefold higher sgp130-Fc protein level (8,800 ng/ml).

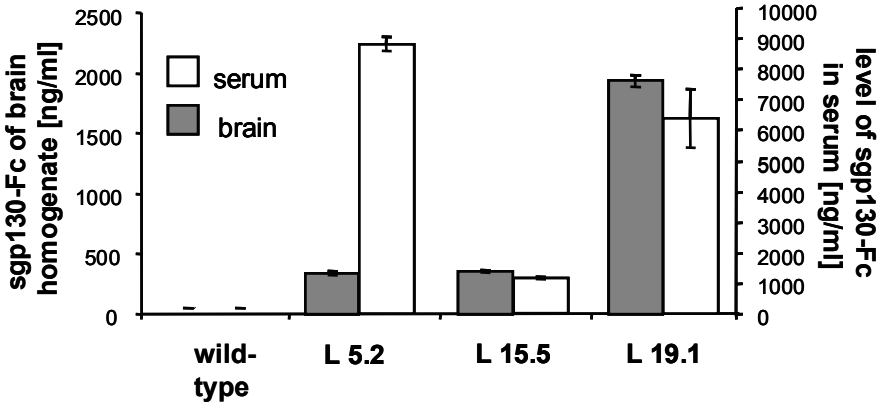

Figure 34 Protein levels of sgp130-Fc in brain homogenates and serum after perfusion for a mouse of each established gfa2–sg130-Fc line 5.2 (backcross 7), 15.5 (backcross 6) and 19.1 (backcross 5), measured by gp130 ELISA. A non-transgenic wildype animal served as negative control. Note the different division of y-axes. Data represent mean values ± S.D. (triplets).

Table 2 Relative comparison of sgp130-Fc levels between brain and serum of the 3 established gfa2–sgp130-Fc mouse lines.

	line 5.2	line 15.5	line 19.1
sgp130-Fc in brain	++	++	+++
sgp130-Fc in serum	+++	+	++

+++ abundant expression; ++ moderate expression; + low expression

3.2.4.5 Transgenic protein expression in gfa2–sgp130-Fc mice in the brain after several steps of backcrossing

It became evident that the absolute sgp130-Fc amounts of the brain changed in the course of backcrossing. The gfa2–sgp130-Fc lines 5.2 and 15.5 displayed reduced transgenic protein expression in brain homogenates, starting from about 700 ng/ml in the founder mice (3.2.4.1, Fig. 25, Table1) and reaching about 350 ng/ml after backcross 6 and 7, respectively (3.2.4.1, Fig. 34, Table 3). Conversely, in brain homogenates of line 19.1 after the 5th backcross sgp130-Fc levels at about 1,900 ng/ml were detected (3.2.4.4, Fig. 34, Table 3), which increased in comparison to the founder animal at about 1,200 ng/ml (3.2.4.1, Fig. 25, Table 1).

Considering that a molar excess of sgp130-Fc (at least 10-fold) is needed to counteract IL-6 trans-signalling [115], molar ratios of the relevant components/molecules were determined (Table 3), as before for the founder mice (3.2.4.1, Table 1). Again, 15 ng/ml msIL-6R (1.875 $\times 10^{-10}$ M), the crucial component of trans-signalling was set as the value to relate to. The molar amounts of sgp130-Fc were calculated from gp130 ELISA measurements of brain homogenates of the 3 established gfa2–sgp130-Fc lines (Table 3). A molar excess of sgp130-Fc was present in the brain of the transgenic lines: about 14-fold in line 5.2 (7th backcross) and 15.5 (6th backcross), and 79-fold in line 19.1 (5th backcross).

Table 3 Protein levels of sgp130-Fc in brain homogenates of the 3 established gfa2–sgp130-Fc mouse lines 5.2 (backross 7), 15.5 (backcross 6) and 19.1 (backcross 5) measured via gp130 ELISA and calculated molarities. Values are means. Protein levels were measured in triplets. Molar relations of sgp130-Fc to msIL-6R were calculated for 15 ng/ml msIL-6R as measured in ELISA.
(1 M sgp130-Fc=130,000 g/l; 1 M sIL-6R=80,000 g/l).

tg line	**L 5.2**	**L15.5**	**L19.1**
sgp130-Fc of brain homogenate [ng/ml]	340.02	357.19	1938.79
sgp130-Fc of brain homogenate [mol/l]	2.616×10^{-9}	2.748×10^{-9}	1.491×10^{-8}
molar factor sgp130-Fc : msIL-6R	13.95	14.65	79.54

4 Discussion

4.1 IL-6 trans-signalling and sleep

4.1.1 Effects of Hyper-IL-6 on sleep-wake behaviour

Several previous publications have demonstrated the sleep-modulatory properties of IL-6 (see 1.7). IL-6 responses are transmitted either via the classic or the trans-signalling pathway (see 1.2, 1.3). This is the first study to systematically investigate the influence of IL-6 trans-signalling on sleep regulation/modulation in rats by means of the designer cytokine Hyper-IL-6. Hyper-IL-6 is the fusion protein of the human IL-6 connected by a flexible peptide linker to the human sIL-6R, which mimics the trans-signalling pathway [70].

As gp130 is ubiquitously expressed, including in the brain [312], Hyper-IL-6 can activate virtually all cells within the brain. The cellular response to Hyper-IL-6 in primary rat astrocytes and microglia was confirmed in this work by assessing the phosphorylation of the transcription factor STAT3 upon Hyper-IL-6 challenge using Western blot analysis (3.1.1, Fig. 8). The reactivity of primary rat hippocampal neurons to Hyper-IL-6 was initially shown by Sun *et al.* [273]. The cytokine samples of Hyper-IL-6 utilised in the animal experiments were verified to be biologically active and to act specifically via the transmembrane receptor gp130 (3.1.1, Fig. 9). To date there has been no publication demonstrating cellular activation independent of gp130. The high specificity and wide reactivity of Hyper-IL-6 was strengthened in the present work.

The i.c.v. administration of Hyper-IL-6 (100 and 500 ng) shortly prior to the dark period (dark onset), had no impact on non-REM sleep in rats (3.1.2, Fig. 12). Rats that received an i.c.v. injection of 500 ng rat IL-6 at dark onset displayed enhanced non-REM sleep initially, that reduced thereafter, and a fragmentation of sleep [99]. However, the amount of non-REM sleep was not altered by antagonising IL-6 via monoclonal or polyclonal antibodies [99] as well as by the lack of IL-6 in IL-6 knock-out mice [174]. Therefore Morrow *et al.* [174] proposed that IL-6 is not involved in the regulation or modulation of non-REM sleep. These findings were supported by the present study. In contrast, a subcutanous administration of IL-6 to humans revealed an increase of SWS (non-REM sleep) during the second part of the sleep period [262]. The route and time of application, the actual doses reaching the brain as well as species differences might be the variables leading to different outcomes of the various studies.

Interestingly, i.c.v. Hyper-IL-6 application (500 ng) had a quantitative (increased amount) and qualitative (reduced EEG power density) effect on REM sleep (3.1.2, Fig. 12; 3.1.3., Fig. 14). An elevation in REM sleep duration predominately occurred during the light phase, which is the main sleeping period for rats. This delayed effect of Hyper-IL-6 18–20 h after injection (late part of sleep) might relate to an indirect sleep-promoting effect of Hyper-IL-6, including activation of brain cells as astrocytes, glia cells and neurons and subsequent translation of further substances mediating the observed REM sleep increase (see 4.1.4). This effect was somewhat surprising since IL-6 KO mice show a 30% increase in REM sleep, mainly during the light period [174]. In contrast, the effect on non-REM sleep elicited by rat IL-6 injection into rats (see above) was not accompanied by changes of REM sleep [99]. In the same way, the effects of exogenous IL-6 application on human REM sleep remain ambiguous. Whereas one publication displayed suppression of REM sleep after a s.c. injection [262], another study did not report changes after an intranasal administration [16]. However, in accordance with the present study, some previous reports point to a role of IL-6 and/or sIL-6R for REM sleep. It has been found that peripheral circulating levels of IL-6 in humans were higher during REM sleep and light sleep, but not in deep SWS [224]. In addition, the human study of Dimitrov *et al.* [56] revealed an association between high amounts of REM sleep, present especially at the late part of nighttime sleep, and high levels of the sIL-6R, which is the receptor mediating the IL-6 trans-signalling pathway. These results might support the current finding herein that Hyper-IL-6 and thus trans-signalling has increased REM sleep during the late part of the rat's sleeping period.

4.1.2 The roles of IL-6 trans-signalling versus classic signalling affecting brain functions

The cytokine IL-6 is produced within the brain by astrocytes, microglia and neurons [160, 230, 237, 243, 247, 254, 295]. In addition to this brain-born IL-6, also peripheral circulating blood-borne IL-6 can signal the brain to affect CNS function as sleep-wake behaviour. Several routes for peripheral cytokines to reach the brain have been delineated so far (reviewed in [107, 133, 216]). They can act directly from the blood on brain areas which lack the BBB. Such areas are termed circumventricular or periventricular organs. Among them are e.g. the pineal gland, the posterior pituitary, the subfornical organ and the median eminence of the hypothalamus, and mostly the areas surrounding the ventricles 3 and 4. Crossing the BBB via active transport and stimulation of afferent nerves like the vagus nerve are further possibilities for cytokines to communicate from the periphery to the CNS. In order to affect brain functions even low levels of circulating cytokines are sufficient [216].

For the activation by IL-6, the IL-6 receptor in membrane-bound (classic signalling) or soluble form (trans-signalling) is required. Whereas the receptor subunit gp130 is ubiquitously expressed throughout the body and also in most of the brain areas [312], the expression of the IL-6R is highly restricted to distinct cell populations, e.g. hepatocytes and lymphocytes ([274], 1.1, 1.3). Within the rat brain, IL-6R mRNA expression has been found in some neuronal lineages, like primary sympathetic neurons [157, 160]. In addition, low levels of IL-6R mRNA were detected in microglia and astrocytes, but not in oligodendrocytes of primary mixed brain cultures of newborn mice [243]. Brain regions in which rat IL-6R mRNA transcripts have been found include hypothalamus, hippocampus, neocortex and cerebellum [75, 76, 102]. IL-6 mRNA was detected to a minor extent in several other brain structures as well [246, 321]. Low levels of IL-6R mRNA were found under basal conditions for instance in the ependymal cells of the ventricles, and in areas lacking the BBB like the subfornical organ and median eminence [288].

However, it should be kept in mind that mRNA expression might not reflect protein expression onto the cell surface. Recently, Helwig *et al.* [94] have analysed rat brain slices via immunohistochemistry after i.c.v. injection of 100 ng labelled IL-6 and found IL-6R protein expression (colocalised with IL-6) in ependymal cells of the third ventricle (periventricular tissue) but not within the brain parenchyma. Moreover, the ependymal cells of the ventricles and circumventricular organs were activated after i.c.v. application of IL-6 (200 ng) into rats, which was detected via *in situ* hybridisation of brain slices for c-fos mRNA [287]. It was further demonstrated that even a systemic IL-6 injection activated sensorial circumventricular organs such as subfornical organ and median eminence (of the hypothalamus). These results suggest that the circumventricular organs (lacking BBB) provide a route for peripheral IL-6 to transmit signals to the brain [287]. Thus IL-6 floating within brain ventricles and peripheral IL-6 could signal through the membrane-bound IL-6R via the classic transduction pathway.

But indeed, it has been shown that many neural cells, e.g. primary rat sympathetic and hippocampal neurons, and newborn rat astrocytes, are only responsive to IL-6 in the presence of the sIL-6R or to Hyper-IL-6 via trans-signalling ([160-162, 249, 273], see 1.3). Hyper-IL-6 can thus activate a broader spectrum of target cells within the brain and has been shown to be far more potent than IL-6.

In sum, considering the spatial restriction of the membrane-bound IL-6R, the findings mentioned above suggest that IL-6 activity within the brain parenchyma requires the trans-signalling pathway. Accordingly, the different properties of IL-6 classic and trans-signalling might explain the seemingly contradictory effects between exogenous IL-6 and Hyper-IL-6 on

sleep behaviour (see 4.1.1). They imply, that IL-6 together with the sIL-6R (mimicked by Hyper-IL-6) may affect areas of the brain that would remain unresponsive to IL-6 alone and therefore may create different biological outputs such as the herein observed supportive effect on REM sleep.

4.1.3 The role of IL-6 trans-signalling in sleep under disease conditions

The sleep-associated increase of the sIL-6R found in healthy humans [56] seems to be of relevance for diseases as well. Under pathological conditions increased levels of both IL-6, mainly released by astrocytes [230, 295] and microglia [254], and sIL-6R were observed [1, 86, 127, 153, 169, 181, 325]. During disease processes an altered sleep behaviour is well documented [50, 53, 66, 107, 121, 134, 176, 285], and thus a link to trans-signalling appears very likely. The main finding of this project, the association of Hyper-IL-6 with REM sleep, is nicely reflected for instance in conditions of major depression and during aging. In these cases, sleep architecture is characterised by an increased percentage of REM sleep [15], and circulating levels of IL-6 and its soluble receptor (sIL-6R) are elevated [153]. These results strengthen the putative involvement of trans-signalling in the modulation of REM sleep during disease conditions. In addition, patients suffering from Parkinson's disease, which is associated with elevated IL-6 levels in cerebrospinal fluid, display daytime sleepiness and a tendency to more REM sleep at sleep onset [53].

Furthermore, expression and activity of the metalloprotease ADAM17 [62], which is responsible for the proteolytic generation of the sIL-6R (trans-signalling), is elevated in the CNS under some pathological conditions including stroke and multiple sclerosis (MS) [173, 323]. These processes promote neural progenitor cell migration and contribute to stroke-induced neurogenesis [173] and might thereby also contribute to altered sleep behaviour during disease conditions.

In this respect, stroke is an interesting case. Stroke is accompanied by a great release of glutamate that triggers neurotoxicity. A few hours after stroke in humans, levels of IL-6 rise in peripheral blood and cerebrospinal fluid [126, 226, 280]. Interestingly, IL-6 plays a neuroprotective role in neurons exposed to glutamate excitotoxicity and thus neuronal protection after cerebral ischemia (stroke) [2]. It is well known that sleep loss increases cytokines like IL-6 (see 1.6.3; 1.7; [108, 302]). A recent study reported that after cerebral ischemia (stroke) in mice, the increased IL-6 mRNA expression caused by experimental sleep deprivation contributed to the attenuation of neuronal cell death [314]. This is remarkable given that the consequences of chronic sleep loss, including elevated cytokine levels, are

normally detrimental. Long-term sleep deprivation may lead to a higher risk developing diseases that are mostly associated with aging, e.g. cardiovascular diseases, diabetes or stroke (see 1.6.3, [241]). For instance, in a recent human study enhanced circulating levels of IL-1β, IL-6, IL-17, CRP and an increased heart rate, which are the risk factors for developing cardiovascular diseases were detected after sleep restriction of 5 nights (to 4 h sleep) [292]. These examples illustrate well both the protective potential of IL-6 when acutely elevated, and the detrimental/degenerative potential when IL-6 is chronically increased during pathological conditions, also in connection to the matter of sleep.

4.1.4 Possibilities for REM sleep modulation by IL-6/sIL-6R

What could be the mechanisms how IL-6/Hyper-IL-6 affects sleep-wake behaviour, particularly REM sleep? In general the model of humoral sleep regulation (Fig. 5) also accounts for IL-6, and partly for REM sleep (see also 1.6.2). The regulation of REM sleep involves a huge network with different generators for the REM sleep signs (e.g. muscle atonia, hippocampal theta waves, cortical activation) with multiple neurotransmitters acting on various sites of the circuits [52]. Briefly, REM sleep is dependent on a reciprocal interaction between monoamine containing REM-off neurons in the brainstem (locus coeruleus (noradrenergic) and raphe nucleus (serotonergic)) and cholinergic REM-on neurons in the pons (lateral dorsal tegmentum and pedunculopontine tegmentum). During REM sleep the aminergic cell activities are drastically reduced or absent (REM-off), whereas the cholinergic cell activities are rather high (REM-on; Wake-REM-on) (ratio 0:0.65). During wakefulness this ratio is 1:1. When REM sleep is absent the cholinergic REM-on neurons are inhibited by the serotonergic and noradrenergic REM-off neurons. At the onset of REM sleep the REM-on neurons become disinhibited as the REM-off neuron activity become silent. The REM-off cell activity may be inhibited by GABA in dorsal raphe and locus coeruleus nuclei [192, 193] and by a reciprocal neuronal activity between serotonergic and GABAergic cells of the dorsal raphe nucleus (rev. in [52]).

It has emerged that neurotransmitters, neuropeptides, hormones, cytokines, etc. interact in a complex net with crosslinks at multiple sites and mutual interactions to affect REM sleep and are in turn affected by sleep, although there are many open questions. Some speculations and hypotheses of mechanisms how the player IL-6 might operate in the modulatory network of REM sleep, are discussed below and illustrated in Fig. 35. Importantly, it should be noted that release of neurotransmitters or sleep modulatory factors at different sites within the brain and networks can result in varying or opposite effects on sleep behaviour, therefore it is difficult

to determine the role for a specific local action of IL-6 and thus more experimental research is strongly necessary.

It was supposed that IL-6 could act directly on sleep-wake regulating neurons [99]. Indeed, IL-6 was shown to influence neuronal firing rates in some brain regions [125, 316]. Further possibilities for an indirect modulation of sleep-wake behaviour by IL-6 include other branches of the network. IL-6 is able to alter expression of neurotransmitters involved in sleep-wake regulation. For instance, GABA release has been demonstrated to be increased by IL-6, which was mediated by prostaglandins [54]. The inhibitory neurotransmitter GABA inhibits wake active neurons and promotes non-REM and REM sleep ([192, 193]; rev. in [268]). IL-6 might be connected to REM sleep modulation by affecting this transmitter. Moreover, IL-6 is involved in glutamatergic neurotransmission, and showed an inhibitory effect on the release of glutamate, which is an excitory wake-promoting neurotransmitter [49, 218]. But in contrast, local activity of glutamate in lateral dorsal tegmentum and pedunculopontine tegmentum is needed for initiation of REM sleep. Also serotonin, whose action is involved in local inhibition of aminergic REM-off neurons during REM sleep, could be influenced by the cytokine IL-6. Several studies indicated the induction of serotonin in the brain by IL-6 [10, 309, 310], in turn serotonin was shown to increase IL-6 mRNA in rat astrocytes [217].

Multiple series of evidence ascribe adenosine, which is omnipresent in the brain, as a sleep regulatory substance for non-REM and REM sleep, providing a metabolism-dependent regulation (rev. in [12, 195, 268]). Both A1 and A2A adenosine receptor subtypes mediate the well-documented sleep inducing effect of adenosine. *In vitro* and *in vivo* studies in mice demonstrated enhanced expression of the neuronal adenosine A1 receptor (hippocampus, cortex, astrocyte cultures) and signalling induced by IL-6 [18, 19]. NO may lead to accumulation of adenosine as determined in rat forebrain neurons in vivo [235]. Likewise, NO has a vital role in REM sleep modulation (rev. in [195]). It has been found that primarily the neural NO synthase is responsible for REM sleep production, not the inducible NO synthase [42]. To promote REM sleep in brain stem, NO may stimulate acetylcholine release [143], may modulate serotonergic activity in raphe [35] and noradrenaline release in pedunculopontine area [129].

As mentioned above all the sleep modulatory components are interweaved with each other. Adenosine is considered an intermediary for the sleep regulatory effects of prostaglandin D2, as suggested for non-REM sleep induction at the VLPO [91]. Prostaglandins, which are eicosanoids, particularly prostaglandin D2 have been shown to promote both non-REM and

REM sleep under normal as well as under some pathological conditions [91]. Similarly, prostaglandin E2, which is a major mediator of the fever response, is implicated in sleep regulation, too [164]. It has been found that central and peripheral injection of IL-6 increased prostaglandin E2 in cerebrospinal fluid, which was inhibited by application of inhibitors for cyclooxygenase (an enzyme involved in the synthesis of prostaglandin E2) [57]. The cyclooxygenase can also be activated e.g. by NO [122], underlining the complex interplay.

Another well-described factor for promoting REM sleep is GH, a member of the somatotropic axis that regulates tissue metabolism, whose release from the pituitary is stimulated by GHRH (rev. in [195, 268]). Exemplary, systemically GH-injected animals and transgenic mice overexpressing GH displayed much higher amounts of REM sleep [60, 87]. In rat pituitary cells, IL-6 promoted GH expression and secretion [80] and thus could increase REM sleep. Likewise, the neuropeptides prolactin and the vasoactive intestinal polypeptide (VIP) also belong to the well-known REM sleep-promoting substances (rev. in [268]). Prolactin is secreted mostly during the second half of human sleep, which is REM rich [242, 306]. Further, a systemic or hypothalamic injection increased REM sleep during the light period (sleep) in rats [231]. Contrary, prolactin-deficient mice have reduced REM sleep [196]. IL-6 has been shown to stimulate the release of prolactin from pituitary cells [3, 227]. Similarly, i.c.v. injection of VIP into rats led to enhanced REM sleep [228]. The VIP effect was clearly demonstrated to be mediated via prolactin, whose expression can be induced by VIP [185, 231]. Interestingly, IL-6 together with the sIL-6R induced VIP mRNA expression in sympathetic neurons [44]. In turn, VIP was able to potentiate IL-6 production as determined in osteoblasts [212]. There is also an indirect connection to cytokines, since VIP together with TGFβ promotes differentiation of the IL-17 producing Th17 cells (see below) [317].

The transcription factor NF-κB, which is also constitutively expressed in neurons, serves as a mediator in sleep-wake regulation [43, 118]. NF-κB is activated by adenosine A1 receptor [11] as well as several cytokines and growth factors involved in sleep regulation/modulation as IL-1β, TNFα, NGF [43, 144, 155, 319]. In turn, as a feedback loop, NF-κB stimulates expression of the adenosine A1 receptor [88, 191], enzymes for syntheses of prostaglandins D2 and E2 [9, 190, 318] and a number of sleep modulatory cytokines such as IL-1β and TNFα, and NGF [6, 204]. Interestingly, knock-out mice for the p50 subunit of NF-κB spent more time in non-REM as well as in REM sleep under normal conditions, but REM sleep was reduced after LPS (inductor of NF-κB) challenge [112]. Ihaveri *et al.* highlighted the role of NF-κB in influencing sleep through various sleep regulatory/modulatory substances during health and disease [112]. Furthermore, NF-κB is a STAT3 target gene induced by IL-6 [258]

and in turn NF-κB also induces IL-6 [145], thus providing an additional possibility to influence (REM) sleep.

Cytokines and growth factors within the brain work together in a large network in synergistic or antagonistic manner with overlapping and feedback activities, including IL-6 with its downstream and upstream actions. IL-6 is supposed to act downstream of IL-1β and TNFα in the cytokine cascade and can build up a negative feeback loop on TNFα [68, 201, 260, 305]. In addition, IL-6 in association with TGFβ promotes the development of the IL-17-secreting Th17 cells [17, 296]. REM sleep deprivation for 72 h in rats led to inflammation and an elevation of IL-17 in blood [322]. In this respect, IL-17 might be a candidate to participate in sleep modulation at least under pathological conditions, which has yet to be experimentally proven. Induction of REM sleep (and non-REM) was also shown for NGF in rabbits and cats [275, 320], which can be triggered by several cytokines [324]. IL-6 in conjunction with sIL-6R (or Hyper-IL-6) has stimulated synthesis of NGF mRNA and protein release in primary rat astrocytes of cortex and hippocampus in a dose-dependent manner, which was strictly regulated by the sIL-6R [161, 204]. NGF is associated with other sleep modulatory substances. In human microglial cells it was shown that an adenosine-induced NGF mRNA and protein release occurred via stimulation of adenosine A2A receptor [92].

There are likewise various sleep inhibitory molecules acting on different levels of the sleep modulatory machinery. They involve hormones of HPA axis as cortisol, ACTH, CRH [156, 299], and the non-REM and REM sleep inhibitory cytokines, with IL-4 and IL-10 [136] for instance inhibiting NF-κB [47, 311].

Moreover, focussing on the overall regulation of sleep, the timing of the sleep-wake cycle is coordinated by a circadian control [22, 24]. The secretion of sleep regulatory/modulatory components is also controlled by the circadian clock. The circadian master (core) clock is located in the SCN of the hypothalamus that receives information via a wide range of neurotransmitters and mediators from various regions of the brain [170]. Interestingly, IL-6 has been detected in the rat SCN [81]. Noteworthy, cytokines are capable of influencing core clock or clock-controlled genes. For example, TNFα and IL-1β inhibited expression of some clock genes in NIH-3T3 cells, whereas IL-6 showed no effect in that study [40]. In contrast, expression of the core clock gene Per1 was induced by IL-6 in a human hepatoma cell line [177]. The basic helix-loop-helix transcription factor Dec1 (=Stra13), which is expressed in the SCN, belongs to the regulators of the mammalian clock [101]. It suppresses the activation of the Per1 promotor [101], the clock-controlled genes Dbp and Rev-erbα and activate the core clock gene Cry1 [130]. The expression of Dec1 mRNA in a human NK-cell line

increased after stimulation with several cytokines such as IL-2, IL-12, TNFα, TNFβ, and also IL-6 [111].

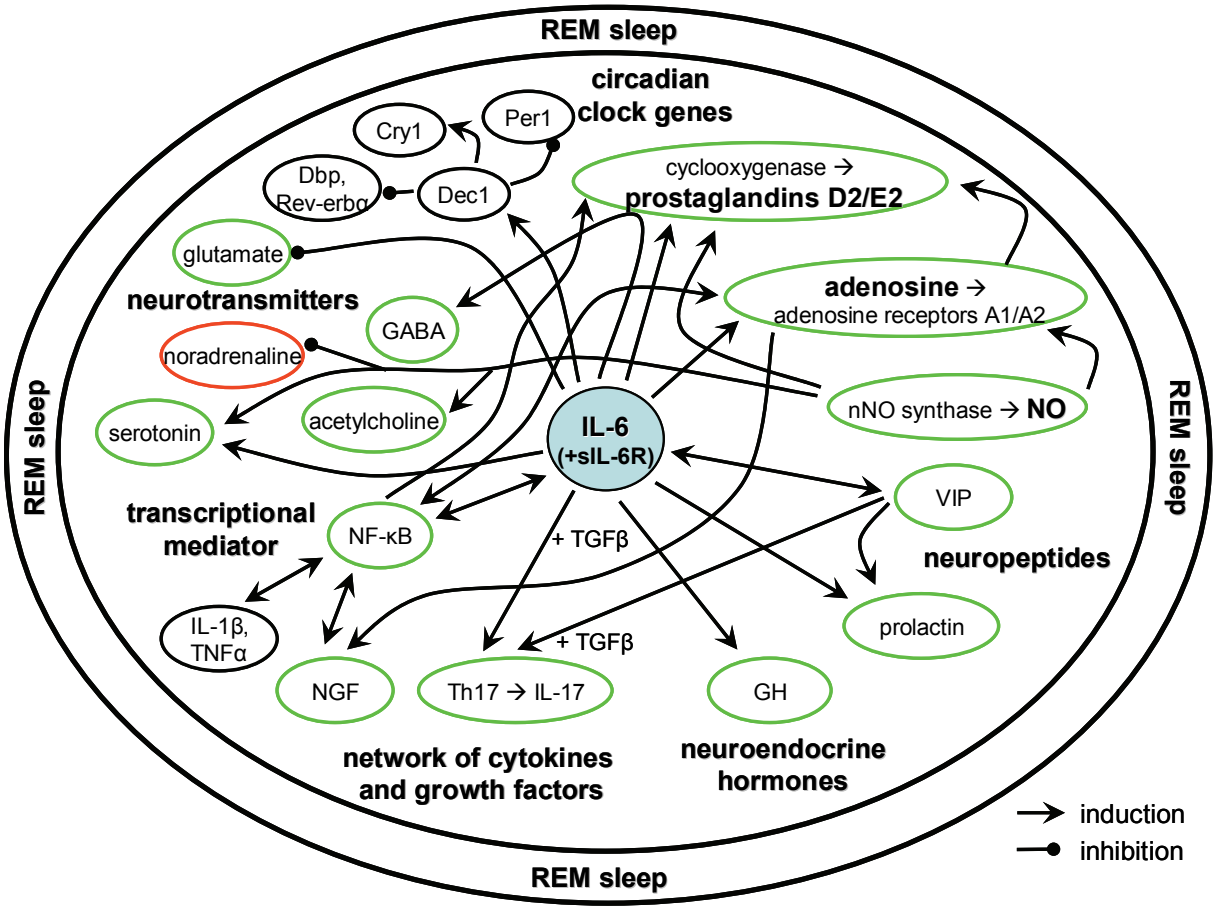

Figure 35 Some speculations for REM sleep support by IL-6/sIL-6R operating in the complex REM sleep regulatory/modulatory network. IL-6 has been shown to influence the designated substances, but not yet in connection to REM sleep and corresponding brain areas. Green marked substances have been shown to support REM sleep. Red marked factor is downregulated during REM sleep. Circadian control belongs to an overall sleep regulatory mechanism. For further details see 4.1.4.

4.1.5 Conclusions

Taken together, this study defines a REM sleep-promoting role of IL-6 trans-signalling (see 4.1.1), which might have a crucial meaning for normal sleep-wake behaviour but especially for sleep in certain pathologies (see 4.1.3). Regardless, single cytokines never act alone but in a complex network associated with other cytokines, neurotransmitters, hormones and cellular molecules (see 4.1.4). In this context, IL-6 would be united with the other "sleep factors" in the complexicity of REM sleep inducing and maintaining actions. The sleep regulatory/modulatory substances presumably influenced by IL-6 might be amplified when

IL-6 acts together with the sIL-6R. Thus IL-6/sIL-6R or Hyper-IL-6 could stimulate a broader range of cells including areas of REM sleep generation (see 4.1.2). Nevertheless, the suggestions that argue the contributed mechanisms for REM sleep modulation by IL-6 should be interpreted cautiously, because they have not yet been experimentally proven.

4.2 Generation of gfa2–sgp130-Fc transgenic mice: Selective blockade of IL-6 trans-signalling in the brain

IL-6 trans-signalling-mediated responses have been implicated in the pathophysiology of inflammatory, autoimmune or degenerative disorders of the periphery and brain, and might be involved in sleep modulation (see 1.1, 1.5, 1.7). For further investigations in these research fields, transgenic mice (to date 3 lines at heterozygous state on 8th backcross to C57BL/6N) expressing sgp130-Fc specifically within the brain were generated and initially characterised.

Soluble gp130 is the natural inhibitor of IL-6 trans-signalling without affecting the membrane-bound IL-6R-triggered responses (classic signalling). The molecular tool sgp130-Fc (see 1.4) is a dimer of the extracellular portion of human gp130 connected by the constant portion of a human IgG1 antibody (Fc). It appeared to be 10-fold more antagonistically active than the monomeric sgp130 [115]. Likely sgp130-Fc rather reflects the situation of preformed gp130-dimers on living cells [281] that facilitates the ligand/receptor complex formation to induce intracellular signalling events (see 1.2, 1.3). Since sgp130-Fc competes with its membrane-bound equivalent for binding of IL-6/sIL-6R, a sufficient excess of sgp130-Fc (transgenically expressed) is required to block IL-6/sIL-6R-mediated cellular responses ([115], see 1.4). Human sgp130-Fc is able to inhibit the complex of murine IL-6/sIL-6R, because of the special species specificity within the IL-6 receptor system. Murine IL-6 only binds the murine IL-6R [293], whereas murine sIL-6R can associate with murine and human IL-6 as well as with murine and human sgp130 [115].

In the present study sgp130-Fc transgenic mice were generated under transcriptional control of the human gfa2 promoter. The gfa2 promoter is a 2.2 kb fragment of GFAP (glial fibrillary acidic protein) and its expression is mainly restricted to astrocytes, which are the major cell population in the CNS [30, 271]. Up to now, the gfa2 promoter has been used successfully in several transgenic mice to specifically target gene expression to astrocytes. For instance transgenic mice were generated expressing proteins including LacZ [29], TGFβ1 [77], somatostatin [250], tau [71], ApoE3, 4 [272], ApoE [257] or angiotensinogen [172]. The

gfa2–sgp130-Fc transgene cDNA construct (see 3.2.1, Fig. 17) was inserted by the method of microinjection into pronuclei of fertilised oocytes, which randomly incorporated into the mouse's chromosomes. It comprises the 2nd rabbit β-globin intron between promoter and the codon-optimised sgp130-Fc cDNA. This type of composition showed a stronger protein expression in an astrocytic cell line than the location of the intron behind the sgp130-Fc cDNA (3.2.2, Fig. 18), again supporting the finding that the insertion of a heterologous intron between promoter and coding region can improve expression [209, 220]. Rabe *et al.* have already demonstrated that the codon-optimised version of the sgp130-Fc cDNA led to a higher protein expression [220]. Accordingly, protein expression has additionally increased by locating the intron upstream of the sgp130-Fc cDNA [220], as utilised herein.

The generated gfa2–sgp130-Fc mice of the present project successfully expressed transgenic protein in the brain, as shown by Western blot analyses and ELISA. The sgp130-Fc protein levels of the founder mice that created the ancestors of different lines (5.2, 15.5, 19.1) ranged from about 750 to over 1,000 ng/ml brain homogenate (Fig. 25). A correlation between the relative copy numbers detected by Southern blot analyses (Fig. 21) and protein expression was not observed. This often reported finding may be attributable to the randomly selected site of integration of the transgene, which can affect the pattern and strength of expression [30]. Because sgp130-Fc is a soluble protein, its secretion was confirmed. Primary astrocytes derived from the cortices of transgenic mice released the transgenic sgp130-Fc into the medium (Fig. 27). That additionally identified the cortex as one of the regions of expression.

The capability of sgp130-Fc to bind the complex of IL-6/sIL-6R that defines its biological function was verified by an *ex vivo* binding/immunoprecipitation test (3.2.4.2, Fig. 29). Accordingly, sgp130-Fc present in transgenic brain homogenates precipitated the added Hyper-IL-6 (IL-6 coupled to sIL-6R, [70]), revealing the biological activity of the transgenic protein. This was expected, because sgp130-Fc of transgenic mice based on the same expression construct composition but with a PEPCK promoter, which induce gene expression mainly in liver and kidney (protein in circulation), had been shown to be functionally active via the same test. The transgene functionality in these mice was additionally reflected e.g. in reduced acute phase response after Hyper-IL-6 challenge, due to inhibition of IL-6 trans-signalling by the transgenic protein sgp130-Fc [220]. Blockade of IL-6 trans-signalling is only possible if a molar excess of sgp130-Fc is available. Jostock *et al.* [115] revealed that a fivefold molar excess was sufficient to completely inhibit Hyper-IL-6-mediated cell proliferation in BaF/3-gp130 cells (for information to the cells, see 3.1.1). Proliferation of BaF/3-gp130 cells induced via IL-6 plus sIL-6R has been blocked by a 10-fold molar excess

of sgp130-Fc versus sIL-6R [115]. Considering these results one could assume that at least a 10-fold molar excess of transgenic sgp130-Fc proteins would be sufficient to suppress IL-6 trans-signalling in the brain of gfa2–sgp130-Fc mice. Molar ratios between sgp130-Fc and sIL-6R were calculated from the sgp130-Fc protein levels measured in brain homogenates of different founder animals, under determination of the endogenous level of the murine sIL-6R (see 3.2.4.1). For the founder mice 5.2, 15.5 and 19.1 a molar excess of sgp130-Fc has been calculated to be 29- to 50-fold, thus more than a 10-fold excess (Table 1). Consequently, the central blockade of sIL-6R-mediated responses in gfa2–sgp130-Fc mice appears very likely.

Ultimately, it turned out that in the course of backcrossing the absolute protein amount of sgp130-Fc measured in brain homogenates of lines 5.2 and 15.5 was reduced (Fig. 34), compared to the corresponding founder mice (Fig. 25). Regardless, these sgp130-Fc levels still exceed the 10-fold molar excess that would be theoretically needed to suppress IL-6 trans-signalling-driven responses ([115], 3.2.4.4, Table 3). There are cases known were transgenic expression strongly varied between parental mice and their offspring and even under individual animals of a litter (not found in gfa2-sgp130-Fc mice) with no clear trend. Explanations for that phenomenon might be possible changes in DNA methylation leading to suppression of gene activity or alternatively genomic instability with deletions of some copies from tandem arrays or transgene rearrangement. Also cessation of transgene activity has been observed once, which never returned in subsequent offspring [208]. Therefore, analyses of sgp130-Fc protein levels are necessary in each newborn mouse generation. Moreover, after the 10^{th} backcross onto C57BL/6N, transgenic mice can be breed to homozygousity. Since homozygous animals carry their transgene on two alleles, an even higher sgp130-Fc protein production is expected.

The expression patterns of sgp130-Fc mRNA of several tissues were assessed by Northern blot analyses and the highly sensitive RT-PCR (3.2.4.3, Fig 30, Fig. 31). These methods exhibited expression predominantly in the brain but with low amounts present in lung, spleen, liver and kidney (the 2 latter only in RT-PCR). Most publications about transgenic mice under transcriptional control of a GFAP promoter reported activity of the appropriate transgene almost exclusively in astrocytes throughout the brain (rev. in [271]). But apart from astrocytic expression, which can also occur in spinal cord astrocytes [71], activity of gfa2-driven transgenes has also been directed to neurons of different brain regions, non-myelinating Schwann cells and reactive Müller cells in several cases (rev. in [30, 271]. The literature pointed out that outside of the brain GFAP promoter-driven genes were individually low expressed ectopically in tissues such as liver, heart, lung, kidney, spleen, skeletal muscle and

thymus (rev. in [271]). For example in gfa2–angiotensinogen transgenic mice, the transgenic protein was mainly localised in astrocytes of all brain regions, but additionally in neurons of the subfornical organ. Although angiotensinogen mRNA was found in liver, protein plasma levels remained low [172]. These findings of transgenic expression on the mRNA and/or protein level (but rather low) outside of the brain are consistent with the herein observed expression pattern for the gfa2–sgp130-Fc mouse. Su *et al.* [271] surmised that ectopic expression seems to be more frequent when an intron is located between promoter and the synthesised transgene, which was employed herein as well. Considering the presence of sgp130-Fc mRNA in liver, the question arose whether transgenic protein would be observed in circulation. Indeed, sgp130-Fc protein was detected in sera of all of the three established transgenic mouse lines, but at varying levels (3.2.4.4, Fig. 33, Fig. 34). It became evident that the transgenic protein contents of the brain did not correlate with the levels found in the serum. Although the line 5.2 showed the highest sgp130-Fc protein level in serum (about 8,800 ng/ml), only moderate amounts were quantified in brain homogenate (about 340 ng/ml). Similar moderate amounts in brain homogenate displayed line 15.5 (approximately 360 ng/ml), which contained much less sgp130-Fc protein in the serum (1,100 ng/ml) than line 5.2. With the highest protein levels of transgenic protein in the brain (1,900 ng/ml brain homogenate), line 19.1 revealed only a moderate serum level (6,400 ng/ml). These results could be related to a different magnitude of expression outside the brain between the mouse lines. Interestingly, the literature pointed out that the distribution and strength of non-astrocytic and ectopic expression respectively, may vary strongly between different lines of a transgenic strain, which was presumed to depend likely on both the transgene integration site and the expressed sequence [271]. In consequence, the transgene expression patterns in various organs need to be clarified for each established gfa2-sgp130-Fc line (5.2, 15.5, 19.1), which has yet to be performed in future.

4.3 Future directions

In this work two different approaches were taken to address the role of IL-6 trans-signalling for sleep-wake behaviour. Initially, the REM sleep-promoting effect of i.c.v. injected Hyper-IL-6 in rats was elicited by a dose of 500 ng/rat but not by 100 ng/rat at dark onset. To further reinforce this recent finding it would be reasonable to conduct such a sleep recording experiment with additional cytokine dosages between 100 and 500 ng/animal as well as above

to test dose-response relations. In order to expand the relevance of Hyper-IL-6 treatment on sleep, the same study should be achieved at light onset. Furthermore, it would be tempting to establish, whether sgp130-Fc challenge could abolish the Hyper-IL-6-induced REM sleep increase.

The alternative approach included the central blockade of IL-6 trans-signalling within the brain without affecting classic signalling. That was realised in the herein generated transgenic gfa2–sgp130-Fc mice. In the future, these animals will be subjected to EEG/EMG-recording studies of sleep-wake behaviour, in comparison to wildtype mice, IL-6 KO mice [131], GFAP-IL-6 mice [36] as well as pepck–sgp130-Fc mice (showing systemic expression, but not within brain) [220]. In this respect, it will be interesting to further explore the sleep architecture after sleep deprivation, LPS challenge or Hyper-IL-6 injection. Given the strong mutual connection between diseases and altered sleep (see 4.1.3., 1.6.3), a coupling to a disease/infection model could be valuable. In addition to the sleep recording, also biochemical measurements of sleep modulatory substances (such as GH, VIP, prolactin, IL-17, cortisol) or mRNA expression of sleep-related genes [46] (gene array analyses) could be examined. Since memory and learning is consolidated during sleep [166, 270, 284] and IL-6 is considered to be involved in memory formation and plasticity [5, 7, 16, 166], these issues will be investigated as well. For instance, a recent study demonstrated in IL-6 KO mice impaired hippocampus-dependent and -independent memory processes, a higher susceptibility for stress, as well as reduced exploratory and despair behaviour. These results have been carried out by Morris water maze, novel object recognition memory test, open field, forced swimming test and elevated plus maze [5]. Such test setting will also be utilised for the gfa2–sgp130-Fc mice to explore behaviour and memory in comparison to wildtype animals. As aging is accompanied by cognitive dysfunction [95, 261], memory and behaviour in mice of different ages could be compared.

The cytokine IL-6 exerts multiple and diverse functions in the CNS. It is well characterised for its dual role to be neuroprotective/neurotrophic in normal brain functions but also neuroinflammatory/deleterious (see 1.1, 1.5). Many neural cells only react to IL-6 in the presence of sIL-6R, thereby it was assumed that trans-signalling is of importance in neuronal differentiation and survival responses (see 1.3, [162]). Also the role of trans-signalling in pathophysiological conditions of the brain is of special interest, since IL-6 and/or sIL-6R increase during neuroinflammatory/degenerative disease states, such as MS, stroke and depression (see 1.5, [63, 325]). Consequently, the gfa2–sgp130-Fc mice with the continous inhibition of IL-6 trans-signalling within the brain could aid to clarify the impact of sIL-6R-

transduced IL-6 signalling in animal models of EAE (model of human MS) (see 1.5), stroke (cerebral ischemia) [61, 105], traumatic brain injury [171], gliosis (tumors originated from astrocytes) [38], cytokine-induced depression [148] or viral/bacterial meningitis [186, 325].
For instance, in C57BL/6 mice anti-IL-6R antibody treatment (i.p., peripheral) inhibited the onset of EAE and the induction of MOG-specific Th17 cells [251], whose development is promoted by IL-6 together with TGFβ [17, 296]. Similarly IL-6 KO mice, which are characterised by absence of any IL-6 signalling (peripheral and in the brain), remained resistant to EAE, [65]. Suppression of IL-6 trans-signalling in rats by i.v. (peripheral) application of sgp130-Fc could not prevent but delayed the EAE onset. In the same publication, Linker *et al.* assumed a role for trans-signalling in the early effector phase by regulating the expression of adhesion molecules at the BBB [146]. Although priming of MOG-specific autoreactive T cells, including Th17 cells, occurred in the periphal immune system, the blockade of trans-signalling in the brain of transgenic gfa2–sgp130-Fc mice could give more clues for the entry of autoreactive T cells into the brain and the development of EAE.
Likewise, it is conceivable to crossbreed the gfa2–sgp130-Fc mice with GFAP–IL-6 mice which targeted the expression of IL-6 to astroytes by the 13-kb murine promoter GFAP2 [36]. GFAP–IL-6 mice developed a number of neurodegenerative changes in the brain, that might e.g. contribute to learning deficits of these mice. In addition, a gliosis with strong activation of astrocytes and microglia was found. In spite of the neuroinflammatory state in GFAP–IL-6 animals, the neuroprotective potential of IL-6 has been emphasised by studies of acute traumatic brain injury [38]. How would these outcomes change in GFAP–IL-6/gfa2–sgp130-Fc double transgenic mice; would there be a counterreaction? Resolving these questions will further provide valuable insight for IL-6/sIL-6R-triggered events.
A bidirectional cytokine-mediated complex communication between the brain and the peripheral immune system maintains homeostasis and health. From a dysfunction within these processes, several disorders may evolve, including those with peripheral complications such as the autoimmune disease rheumatoid arthritis [63]. Available data point to an implication of the sIL-6R contributing to arthritic lesions [114]. Inhibition of IL-6 trans-signalling by soluble gp130 in mice led to an improved disease outcome in experimental arthritis [194]. In this respect, studying the effects of the selective blockade of IL-6 trans-signalling in the brain (gfa2–sgp130-Fc mice) on the course of arthritic joint inflammation may offer considerable clues for the IL-6 system within the interplay of CNS and peripheral immune system.

5 Summary

The cytokine Interleukin 6 (IL-6) is involved in many biological functions of the immune system, metabolism and the central nervous system, and in the bidirectional communication between these systems. In the brain, IL-6 is considered to affect the modulation of sleep-wake behaviour and synaptic plasticity. IL-6 is further well characterised for its dual role to maintain homeostasis as well as to contribute to inflammatory and autoimmune processes, both in brain and periphery. Signal transduction of IL-6 is induced by binding of IL-6 to the membrane-bound IL-6 receptor (IL-6R; classic signalling) or alternatively to the soluble form of the IL-6R (sIL-6R; trans-signalling). The membrane-bound glycoprotein 130 (gp130) is the signal-transducing receptor subunit in both signalling modes. Gp130 is ubiquitously expressed throughout the body, whereas IL-6R expression is restricted to distinct cell populations. Within the brain parenchyma the IL-6R is sparsely expressed, and thus the brain is primarily reliant on trans-signalling in its response to IL-6.

As a previous study revealed an upregulation of the peripheral sIL-6R in humans during sleep (Dimitrov *et al.*, 2006), the influence of IL-6 trans-signalling on sleep regulation/modulation was scrutinised in this work initially. The effect of the IL-6 trans-signalling mimetic designer cytokine Hyper-IL-6 (sIL-6R coupled to IL-6) was investigated on sleep architecture of rats by EEG/EMG sleep recordings. Hyper-IL-6 can activate almost all cells of the brain in contrast to IL-6 itself. Results of this present work demonstrated that an intracerebroventricular injection of Hyper-IL-6 (500 ng) before the dark phase into rats led to increased amounts of REM sleep accompanied by a reduced EEG power density. Non-REM sleep was not affected. These data define a new function of IL-6 trans-signalling within the complex network of REM sleep modulation. That might further be of importance for altered sleep behaviour under pathophysiological conditions in which sIL-6R and IL-6 are elevated (e.g. bacterial/viral infections, major depression).

A further approach to explore the impact of IL-6 trans-signalling on brain-regulated functions included the central “knock-out” of this signal transduction mode. For that purpose transgenic mice were generated in this work expressing the competitive antagonist of IL-6 trans-signalling sgp130-Fc under transcriptional control of the astrocyte-specific promoter gfa2 within the brain. The protein sgp130-Fc is a dimer of the human extracellular part of gp130 fused to the Fc-portion of a human IgG antibody. Expression and secretion of sgp130-Fc in the brain of transgenic mice on the protein level could be verified. The biological activity of the transgenic sgp130-Fc protein was confirmed by Hyper-IL-6 precipitation tests. Likewise,

the protein amounts of sgp130-Fc appeared to be sufficient for the blockade of sIL-6R-mediated cellular responses in the brain. The presence of sgp130-Fc mRNA in transgenic animals was detected predominantly in the brain. However, sgp130-Fc was also found in sera of the three different established mouse lines at varying protein levels, which did not correlate with the amounts observed within the brain.

The gfa2–sgp130-Fc transgenic mice generated in this work offer an useful "*in vivo* tool" to further study the relevance of IL-6 trans-signalling for sleep-wake behaviour, memory consolidation, and models of peripheral and neuropathological inflammation.

6 Zusammenfassung

Das Zytokin Interleukin 6 (IL-6) ist an vielfältigen Funktionen des Immunsystems, des Stoffwechsels und des Zentralnervensystems beteiligt, sowie an der bidirektionalen Kommunikation zwischen diesen Systemen. Im Gehirn beeinflusst IL-6 die Modulation von Schlaf-Wach-Verhalten und die synaptische Plastizität. Charakteristisch für IL-6 ist weiterhin dessen duale Rolle bei der Aufrechterhaltung der Homeostasis einerseits aber auch bei der Förderung von entzündlichen und autoimmunen Prozessen andererseits, sowohl im Gehirn als auch in der Peripherie. Die Signaltransduktion von IL-6 wird durch Bindung des IL-6 an den membranständigen IL-6-Rezeptor (IL-6R; klassisches *Signalling*) oder alternativ an die lösliche Form des IL-6R (sIL-6R; IL-6-*Trans*-*Signalling*) induziert. Das membrangebundene Glykoprotein 130 (gp130) ist die signalweiterleitende Rezeptor-Untereinheit beider *Signalling*-Arten. Gp130 wird ubiquitär im Körper exprimiert, wohingegen die Expression des IL-6R auf bestimmte Zellpopulationen begrenzt ist. Im Gehirnparenchym ist der IL-6R wenig exprimiert, deshalb ist das Gehirn in seiner Reaktion auf IL-6 vornehmlich vom *Trans*-*Signalling* abhängig.

Eine vorherige Studie beschrieb die Erhöhung des peripheren sIL-6R im Menschen während des Schlafes (Dimitrov *et al.*, 2006). Deshalb wurde in der hier vorliegenden Arbeit erstmals der Einfluss des IL-6-*Trans*-*Signalling* auf die Schlaf-Modulation erforscht. Die Wirkung des IL-6-*Trans*-*Signalling* imitierenden Designer-Zytokins Hyper-IL-6 (sIL-6R gekoppelt an IL-6) auf das Schlaf-Profil von Ratten wurde in EEG/EMG-Schlaf-Messungen untersucht. Hyper-IL-6 kann fast alle Zellen des Gehirns aktivieren, im Gegensatz zu IL-6 allein. Ergebnisse dieser Arbeit zeigten, dass eine intracerebroventrikuläre Injektion von Hyper-IL-6 (500 ng) kurz vor der Dunkelphase in Ratten den Anteil an REM-Schlaf erhöhte, begleitet von einer reduzierten EEG-Power-Spektrum-Dichte. Dabei wurde der Non-REM-Schlaf nicht beeinflusst. Diese Daten definieren eine neue Funktion der IL-6-Trans-Signaltransduktion innerhalb des komplexen Netzwerks der REM-Schlaf-Modulation. Das könnte ebenso bei pathophysiologischen Zuständen, in denen der sIL-6R sowie IL-6 erhöht sind (z.B. bakterielle/virale Infektionen, Depression), von Bedeutung sein.

Ein weiterer Ansatz, um die Auswirkung der IL-6-Trans-Signaltransduktion auf Gehirn-gesteuerte Prozesse zu untersuchen, beinhaltete der zentrale „Knock-out“ dieses Signaltransduktionsweges. Dafür wurden im Rahmen dieser Arbeit transgene Mäuse generiert, die den kompetitiven Antagonisten des IL-6-*Trans*-*Signalling* sgp130-Fc im Gehirn unter transkriptioneller Kontrolle des astrozytenspezifischen Promotors gfa2 exprimieren. Das

Protein sgp130-Fc ist ein Dimer des humanen extrazellulären Teiles von gp130, das über den Fc-Teil eines humanen IgG-Antikörpers verbunden ist. Die Expression und Sekretion von sgp130-Fc konnnte im Gehirn transgener Mäusen auf Proteinebene nachgewiesen werden. Die biologische Aktivität des transgenen sgp130-Fc-Proteins wurde in Hyper-IL-6-Präzipitationsversuchen bestätigt. Zudem erschienen die Proteingehalte an sgp130-Fc für die Hemmung sIL-6R-vermittelter zellulärer Reaktionen im Gehirn ausreichend zu sein. Das Vorhandensein von sgp130-Fc-mRNA transgener Tiere wurde vorwiegend im Gehirn detektiert. Dennoch wurde sgp130-Fc auch in Seren der drei etablierten Mauslinien mit unterschiedlichen Proteingehalten gefunden, die nicht mit den Mengen im Gehirn korrelierten.

Die in dieser Arbeit erzeugten gfa2–sgp130-Fc-transgenen Mäuse stellen ein geeignetes „*in vivo*-Werkzeug“ dar, um die Bedeutung des IL-6-*Trans-Signalling* für das Schlaf-Wach-Verhalten, die Gedächtniskonsolidierung sowie Modelle peripherer und neuropathologischer Entzündungen eingehend zu studieren.

7 References

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8 Appendix

8.1 Abbreviations

°C	Celsius centigrade
µg	microgram
µl	microlitre
A	adenine
ACTH	adrenocorticotropin hormone
ADAM	a destintegrin and metalloprotease
Amp	ampicillin
APS	ammoniumperoxodisulfate
ATCC	*American Type Culture Collection*
ATP	adenosine 5'-triphosphate
BBB	blood brain barrier
bp	base pairs
BSA	bovine serum albumine
C	cytosine
CBM	cytokine binding module
CD	cluster of differentiation
cDNA	complementary DNA
Ci	Curie
CIAP	calf intestine alkaline phosphatase
CLC	cardiotrophin-like cytokine
cm	centimetre
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRH	corticotropin releasing hormone
CRP	C-reactive protein
CT-1	cardiotrophin 1
C-terminus	carboxy-terminus
dATP	desoxy adenosine triphosphate
DEPC	diethylpyrocarbonate

DMEM	Dulbeccos modified eagle medium
DNA	desoxyribonucleic acid
dNTP	2'-desoxyribonucleotide-5'-triphosphate
E.coli	*Escherichia coli*
e.g.	for example
EAE	experimental autoimmune encephalitis
EDS	excessive daytime sleepiness
EDTA	ethylenediaminetetraacetic acid
EEG	electroencephalogram
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
EMG	electromyogram
Epo	erythropoietin
ERK	extracellular signal regulated kinase
et al.	et alteri
FCS	fetal calf serum
FFT	fast Fourier transformation
Fig.	figure
FN III	fibronectin type III
G	guanine; glycine
g	gram; gravitational constant (=9.81 m/s^2)
GABA	gamma-amino butyric acid
GDP	guanosine triphosphat
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GH	growth hormone
GHRH	growth hormone-releasing hormone
gp130	glycoprotein 130
Grb2	growth factor receptor bound protein
GTP	guanosine diphosphat
h	hour
HPA	hypothalamic-pituitary-adrenal
Hyper-IL-6	Hyper-Interleukin 6
Hz	Hertz

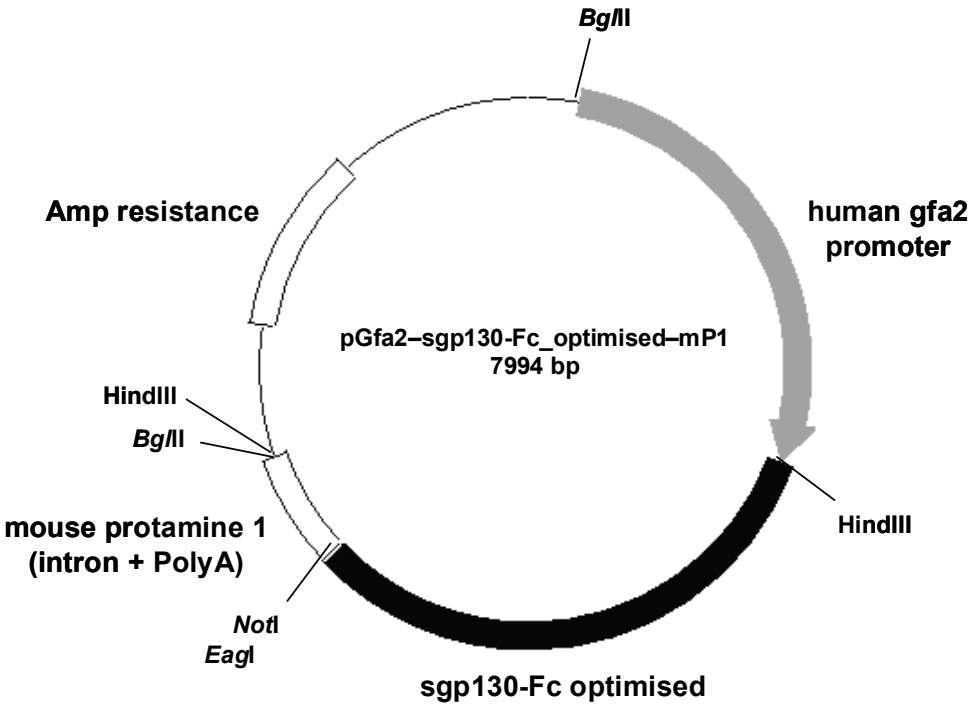
i.c.v.	intracerebroventricular
i.p.	intraperitoneal
ICAM	intracellular adhesion molecule
IFN	interferon
IGF	insulin-like growth factor
IgG	immunglobulin G
IL	interleukin
IL-1RA	interleukin 1 receptor antagonist
IL-6	interleukin 6
IL-6R	interleukin 6 receptor alpha
IP	immunoprecipitation
JAK	Janus kinase
kb	kilobase pairs
kDa	kilo-Dalton
kg	kilogram
KO	knock-out
l	litre
L	leucine
LB	Luria Bertani (medium)
LIF	leukemia inhibitory factor
LPS	lipopolysaccharide
M	molar
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
mg	milligram
min	minute
mJ	millijoule
ml	millilitre
mM	millimolar
mmol	millimol
mRNA	messenger RNA
MS	multiple sclerosis
msIL6-R	murine soluble interleukin 6 receptor
mV	millivolt

NF-κB	nuclear factor kappa B
ng	nanogram
NGF	nerve growth factor
NK	natural killer
nm	nanometre
NO	nitric oxide
non-REM	non rapid eye movement sleep
NP	neuropoietin
N-terminus	amino terminus
OD	optical density
OSM	oncostatin M
P	phosphor; phosphate
PAGE	polyacrylgelelectrophoresis
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline including Tween-20
PCR	polymerase chain reaction
PEPCK	phosphoenolpyryvate carboxygenase
Per1	period 1 (a circadian clock gene)
PIAS	protein inhibitors of activated STAT
POD	peroxidase
PolyA	polyadenylation site
P-STAT3	phospho-STAT3 (phosphorylated STAT3)
PVDF	polyvinylidene fluoride
R	receptor; arginine
REM	rapid eye movement
RNA	ribonucleic acid
rpm	rounds per minute
rRNA	ribosomal RNA
RT-PCR	reverse transcriptase polymerase chain reaction
s	second
S	serine
s.c.	subcutanous
S.D.	standard deviation
S.E.M.	standard error of mean

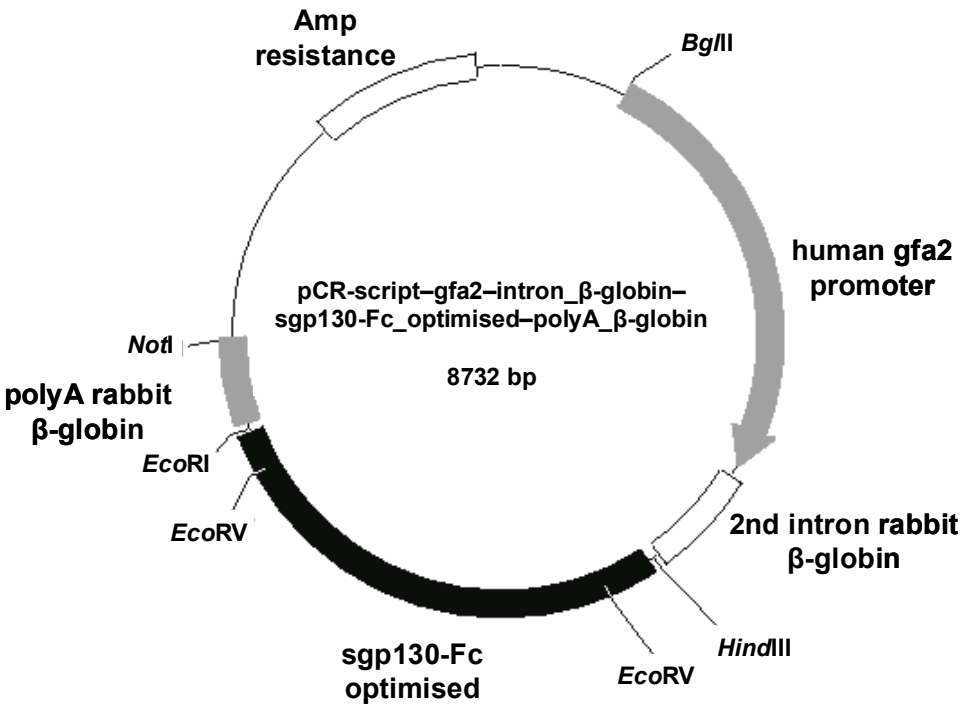
SCN	suprachiasmatic nucleus
SDS	sodium dodecyl sulfate
sgp130-Fc	soluble glycoprotein 130-Fc
SH2 domain	src homology 2 domain
SHP-2	src homology protein 2 tyrosine phosphatase 2
sIL-1R	soluble IL-1 receptor
sIL-6R	soluble interleukin 6 receptor
SN	supernatant
SOCS	suppressor of cytokine signalling
SOS	son of sevenless
SRS	sleep regulatory substance
STAT (Stat)	signal transducer and activator of transcription
sTNFR	soluble TNF receptor
SWS	slow wave sleep (in humans)
T	thymine
Taq	*Thermophilus aquaticus*
TBS	tris buffered saline
TBS-T	tris buffered saline including Tween-20
TEMED	N,N,N',N'-tetramethylenediamine
TF	transcription factor
tg	transgenic
TGF	transformic growth factor
TNF	tumor necrosis factor
U	Unit, Uracil
UV	ultraviolet
V	volt
VCAM	vascular cell adhesion molecule
VIP	vasoactive intestinal polypeptide
VLPO	ventrolateral preoptic (hypothalamic region)
W	tryptophan
Wake	wakefulness
X	an unspecified amino acid
Y	tyrosine

8.2 Vector maps

A pGfa2–sgp130-Fc_optimised–mP1



B pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin



8.3 Sequences

A Nucleotide sequence of the gene expression cassette cutted by *Bgl*II from the vector pGfa2–sgp130-Fc_optimised–mP1

```
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 351 GGCGCAGTGG CGCACAACTG TAATTCCAGC ACTTTGGGAG GCTGATGTGG

 401 AAGGATCACT TGAGCCCAGA AGTTCTAGAC CAGCCTGGGC AACATGGCAA

 451 GACCCTATCT CTACAAAAAA AGTTAAAAAA TCAGCCACGT GTGGTGACAC

 501 ACACCTGTAG TCCCAGCTAT TCAGGAGGCT GAGGTGAGGG GATCACTTAA

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1351 TCCAGTGGGA GACGGAGGGG TGGCCAGGGA AACGGGGCGC TGCAGGAATA

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1851 ATCAAAAAGT TGGAAAGCAG GTCAGAGGTC ATCTGGTACA GCCCTTCCTT

1901 CCCTTTTTTT TTTTTTTTTT TTGTGAGACA AGGTCTCTCT CTGTTGCCCA

1951 GGCTGGAGTG GCGCAAACAC AGCTCACTGC AGCCTCAACC TACTGGGCTC

2001 AAGCAATCCT CCAGCCTCAG CCTCCCAAAG TGCTGGGATT ACAAGCATGA

2051 GCCACCCCAC TCAGCCCTTT CCTTCCTTTT TAATTGATGC ATAATAATTG

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                          HindIII
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2651 TACTTCCACG TGAACGCCAA CTACATCGTG TGGAAAACCA ACCACTTCAC

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2951 AACTTCACCC TGAAGTCTGA GTGGGCCACC CACAAGTTTG CCGACTGCAA

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3051 ACTTCGTGAA CATCGAGGTG TGGGTGGAGG CTGAGAATGC TCTGGGCAAG
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3251 TACAACATCC AGTACAGGAC CAAGGATGCT TCTACCTGGT CTCAGATCCC
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4801 TGGCCAGCCC GAGAACAATT ACAAGACCAC CCCTCCTGTG CTGGATTCTG

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           NotI
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```

B Nucleotide sequence of the transgene expression cassette cutted by *Bgl*II and *Not*I from the vector pCR-script–gfa2–intron_β-globin–sgp130-Fc_optimised–polyA_β-globin used for microinjection to generate transgenic mice

```
cut BglII - 666 GATCT GAGCTCCCAC CTCCCTCTCT GTGCTGGGAC
 701 TCACAGAGGG AGACCTCAGG AGGCAGTCTG TCCATCACAT GTCCAAATGC
 751 AGAGCATACC CTGGGCTGGG CGCAGTGGCG CACAACTGTA ATTCCAGCAC
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 901 AGCCACGTGT GGTGACACAC ACCTGTAGTC CCAGCTATTC AGGAGGCTGA
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                                      HindIII
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EcoRV

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3951 AATGAGGGCA AGAAGATGAG GTGTGAGTGG GATGGCGGCA GAGAGACACA

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5901 CTGGATTCTG ACGGCTCCTT CTTCCTGTAC TCCAAACTGA CCGTGGACAA

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          EcoRI
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6351 GAAAAGCCTT GACTTGAGGT TAGATTTTTT TTATATTTTG TTTTGTGTTA

6401 TTTTTTTCTT TAACATCCCT AAAATTTTCC TTACATGTTT TACTAGCCAG

6451 ATTTTTCCTC CTCTCCTGAC TACTCCCAGT CATAGCTGTC CCTCTTCTCT

6501 TATGGAGATC CCTCGACGC - cut NotI
```

8.4 Publications

May, U., T. Schiffelholz, P.C. Baier, J.M. Krueger, S. Rose-John, and J. Scheller. 2009. IL-6-trans-signalling increases rapid-eye-movement sleep in rats. Eur J Pharmacol. 613:141-5.

Baier, P.C., **U. May**, J. Scheller, S. Rose-John, and T. Schiffelholz. 2009. Impaired hippocampus-dependent and -independent learning in IL-6 deficient mice. Behav Brain Res. 200:192-6.

Rabe, B., A. Chalaris, **U. May**, G.H. Waetzig, D. Seegert, A.S. Williams, S.A. Jones, S. Rose-John, and J. Scheller. 2008. Transgenic blockade of interleukin 6 transsignaling abrogates inflammation. Blood. 111:1021-8.

Koch, M., **U. May**, S. Kuhns, H. Drechsler, N. Adam, K. Hattermann, S. Wirtz, S. Rose-John, and J. Scheller. 2007. Interleukin 27 induces differentiation of neural C6-precursor cells into astrocytes. Biochem Biophys Res Commun. 364:483-7.

8.5 Curriculum vitae

Name: Ulrike May
Geburtsdatum: 10.11.1978
Geburtsort: Ludwigslust
Staatsangehörigkeit: deutsch

Wissenschaftlicher Werdegang:

2005 – 2009: Anfertigung der experimentellen Arbeiten zur vorliegenden Dissertation in der Arbeitsgruppe Prof. Rose-John/Prof. Jürgen Scheller am Biochemischen Institut der Medizinischen Fakultät an der Christian-Albrechts-Universität zu Kiel;
April-Mai 2008: 2-monatiger Forschungsaufenthalt in der Arbeitsgruppe Prof. James Krueger an der Washington State University (Pullman, WA, USA), Department of VCAPP, College of Veterinary Medicine

2002 – 2003: Diplomarbeit in der Abteilung Mikrobiologie am Fachbereich Biowissenschaften der Universität Rostock in der Arbeitsgruppe Prof. Hubert Bahl zum Thema: „Entwicklung eines ELISA-Testes zur Bindungsanalyse von SLH-Domänen aus *Thermoanaerobacterium thermosulfurigenes* EM1 an die Zellhülle“ mit Abschluss Diplom-Biologin

1997 – 2002: Studium der Biologie an der Universität Rostock; Diplomprüfungen in den Fächern Mikrobiologie, Molekularbiologie/Genetik, Immunbiologie und Medizinische Mikrobiologie

1991 – 1997: Besuch des Elbe-Gymnasiums in Boizenburg/Elbe mit Abschluss Abitur

1985 – 1991: Besuch der Polytechnischen Oberschule IV in Boizenburg/Elbe

Danksagung

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Eidesstattliche Erklärung

Hiermit versichere ich, Ulrike May, an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur mit Hilfe der angegebenen Hilfsmittel und Quellen unter Anleitung meiner akademischen Lehrer angefertigt habe.

Diese Dissertation wurde bisher an keiner anderen Fakultät vorgelegt.

Ich erkläre, kein anderes Promotionsverfahren ohne Erfolg beendet zu haben und dass keine Aberkennung eines bereits erworbenen Doktorgrades vorliegt.

Die vorliegende Arbeit entstand unter Einhaltung der Regeln guter wissenschaftlicher Praxis der Deutschen Forschungsgemeinschaft.

Kiel, den 26.04.2010

Ulrike May