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Narcoleptic episodes of laboratory rodents:

Development of therapeutic strategies and of a rat model

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ABSTRACT

Narcolepsy is a neurodegenerative disease with extensive daytime sleepiness, hypnagogic hallucinations and cataplexy. Narcolepsy is connected to the loss of orexin neurons in the lateral hypothalamus. The present dissertation summarizes two projects with different animal models. Narcoleptic episodes of orexin-deficient mice were treated with potential pharmacological agents (project 1). The development and evaluation of a narcoleptic rat model by stereotactic injections of neurotoxins in the lateral hypothalamus (project 2).

The orexin-deficient mice with a prepro-orexin gene deletion develop very similar symptoms to human narcoleptic patients. However, even though cataplexy in narcolepsy patients is treated since decades with antidepressant, it is still unclear via which pharmacological mechanisms they mainly exert their effects. Since antidepressants support monoaminergic function, important roles of the brain norepinephrine and serotonin systems were suggested. In project 1, orexin-deficient mice were treated with selective serotonin reuptake inhibitor and selective norepinephrine reuptake inhibitor to supress narcoleptic episodes. The data showed that narcoleptic episodes can be more efficiently suppressed by adrenergic stimulation than by serotonergic stimulation.

In project 2, a targeted neurotoxin, anti-Ox2R-SAP, was administered in the lateral hypothalamus to develop and evaluate a rat model. A reduction of orexin neurons was obtained. However, this reduction was unselective and went along with a loss of MCH neurons. A group of rats with a high number of orexin neurons (group α) and a group with an effective lesion of orexin neurons (group β) were compared irrespectively of the treatment in various behavioral paradigms. For instance, body weight increase was diminished significantly in group β. Notably, both groups did not express narcoleptic episodes.

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SCHLÜSSELWORTE

Orexin, narkoleptische Episoden, Orexin-KO Mäuse, SSRI, NRI, anti-Ox2R-SAP, Rattenmodell, LH, stereotaktische Operation

ÜBERSICHT:

Die neurodegenerative Erkrankung Narkolepsie zeichnet sich durch den Verlust von Orexin-produzierenden Neuronen aus. Neben der verstärkten Tagesschläfrigkeit ist Kataplexie das krankheitsdifferenzierende Symptom, welches sehr oft durch starke emotionale Erregung ausgelöst wird und mit minutenlangem Verlust des Muskeltonus einhergeht. Ziele der vorliegenden Arbeit waren es, verschiedene potentielle pharmakologische Behandlungen von narkoleptischen Symptomen in einem bereits etablierten Maus-Modell für Narkolepsie zu vergleichen (Projekt 1), sowie ein mögliches Narkolepsie-Modell in der Laborratte zu evaluieren (Projekt 2).

Obwohl Antidepressiva bereits seit langem zur Therapie von narkoleptischen Symptomen eingesetzt werden, gibt es immer noch Unklarheiten zur Pathophysiologie. Da Antidepressiva vor allem die monoaminergen Transmitter im Gehirn modulieren, wurde der Effekt von selektiven Wirkstoffen untersucht. Im Projekt 1 erhielten prepro-Orexin-Knockout-Mäuse einen selektiven Serotonin-Wiederaufnahmehemmer oder selektiven Noradrenalin-Wiederaufnahmehemmer zur Unterdrückung der narkoleptischen Episoden. Zusammenfassend ließen sich narkoleptische Episoden sowohl durch noradrenerge als auch serotonerge Stimulation potent unterdrücken. Die noradrenerge Stimulation war im Vergleich bereits bei niedrigeren Dosierungen effektiv.

Im Projekt 2 wurde durch stereotaktische Injektion von anti-Ox2R-SAP ein Narkolepsie-Modell in Laborratten entwickelt. Die immunhistochemische Kontrolle ergab eine unselektive Schädigung der Orexin- und MCH-Neurone im LH. Nach Einteilung der Tiere in eine Gruppe mit vielen Orexin-Neuronen (group α) und großen Neuronenverlust (group β) konnten dennoch Verhaltensexperimente ausgewertet werden. Beispielweise gibt es einen signifikanten Unterschied bei der Gewichtszunahme. Narkoleptische Episoden traten in beiden Gruppen nicht auf.

I. Table of Contents

II. List of Abbreviations

anti-Ox2R-SAP	Orexin-2 receptor antibody attached to saporin
ARAS	Ascending reticular activating system
ARC	Arcuate nucleus, tuberal region and medial cluster of the hypothalamus, influences feeding behavior
BSA	Bovine serum albumin
Cir	Cirazoline
CNS	Central nervous system
CS	Conditioned stimulus
CSF	Cerebrospinal fluid, taken by lumbar puncture
DA	Dopamine
DAG	diacyl glycerol, a second messenger signaling lipid produced by enzyme phospholipase C activity
DMH	Dorsomedial hypothalamic nucleus, tuberal region and medial cluster of the hypothalamus, influences behavioral circadian rhythm as blood pressure and heart rate
DORA	Dual orexin receptor antagonist (competitive on Ox1R and Ox2R)
DRN	Dorsal raphe nucleus, serotonergic nucleus; part of the ARAS
DSM-5	Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition 2013
ECG	Electrocardiography
EDS	Excessive daytime sleepiness, main symptom of narcolepsy
EEG	Electroencephalogram
EMA	European Medicines Agency; European Union agency for the evaluation and approval of drugs and medicinal products
EMG	Electromyogram
ER	endoplasmic reticulum, a type of organelle which regulates the intracellular calcium ion concentration
ERK	extracellular signal-regulated kinases, protein kinases which are part of the intracellular signalling
Esc	Escitalopram
ESS	Epworth Sleepiness Scale
eVLPO	extended part of the VLPO; see VLPO
FDA	Food and Drug Administration
g	Gram
$G_{i/O}$ & G_q	heterotrimeric G protein alpha subunits
GABA	γ-Aminobutyric acid; main inhibitory neurotransmitter in the mammalian brain
$GABA_B$	GABA-B-receptor
GHB	γ-Hydroxybutyric acid
GHBR	γ-Hydroxybutyrate receptor (GPR172A); a GPCR
GIRK	membrane-located G-protein-coupled inwardly-rectifying potassium channel
GPCR	G-protein-coupled receptor
Hcrt1	see OxA
Hcrt2	see OxB
HcrtR1	see Ox1R
HcrtR2	see Ox2R
HLA	Human leucocytes antigen
ICD-10-GM	International Classification of Disease German Modification, Tenth Edition 2017

ICSD-3	International Classification of Sleep Disorders, Third Edition 2014
ICV	Intracerebroventricular
IgG-SAP	Rabbit IgG antibody attached to saporin
INN	International Nonproprietary Name
IP_3	inositol trisphosphate,an inositol phosphate signalling molecule
LC	Locus coeruleus, norepinephrine nucleus; part of the ARAS
LDT	Laterodorsal tegmental nucleus, cholinergic nucleus; part of the ARAS
LH	Lateral hypothalamus, part of the anterior, tuberal and posterior region as a lateral cluster of neurons containing Orexin/ Hypocretin neurotransmitters
lx	Lux, SI derived unit of illuminance
MAO_B	Monoamine oxidase inhibitors, Type B is more selective for dopamine reduction to 3,4-Dihydroxyphenylacetic acid
MCH	Melano-corticotropes hormone
mg	Milligram
ml	Millilitre
min	Minutes
MRN	Median raphe nucleus, serotonergic nucleus; part of the ARAS
MSLT	Multiple sleep latency testing
MWT 20	Maintenance wakefulness test in five trials of 20 min
Ncl	Nucleus, group of neurons in the CNS
NDRI	Norepinephrine–dopamine reuptake inhibitor
NGS	normal goat serum
NPY	Neuropeptid Y, neurotransmitter secreted by the ARC and PVN which has amongst others a major influence on food intake and obesity
NRI	Norepinephrine reuptake inhibitor
NREM	Non rapid eye movement sleeping stage
NSCC	non-selective cation channels mostlylocated in the plasma membrane
OTC	Over-the-counter drugs, non-prescription drug
OxA	Orexin A/ Hypocretin1; both names for the same neurotransmitter, which was discovered simultaneously in 1998 by two different scientist groups (Lecea et al. and Sakurai et al.)
OxB	Orexin B/ Hypocretin 2
Ox1R = HcrtR1	Orexin 1 receptor; GPCR with affinity OxA > OxB
Ox2R = HcrtR2	Orexin 2 receptor; GPCR with affinity OxA = OxB
Praz	Prazosin
PBS	Phosphate buffer solution (0.1 mol L^{-1})
PH	Posterior hypothalamus, posterior region and medial cluster of the hypothalamus
PIP_2	phospholipid which is located in the plasma membrane and reactant of the enzyme phospholipase C
PLC	enzyme phospholipase C, a membrane-bound enzyme which produces the second signalling messengers IP_3 & DAG
PPT	Pedunculopontine tegmental nucleus cholinergic nucleus; part of the ARAS
pre-LC	pre-locus coeruleus; a nucleus rostral of the LC
PSG	Polysomnography
PSQI	Pittsburgh Sleep Quality Index
PVN	Paraventricular nucleus, anterior region and medial cluster of the hypothalamus
Reb	Reboxetine
REM	Rapid eye movement sleeping stage

RIP	ribosome inactivating protein; group of plant proteins like saporin, which inhibits the eukaryotic protein synthesis
SBX	Sodium oxybate (sodium γ-hydroxybutyrate); sodium salt of γ-hydroxybutyric acid (GHB)
SCN	Suprachiasmiatic nucleus, anterior region and medial cluster of the hypothalamus, circadian pacemaker
SORA-1R	Selective orexin receptor antagonist Ox1R affinity Ox1R >> Ox2R
SORA-2R	Selective orexin receptor antagonist Ox2R affinity Ox1R << Ox2R
SOREMP	Sleep-onset REM-sleep periods
SSRI	Selective serotonin reuptake inhibitor
SSNRI	selective serotonin/-norepinephrine reuptake inhibitor
TASK	two-pore-domain potassium channel are regulated by signalling lipids, oxygen tension, mechanical stretch and G-proteins
TCA	Tricyclic antidepressants
TMN	Tuberomammillary nucleus, posterior region and lateral cluster of the hypothalamus, histaminergic nucleus; part of the ARAS
TNFR2	Tumor necrosis factor receptor 2
TRH	Thyrotropin-releasing hormone
TrpC	transient receptor potential channel, diacylglycerol-sensitive cation channel; subfamilies TrpC3 and TrpC6 are activated by PLC or Calcium store depletion
US	Unconditioned stimulus
vlPAG	ventrolateral part of the periaqueductal grey matter; descending center of autonomic function, motivated behavior and behavioural responses to threatening stimuli
VLPO	Ventrolateral preoptic nucleus, also known as the intermediate nucleus of the preoptic area, anterior region and preoptic cluster of the hypothalamus, inhibits the ARAS
VMN	Ventromedial nucleus, tuberal region and medial cluster of the hypothalamus, influences safety behavior
VTA	Ventral tegmental area, dopaminergic nucleus

1. General Introduction

1.1. Sleep

From a provocative viewpoint sleep is the usual, repetitive, boring and useless part of our life, but that is merely the tip of the iceberg. A closer look reveals the huge influence of night sleep on our daily life and is shown to be inexorable if we are not getting any. The protagonist in William Shakespeare's Henry IV quotes this conflict: "O sleep, O gentle sleep, nature's soft nurse, how have I frightened thee, that thou no more will weigh my eyelids down, and steep my senses in forgetfulness?". In fact, about 30% of the adult population experience poor sleep quality and insomnia (Bahr, 1983; Barclay et al., 2011). In Germany a newly published study by a health insurance company confirms the poor sleep quality of one third of its policyholders (Voermans et al., 2017).

In a descriptive manner, sleep is a relaxation of body and mind with diminished perception, paralysis of voluntary muscles and altered brain activity. Lacking this relief, sleep disorders occur under emotional and stressful circumstances – for example as a risk factor for suicidal ideation (Perlis et al., 2016). Sleep disorders are often associated with negative life-changing events which are defined as independent (e.g. death of a close relative, victim of robbery) or dependent events (e.g. financial or relationship problems, depression) (Gregory et al., 2006; Mezick et al., 2009).

Sleep has obviously beneficial impact on our well-being and intellectual potential (Adam and Oswald, 1984). Brain development in newborn mammalians requires sufficient sleep and regeneration (Maquet, 2001; Graven and Browne, 2008; Mindell and Lee, 2015; Simon et al., 2017). Even small disturbances of sleep like snoring reduces cognitive development (Piteo et al., 2011). A hypothesis of (Tononi and Cirelli, 2006) suggests that slow wave sleep influences the strength of synaptic bonding (Urbain et al., 2013; Galer et al., 2015). Paradox sleep (REM) may be important to strengthen new brain circuits. Furthermore a potential impact on metabolic recovery, on the function on the immune system and on the consolidation of memory are discussed (Bryant et al., 2004; Cipolli et al., 2013). With sleep, even gender matters. It has been published that women's sleep needs to be longer in order to be healthy (Darnall and Suarez, 2009). Especially REM sleep is reported to be a housekeeping process which removes the disturbance of parasitic modes which occur after growth of the brain or experience (Crick and Mitchison, 1983). Mutual effects of sleep and learning have been discovered in 2006 by Fogel and Smith. This research revealed that following a simple motor procedural learning period the duration of Stage 2 sleep, i.e. a NREM sleep phase, is increased (Fogel

and Smith, 2006). For instance Alzheimer's dementia patients show deprivation of short-term memory and significant sleep disturbance (Vitiello et al., 1990; McCurry et al., 2000).

Due to the basal role of sleep, insomnia is often a premonitory symptom as well as an aggravating factor in diseases like anxiety disorders, depression, obstructive sleep apnea, paraneoplastic syndrome, etc. (Die Trill, 2013; Kasper et al., 2015; Winokur, 2015). Daily routines are largely affected by insomnia and result in decreased performance at work (Pilcher and Huffcutt, 1996). A study of physicians suffering from insomnia revealed decreased clinical performance while operating, administering anesthesia or managing medical crises (Weinger and Ancoli-Israel, 2002).

But not only the absence of sufficient sleep is unhealthy. Kaneita hypothesized that depression is associated with the right amount of sleep. Less than 6 hours and more than 8 hours' sleep daily is associated with depression (Kaneita et al., 2006). The implication for general physicians must be to strictly assess sleep quality, duration and timing and state of mind as an early warning system for the sake of public health.

1.2. **Physiology of sleep**

According to Rechtschaffen and Kales, sleep can be categorised into five definable stages (Kales et al., 1968). They can be measured by means of an electroencephalogram (EEG), monitoring of eye movement and an electromyogram (EMG).

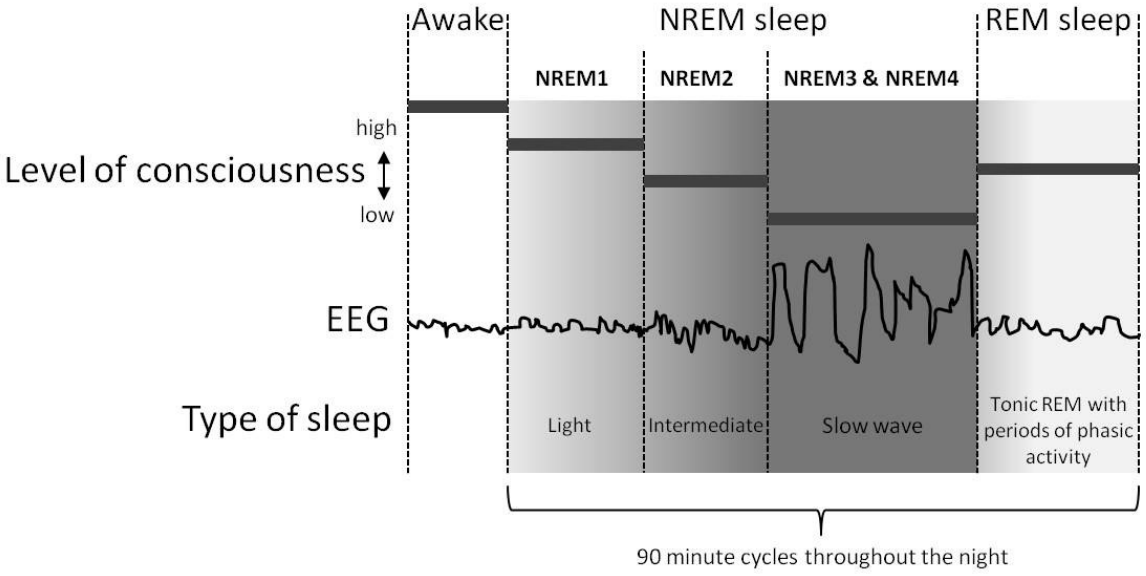

Fig. 1 Different sleep stages adapted from (Bryant et al., 2004)

Sleep is definable in a part with rapid eye movements (REM) and stages without rapid eye movement (NREM). REM sleep is often referred to paradox sleep. The physiological sleep (NREM sleep and REM sleep) is divided into five different stages which usually occur in an ordered sequence. The first phase begins shortly after falling asleep as a superficial sleeping stage 1 (NREM1). An EEG reveals less α-waves (8 – 13 Hz) and a progression of slower frequency like θ-waves (4 – 7 Hz). Subsequently in stage 2 (NREM2), the real moment of falling asleep, peaks like K-complexes and sleep spindles are visible in the EEG characteristic. The third stage NREM3, indicates the period of deep or slow wave sleep. In this phase, EEG shows more frequent δ activity (0.5 – 3 Hz) with an increased EEG signal. The first REM sleep stage occurs approximately 70 to 90 min after falling asleep. As the name indicates, this phase is represented by rapid eye movement. Furthermore, muscle tone is reduced whilst heart beats and breathing frequency are increased at the same time. This stage resembles the NREM1 phase and can usually be followed by awakening or returning to NREM1 (Rechtschaffen and Kales, 1968; Bryant et al., 2004). These 90 min cycles are repeated four to five times every night in an interlaced order. NREM3 stages are more dominant at the beginning of the sleeping period and REM stages at the end are more dominant. In newborns and children, the fraction of REM sleep is higher than 50% of the sleeping time, which indicates the enormous impact on brain development. In adulthood, REM sleep is reduced to 20% and NREM2, NREM3 and NREM4 slow wave sleep is reduced as well (Graven and Browne, 2008).

1.3. **Classification of sleep disorders**

A study by Foley et al. on sleep habits in elderly patients revealed that less than 16% had no sleep disturbances at all (Foley et al., 1995). Some authors report of a prevalence of 10 – 40% of the population with sleeplessness (Simon and VonKorff, 1997). In addition to the neurological effects mentioned above, these disturbances also have metabolic consequences (Kripke et al., 2002; Knutson et al., 2007).

Sleep impairments are classified in awakening or falling asleep disorders (insomnia), non-recreative nightsleep (parasomnia) and extensive daytime sleepiness with augmented necessity of sleep (hypersomnia). Three different classification systems are in use which characterize sleep impairment. The DSM-5 (Diagnostic and statistical Manual of Mental Disorders, American Psychiatric Association), the ICSD-3 (International Classification of Sleep Disorders) and the ICD-10 (International Statistical Classification of Disease and Related Health Problems) underline the difficulty of comprehending symptoms of sleep disorders.

According to the DSM-5 categorization model, ten conditions are distinguished which manifest themselves as disturbed sleep and by causing distress as well as impairment in daytime functioning

(Winokur, 2015). The conditions are insomnia, hypersomnolence, narcolepsy, breathing-related sleep disorders, circadian rhythm sleep-wake disorders, non-rapid eye movement (NREM) sleep arousal disorders, nightmare disorder, rapid eye movement (REM) sleep behavior disorder, restless leg syndrome and substance-induced sleep disorder (Thorpy, 2012).

The International Classification of Sleep Disorders (ICSD-3) combines seven major categories (insomnia, sleep-related breathing disorders, central disorders of hypersomnolence, circadian rhythm sleep/-wake disorders, parasomnias, sleep-related movement disorders and other sleep disorders). Compared to the DSM-5, which is a diagnostic classification for psychiatrists, the ICSD is open to every medical speciality concerned with sleep behavior diseases (International Classification of Sleep Disorders, 2014). It is a very elaborately characterization with disadvantages in clinical practice. Due to its claim to map all sleep disorders and complexity the usage for non-sleep specialists is challenging.

The recent international classification of disease in Germany (ICD-10-GM) separates non-organic sleep disorders (F51.-) from neurological sleep disorders (G47.-) in addition to the intoxication of sedative drugs (T42.-) (Deutsches Institut f. medizinische Dokumentation u. Information (DIMDI), 2016).

The precise anamnesis and diagnostic differentiation is therefore important for the treatment of patients with sleep disorders. The development, recent sleeping habits and familial background on sleep impairments are fundamental for the evaluation of an objective assessment of the habits. These can be obtained by using the Pittsburg sleep quality index (PSQI) or the Epworth sleepiness scale (ESS) as questionnaires. Subjective evaluation with a sleep diary should not be deployed to determine the disturbance (Buysse et al., 1991; Johns, 1991). The examination under polysomnography is the first line approach to rule out organic dysfunctions. An EEG, EMG, electrocardiography (ECG) and video monitoring are usually applied in these settings.

Although the impact of disturbed night sleep is well-known, physicians are caught between a rock and a hard place treating patients with sleep-related disorders. Although in an industrial society insomnia is a common symptom for patients which is addressed by many pharmacological and behavioral psychological approaches, long-term treatment still is an ongoing challenge.

1.4. **Narcolepsy**

Compared to insomnia narcolepsy is a rare sleep disorder. Some authors reported that 0.02%–0.18% of the populations of the US and Western European populations suffer from narcolepsy (Abad and Guilleminault, 2017). Others state that, narcolepsy occurs in one individual in every 2000 with a 40-fold higher probability if a first-degree relative is affected (Scammell, 2015). Even though, monozygotic twins are affected in 25% of cases if one of them is diagnosed positively. Nevertheless, an association with human leucocytes antigen (HLA) class II DR2 and DQ1 was found (Matsuki et al., 1987; Hublin et al., 1994). DR2 HLA is found in 100% Japanese, 90 – 95% of white and 60% of African American narcoleptic patients. A DQ1 subtype allele, DQB1*0602, is found in 88 – 98% of narcoleptic individuals. On the other hand, this genetic evidence is not suitable for screening the population because more than 99% of the DQB1*0602 conductors have no narcolepsy or any symptoms related to the disease (Mignot et al., 1997). A novel hypothesis suggests the influence of an autoimmune reaction on the disease. An environmental factor as birth order is significantly associated with narcolepsy risk. Third-born or later are more likely to be affected by the disease than second-born and first-born (Watson et al., 2012). In the Japanese population an association between tumor necrosis factor receptor 2 (TNFR2) gen and narcolepsy is discussed (Hohjoh et al., 2000; Scammell, 2003). Newly published reports state that narcolepsy with cataplexy is associated with the increase and activation of regulatory T cells and global T cell activation (Scammell, 2003; Wing et al., 2011; Lecendreux et al., 2017).

Although genetic links have been discovered, narcolepsy is a clinical diagnosis. In first attempts the disease was described by the Frenchman Jean Baptiste Edouard Gélineau (1828-1906) in 1880 and the German Karl Friedrich Otto Westphal (1833-1890) in 1877. Gélineau read a report of a patient with an irresistible and incessant propensity to sleep. In a case study, Gélineau described a 38-year old man with very frequent narcoleptic sleep attacks which add up to 200 attacks per day. A wide array of intense emotional states played a prominent role in triggering his sleep attacks. He was the first to figure out that emotions are triggering the sleep attacks and named the disease narcolepsy. Westphal presented two cases of narcolepsy-cataplexy at the Berlin Medical and Psychological Society meeting in 1877. He described that the patients lost muscle tone but did not lose consciousness during cataplectic attacks and had night-time sleeplessness. Furthermore, he suggested a familial link, because the mother of his patient had similar cataplexy attacks. Besides the excessive daytime sleepiness (e.g. sleep attacks) the eye catching symptom for Gélineau and Westphal was the sudden loss of gravity muscle tone. Those cataplectic attacks were first named by Henneberg in 1916 as “cataplexy” (Schenck et al., 2007). Later on, after the development of sleep surveillance with EEG and EMG it was discovered that sleep attacks and cataplectic attacks occur

often simultaneously and are not distinguishable without EEG and EMG. The early REM onset as another main symptom was first described in 1960 (Vogel, 1960).

Classically narcolepsy is described as a tetrad of symptoms consisting of excessive daytime sleepiness (EDS), cataplexy, sleep paralysis and hypnagogic hallucinations (Yoss and Daly, 1957). Even though there are more symptoms as described above, not all of them appear in every narcoleptic patient. The fear of night-time hallucinations is associated with sleep onset insomnia, frequent awakenings are caused by periodic limb movement or nightmares. Moreover automatic behavior and sleep apnea has been reported (Stores, 1999). In children it has been reported that facial grimaces, cataplectic facies, occur (Serra et al., 2008). This automatic behavior includes jaw opening, eyelid drooping, head rolling, or tongue thrusting movements (Pizza et al., 2013). Further comorbidities are obesity and sleep apnea (Inocente et al., 2013).

The four main symptoms are likely but not mandatory for the diagnosis of narcolepsy (Yoss and Daly, 1960). Based on the data of a UK national study by Morrish et al., it was reported that about 98% of patients suffer from EDS and 90% from cataplexy. They also described a mean time gap of 15 years with a range from 1 to 61 years in between first symptoms and receiving the diagnosis narcolepsy. The worsening of cataplexy had an especially significant impact on the likelihood of diagnosis (Morrish et al., 2004).

The diagnosis according to DSM-5 requires at least one other symptom or diagnostic hint besides EDS. The secondary criteria are the occurrence of cataplexy, REM sleep latency ≤15 minutes on nocturnal polysomnography (PSG), mean sleep latency ≤8 minutes on multiple sleep latency testing (MSLT) with ≥2 sleep-onset REM-sleep periods (SOREMPs) or a cerebrospinal fluid (CSF) hypocretin deficiency (Ruoff and Rye, 2016).

ICSD-3 divides narcolepsy into type 1 (narcolepsy with cataplexy) and type 2 (narcolepsy without cataplexy). The criteria are shown in the table below and define the symptoms more accurately. They might therefore be less practical in clinical day-to-day routine (Narcolepsy Link® | Diagnostic Criteria; Vignatelli et al., 2002; Ruoff and Rye, 2016). The classification as narcolepsy with or without cataplexy is consistent in the opinion of the authors who hypothesized that there are insufficient orexin levels in narcolepsy type 2. Cataplexy results from a higher orexin neuron loss in the patients (Scammell, 2003).

Less elaborated is the ICD-10 GM. Narcolepsy and cataplexy are classified as one entity and are coded as G47.4 in the section of neurological sleep disorders. No further marks take account of

narcolepsy without cataplectic attacks (Deutsches Institut f. medizinische Dokumentation u. Information (DIMDI), 2016).

narcolepsy type 1	**criteria A and B must be met**
A	Cataplexy and a mean sleep latency of ≤8 minutes and ≥2 SOREMPs on an MSLT performed according to standard techniques. A SOREMP (within 15 minutes of sleep onset) on the preceding nocturnal PSG may replace one of the SOREMPs on the MSLT.
B	CSF hypocretin-1 concentration, measured by immunoreactivity, is either ≤110 pg·mL^{-1} or <1/3 of mean values obtained in normal subjects with the same standardized assay.
narcolepsy type 2	**criteria A - E must be met**
A	The patient has daily periods of irrepressible need to sleep or daytime lapses into sleep occurring for ≥3 months.
B	A mean sleep latency of ≤8 minutes and ≥2 sleep-onset REM periods (SOREMPs) are found on an MSLT performed according to standard techniques. A SOREMP (within 15 minutes of sleep onset) on the preceding nocturnal PSG may replace one of the SOREMPs on the MSLT
C	Cataplexy is absent.
D	Either CSF hypocretin-1 concentration has not been measured or CSF hypocretin-1 concentration measured by immunoreactivity is either >110 pg·mL^{-1} or >1/3 of mean values obtained in normal subjects with the same standardized assay.
E	The hypersomnolence and/or MSLT findings are not better explained by other causes such as insufficient sleep, obstructive sleep apnea, delayed sleep phase disorder, or the effect of medication or substances or their withdrawal.

Tab. 1 Diagnosis Criteria for typ 1 and 2 narcolepsy according to ICSD-3 (Narcolepsy Link® | Diagnostic Criteria).

In addition to the official criteria there are screening questionnaires such as the “Stanford Center for Narcolepsy Sleep Inventory” a helpful tool for clinical routine in primary care. This questionnaire focuses on distiguishing between cataplexy and physiologic muscle weakness in the general population. Especially muscle weakness during sexual intercourse and during and after athletics activities have a high probability of being non-cataplectic events (Anic-Labat et al., 1999).

There are reports of associated narcolepsy with vaccination (Pandemrix®) of the H1N1 influenza-A-virus (O’Flanagan et al., 2014). The studies have been closely reviewed and some methodical bias was uncovered (Wijnans et al., 2016). Finally a hypothesis revealed a mimic of surface influenza nucleoprotein A and first extracellular domain of Ox2R. Positive patients antibodies against Ox2R were detected in sera of Finnish HLA DQB1*0602. In cases of deficiency of the blood-brain barrier those antibodies may act in CNS and might cause a narcoleptic phenotype (Ahmed et al., 2015). A combination of DQB1*06:02, young age, and particular immune stimuli

increases the risk of narcolepsy (Scammell, 2015). A recent study in Germany again correlates the risk of narcolepsy after vaccination and rules out any other risk factor. Taken together these studies show that following Pandemrix® vaccination the risk for narcolepsy is increased (Oberle et al., 2017; Sarkanen et al., 2018).

1.5. **Orexin/ hypocretin brain system**

The underlying pathophysiology of narcolepsy was unclear for a long time. But in 1998 the orexin/ hypocretin brain system was independently described by two different scientific groups. Both named the neuropeptide differently, therefore “orexin” and “hypocretin” refer to the same substance (Lecea et al., 1998; Sakurai et al., 1998). The lack of orexin-producing neurons resulting in low CSF levels of orexin was the missing link to narcolepsy. Alternatively, a dysfunction of postsynaptic orexin receptors is supposed (Nishino et al., 2000; Mignot et al., 2002; Scammell, 2003; Bourgin et al., 2008).

The hypothalamus is an integrating brain region for various autonomic and endocrine homeostatic processes such as cardiovascular, temperature and abdominal visceral regulation. Small neuropeptides are transmitters in these regulatory circuits. In 1996, Gautvik et al. used linear tag-PCR substraction to identify some of those neuropeptides (Gautvik et al., 1996). One year later, Sutcliffe (1997) and de Lecea (1998) encoded a 130-residue protein called prepro-orexin from this hypothalamus-enriched cDNA (Lecea et al., 1997). Subsequence analysis revealed two neuropeptides, orexin A (OxA or Hcrt1) and orexin B (OxB or Hcrt2). Orexin A is a 33 amino acid residue peptide with two intra-chain disulfide bonds, while orexin B is a linear 28 amino acid residue peptide. They are derived from a precursor, prepro-orexin by proteolytic processing and post-translation modification of a convertase. The physiological concentration of orexin B is age-related approximately 65% of the orexin A concentration, and the degradation in the CSF is much faster (Date et al., 1999; España et al., 2001; Matsumura et al., 2002; Tomasik et al., 2004).

Two G-protein-coupled receptors are found in the human brain. Some regions of the brain have an equal distribution of the Orexin-1- (Ox1R or HcrtR1) and Orexin-2-receptor (Ox2R or HcrtR2). Moreover both neuropeptides, Orexin A und B, which are produced by neurons within the lateral hypothalamus, have different receptor affinities. Orexin A binds to both receptors almost equally whilst slightly favoring Ox2R, whereas Orexin B has a distinct preference for Ox2R. The biophysiological consequences of these binding characteristics are still unclear (Sakurai and Mieda, 2011).

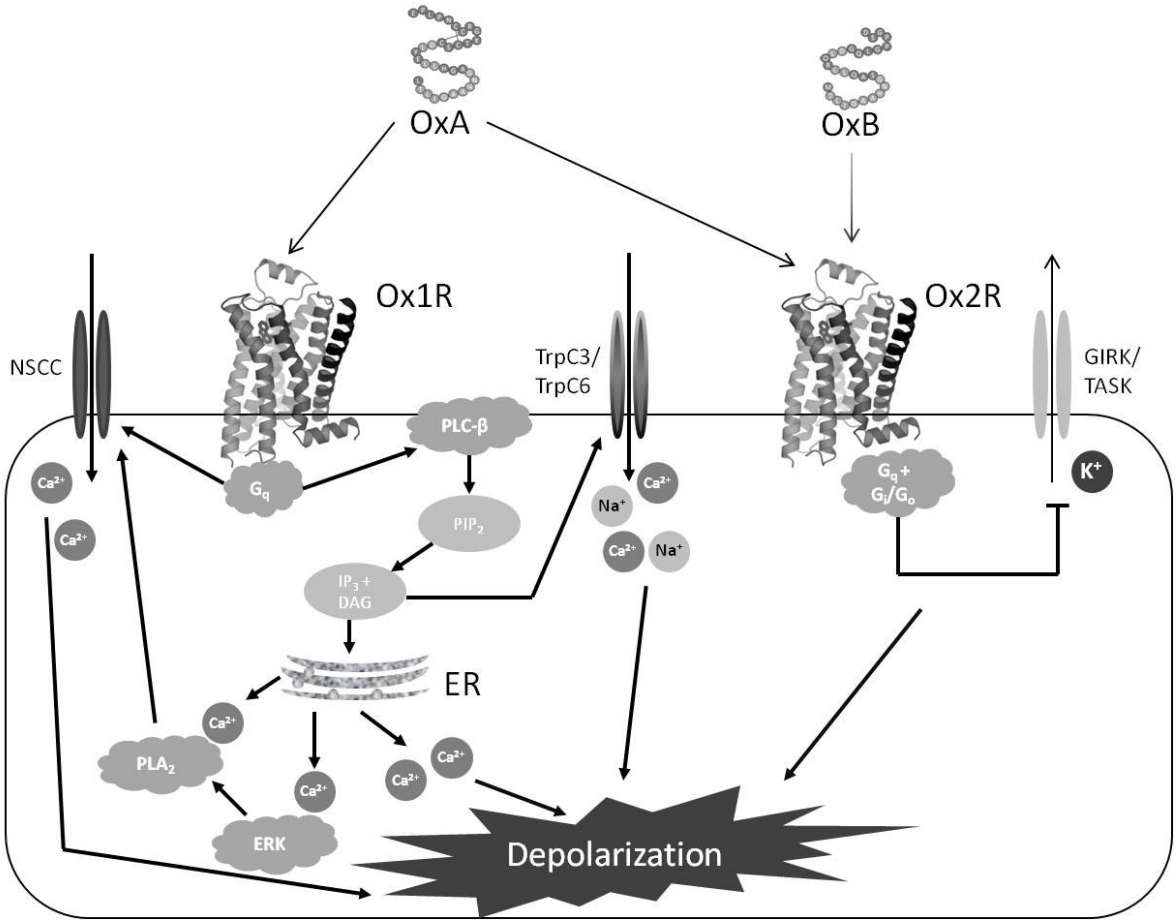

Fig. 2 Different mechanism of Ox1R and Ox2R receptor activation NSCC: PLA_2: PLC:adapted from (Kumar et al., 2016)

Orexin-producing neurons are localized in the lateral and dorsomedial hypothalamus including the perifornical nucleus as shown by studies investigating the distribution of preproorexin mRNA and OxA by immunohistochemistry. The investigation of the orexin neuron system revealed fibers projecting into almost every part of the brain. The orexin neurons project to the cerebral cortex, medial groups of thalamus, circumventricular organs (subfornical organs, area postrema), limbic system (hypothalamus, amygdala, indusium griseum), and brain stem (locus coeruleus, raphe nuclei). The strongest fiber staining in the brain stem was found in the locus coeruleus (LC) whereby the orexin fibers were situated next to the area with noradrenergic cells (Nambu et al., 1999). Orexin neurons receive information from the medial hypothalamus and distributes the integrated information in a similar pattern as melano-corticotropes hormone (MCH) producing neurons (Zheng et al., 2003). Especially neurons in the LC (norepinephrine), tuberomamillary nucleus (TMN, histaminergic), raphe nuclei (DRN, serotonergic), ventral tegmental area (VTA, dopaminergic) and cholinergic as well as non-cholinergic cells of the basal forebrain have sleep-wake regulating properties (Kumar et al., 2016).

In a study by Nishino the CSF level of orexin of nine narcoleptic patients was analyzed and compared to eight controls (Nishino et al., 2000). A lower CSF level of orexin was detected in seven narcoleptic patients. A hypothesis was formulated which suggests that receptor dysfunction or neuropeptide synthesis misconduct, related to reduced CSF orexin level, may result in a narcoleptic phenotype. It is assumed that a HLA-mediated autoimmune destruction is associated with narcolepsy. The two outliner patients in the study mentioned above were clinically different from the rest. In human cases the loss of 90% of orexinergic neurons has been identified although there are cases in the literature with a clinically distinct narcolepsy with the same CSF levels of orexin as unaffected individuals (Lin et al., 1999). Another possible explanation is receptor deficiency (Peyron et al., 2000; Mignot et al., 2002). The Ox2R gene mutation was earlier identified as a cause of canine narcolepsy. The occasional appearance of secondary narcolepsy as outlined in the chapter above following a H5N1 vaccination supports the hypothesis of an autoimmune link (Bassetti et al., 2019). On the other hand rare focal lesions of the posterior hypothalamus subsequent stroke, arteriovenous malformation or hypothalamic sarkoidosis result in narcolepsy (Scammell, 2003).

Cataplexy as a major symptom is characterized by sudden loss of muscle tone in highly emotional situations. In contrast to sleep attacks or usual sleep, in cataplexy consciousness is retained. It has been discussed that cataplexy is a failed expression of sleep paralysis during the day. Evidence for this hypothesis is that potent anticataplectic drugs such as venlafaxine or tricyclic antidepressants are suppressing REM sleep. These anticataplectic drugs enhance the levels of norepinephrine and serotonine which in turn inhibit REM-producing neurons in the dorsal pons (Scammell, 2003).

1.6. **Treatment of narcoleptic symptoms**

In general the therapy of narcolepsy is symptom-based. Due to gaps in understanding the pathoetiology there is no cure for the disease and life-long medication has to be administered. It has been beneficial to combine behavioral and pharmacological interventions. For instances daily power naps or brief naps of 20 minutes twice a day improve alertness and concentration. Some patients require at least one drug, but most of them two or more to lessen symptoms which impair quality of life (Billiard et al., 2006). Psychiatric disorders such as anxiety disorders or depression are more common in patients than in the general public. Besides the core symptoms of narcolepsy, patients struggle with various problems like physical tiredness or unemployment (Daniels et al., 2001). Socio-economic status is affected by sleep attacks and cataplexy due to reduced performance at work and in school. An appropriate therapy improves these manifold consequences of narcolepsy (Dodel et al., 2007).

Central stimulants are prescribed as first-line therapy for EDS, but are associated with addiction and tolerance after prolonged administration. Psychostimulants such as modafinil, its r-enantiomer, armodafinil and methylphenidate, as well as the GABAergic compound sodium oxybate (SBX) are FDA-approved drugs for adults and promote wakefulness (Black et al., 2009). Amphetamine-type stimulants have a high potency of addiction and various side effects such as hypertension, arrhythmia, restlessness, anorexia and disturbance of night-sleep. Due to their hunger-reducing properties, both the abuse and the addiction are likely; the number of prescriptions is therefore diminished nowadays. Methylphenidate is given 10 – 20 mg two times daily and is associated with headache, insomnia and irritability (Jacobsen, 1933; Aran et al., 2010).

Modafinil is a drug with reduced side effects and good potency for reducing sleepiness but unfortunately with no effect on cataplexy compared to amphetamines (Scammell, 2003). The mechanisms of actions is not fully elucidated but a binding on dopamine (DA) transporter, α-adrenergic alteration without receptor binding and reduction of extracellular GABA concentrations are described. Amphetamine-type substances as well as modafinil increase dopaminergic signaling which has wake-promoting effects (Wisor et al., 2001). The usual therapeutic dosage is 100 – 200 mg and may be increased to its maximum of 400 mg modafinil daily. Rash, headache and Stevens-Johnson syndrome are known as side effects. Both modafinil and armodafinil are first-line drugs in the treatment (Nishino and Mignot, 1997; Houghton et al., 2004; Harsh et al., 2006).

Compared to that, sodium oxybate, a gamma-aminobutyric acid derivate, is administered 4.5 g at the beginning and can be increased to double this dose. One major advantage is the potent treatment of both EDS and cataplexy. Some authors also described a reduction of hypnagogic hallucinations and decreased naps during daytime which confirms the appliance of a first-line therapy for narcolepsy and cataplexy (Lammers et al., 1993). While treating with SBX, development of tolerance and interactions with alcohol or other drugs should be disclosed to the patient. In high doses one third of the patients describe dizziness and nausea, more than 10% are obtaining urinary incontinence. Drug abuse may cause vomiting, coma, respiratory depression and death. Withdrawal is associated with anxiety, insomnia and delirium. Comorbidity sleep apnea and obesity must be ruled out before starting a treatment with SBX. The mechanisms of actions are mediated by coupling on the GHB and $GABA_B$-receptor (Xyrem International Study Group, 2005; Ristanovic et al., 2009; Abad and Guilleminault, 2017). Notably, SBX and its derivates are known to the general public as “liquid ecstasy” and have a baleful role as a “date rape drug” (Troester and Pelayo, 2015).

Only a few patients require extra treatment for cataplexy since cataplexy in most cases is mild and less frequent. For those who are treated with an amphetamine, methylphenidate or SBX the anticataplectic effects are often incorporated. In contrast, patients with modafinil as first-line

therapy have no beneficial effect regarding cataplexy. Therefore, a second drug is required to attenuate the cataplexy attacks. Traditionally tricycle antidepressants (TCA) such as clomipramine are administered, but due to their anticholinergic side effects and unspecific binding their usage is diminished. The instant withdrawal of TCA may increase the frequency of cataplexy or even create a status cataplecticus in humans. Modern selective reuptake inhibitors of serotonin (SSRI) and norepinephrine (NRI) may be an alternative. Nowadays selective serotonin/-norepinephrine reuptake inhibitors (SSNRI) such as venlafaxine, or NRIs like atomoxetine and reboxetine, MAO_B-Inhibitor selegiline or SSRIs as citalopram, sertraline, fluoxetine and paroxetine are administered as second-line medications. The usual therapeutic dose for venlafaxine is 37.5 mg and may be raised to 100 mg twice a day. Atomoxetine is given 40-80 mg/day to ameliorate cataplexy but is not FDA-approved for this indication. Nausea, dry mouse, headache, hypertension, tachycardia, liver injury, emergence of psychosis, erectile dysfunction and increased suicide risk in depressed patients are accompanying side effects(Houghton et al., 2004; Abad and Guilleminault, 2017).

Selective MAO_B-blockers like selegiline are administered 10 mg twice a day and have been become later choices for treatment of narcolepsy. They suppress REM-sleep and increase REM-onset latencies on both PSG and MSLT via blockade of DA metabolism (Thorpy, 2007).

SBX and baclofen both affect the $GABA_B$-receptor as agonists. A study comparing the compounds on narcoleptic children leads to significant advantages of SBX on frequency of cataplexy and EDS. Baclofen did not affect these symptoms of narcolepsy (Huang and Guilleminault, 2009). In contrast the r-enantiomer, arbaclofen (STX209; Seaside Therapeutics, Inc.), administered in knockout mice has effects on NREM-sleep, sleep intensity and consolidation (Black et al., 2014).

Promising new targets for narcolepsy are the H_3-Receptor inverse agonist pitolisant (BF2.649, tiprolisant), norepinephrine–dopamine reuptake inhibitor JZP-110 (ADX-N05, Aerial BioPharma) in adults and JZP 13-005 for children[1]. The orexin brain system affects histaminergic neurons in the posterior hypothalamus which control wakefulness, feeding, learning and memory via GPCRs (histamine H1, H2, H3 and H4 receptors). H3 suppresses histamine release due to presynaptic autoreceptor characteristics. Pitolisant may be administered 9 mg at the beginning and maintenance dose of 18-36 mg/day. Side effects are headache, insomnia, tiredness, nausea, anxiety, irritability, vertigo, depression, tremor, dyspepsia, abnormal weight loss and abortion. In Europe the EMA approved the drug for narcolepsy type 1 and type 2 as Wakix® in 2016. Several H_3-antagonists are in clinical trials: PF-03654746 (Pfizer), GSK189254, GSK239512 (GlaxoSmithKline), MK-0249, MK-3134

[1] The abbreviation with two or three letters and attached digits indicates the research code of the substances. The letters characterized often the investigating scientist or company i.e. “BF” = Bioprojet Pharma, “JZP” = Jazz Pharmaceuticals, “ADX” = Addex Therapeutics (Inocente et al. (2012); Ruoff et al. (2016)).

(Merck Sharp & Dohme), JNJ-17216498 (Johnson & Johnson), and ABT-286 (AbbVie). For most of them results have not been published, but for PF-03654746 the results of maintenance of wakefulness tests in five trials of 20 min (MWT 20). MWT 20 do not differ significantly and cataplexy is not suppressed. The results therefore suggest no benefit in the treatment of EDS or cataplexy (Inocente et al., 2012; Abad and Guilleminault, 2017). The NDRI are norepinephrine and DA reuptake inhibitor combined with no effects on serotonin reuptake. The advantage of JZP-110 (ADX-N05) is a mild inhibition of DA reuptake without effects on neither MAO nor direct release of monoaminergic agents. The results are promising regarding improvements in MWT and ESS which encourages a Phase III trial with doses of 75, 150, and 300 mg (Ruoff et al., 2016). Recently the FDA approved JZP-110 which is named soriamfetol (Bassetti et al., 2019).

A narcolepsy-related single nucleotide polymorphism which is located adjacent to carnitine palmitoyltransferase 1B gene has been discovered. The administration of L-carnitine in patients inheriting this polymorphism reduces EDS (Miyagawa et al., 2013). Further investigations on thyrotropin-releasing hormone (TRH) and analogs revealed that cholinergic turnover, dopamine and norepinephrine firing as well as locomotor activity are increased. In addition orexin cells are modulated by TRH. Potential targets for narcolepsy are these derivates of TRH (Ijiro et al., 2015).

The lateral hypothalamus contains melano-corticotropes hormone (MCH) neurons adjacent to orexin producing cells. The MCH neurons discharged during NREM but preferentially during REM sleep. MCH projections stimulate histamine-producing TMN cells and norepinephrine-producing LC cells. In addition, MCH cells release GABA and block wake-promoting circuits. Optogenetic stimulation of MCH cells trigger sleep onset (Bittencourt et al., 1992; Rao et al., 2008; Hassani et al., 2009; Blouin et al., 2013; Konadhode et al., 2013). In conclusion, the MCH receptor is a potential target for the treatment of narcolepsy.

1.7. **Novel treatments for narcolepsy**

The discovery of the orexin brain system and the insight of pathophysiology of narcolepsy offers novel treatment options for sleep related disorders. An antagonism of orexin might be valuable treating insomnia. A replacement of insufficient orexin production is advisable to decrease symptoms of narcolepsy.

Intravenous or intracerebroventricular (ICV) application of OxA is a reasonable approach to cope with neurotransmitter deficiency. But studies revealed small penetration of the blood-brain-barrier and short-term anti-cataplectic effects in animals (Fujiki et al., 2003; Mieda et al., 2004). Intranasal applications are an elegant way to bypass the blood-brain-barrier, therefore OxA may be

administered as nasal spray (Deadwyler et al., 2007). Improvement of REM sleep, but no effects on wakefulness have been disclosed (Baier et al., 2011; Weinhold et al., 2014). Another approach might be the development of orexin analogs with good penetration of the blood-brain-barrier and increased half-life.

There have been attempts to transplant the hypothalamus region of young rats into the midbrain of adult rat (Arias-Carrión et al., 2004). In addition, the same group published preliminary data on the transplant of orexin neurons into LH of anti-Ox2R-SAP lesioned rats. Unfortunately less than 5% of the orexin neurons survived the procedure longer than 36 days post-surgery. In future, stem cell transplantation from orexin-producing neuroblasts is suggested by the authors (Arias-Carrión and Murillo-Rodríguez, 2014).

Gene transfer might be another valuable technique to heal narcolepsy. The application of mouse prepro-Orexin gene by a viral vector into the LH of orexin-deficient mice reduces REM sleep in the second half of the night, while awaking did not differ to wild type mice and cataplexy by 60% compared to wild-type mice (Liu et al., 2008).

Taking account to possible autoimmune mechanism in emergence of narcolepsy immunosuppression holds options for the future. A case report of a narcoleptic patient treated with alemtuzumab (monoclonal anti-CD52, human IgG1κ-antibody) describes a total reduction of cataplexy. The patient suffered under daily cataplectic attacks, when low grade T-cell lymphoma was diagnosed. Under the therapy with alemtuzumab in contrast to other narcoleptic symptoms his cataplexy vanished and did not occur until his death (Donjacour and Lammers, 2012). This individual case might support efforts on investigating new treatment targets.

1.8. **Aim of dissertation**

1.8.1. **Project 1**

The neuropathology of narcolepsy was mainly investigated using dogs with a mutation of the *canarc-1* gene (Lin et al., 1999). Nowadays, orexin knockout mice are commonly used in narcolepsy or sleep related research. Different kinds of knockouts exist. Similar to the dogs there are mouse models with the lack of the gene of the Ox2R or a total depletion of both orexin receptor genes, i.e. the total loss of orexin 1 and 2 receptors (Kisanuki et al., 2001; Willie et al., 2003; Funato et al., 2009). Furthermore there are mouse models displaying the depletion of orexin-containing neurons by prepro-orexin gene replacement which have been used in project 1 in the present study or by orexin-specific expression of ataxin-3 (Chemelli et al., 1999; Hara et al., 2001; Kantor et al., 2009). In addition to these single knockouts there are also mice strains showing combined destruction of the

histaminergic and orexinergic brain system (Bastianini et al., 2015). Very many other combinations are realizable and conceivable to target sleep related diseases. In project 1 of the present MD thesis, orexin-deficient mice with a prepro-orexin gene deletion (B6.129S6-Hcrt^{tm1Ywa}/J) were used (Chemelli et al., 1999).

These animals develop very similar symptoms to human narcolepsy patients, e.g. cataplexy, sleep attacks and REM-onset sleep periods (Scammell et al., 2009). In *canarc-1* mutated dogs, cataplexy can be suppressed by specific monoamine reuptake blockers or tricyclic antidepressants (Mignot et al., 1993a; Nishino et al., 1993). However, even though cataplexy in narcolepsy patients is treated since decades with antidepressant, it is still unclear via which pharmacological mechanisms they mainly exert their effects. Since antidepressants support monoaminergic function, important roles of the brain norepinephrine and serotonin systems, i.e. the locus coeruleus and the nucleus raphe, were suggested. Interestingly, locus coeruleus activity is strongly suppressed during cataplexy whereas the nucleus raphe is still active (Wu et al., 1999), indicating that it is maybe more important to support norepinephrine function than serotonin function. This is supported by the observation that affinity to the norepinephrine transporter predicts the efficiency of antidepressants in treating canine narcolepsy but not the affinity to the serotonin transporter (Mignot et al., 1993a; Nishino et al., 1993).

These findings led to the hypothesis that a specific blockade of the norepinephrine transporter could be much more effective in treating cataplexy than a blockade of the serotonin transporter (Mignot et al., 1993a; Nishino, 2007a). Aim of project 1 was to test this working hypothesis. We therefore treated orexin-deficient mice with either with the specific serotonin reuptake inhibitor (SSRI) escitalopram or the selective norepinephrine reuptake inhibitor (NRI) reboxetine and tested the effects of these treatments on narcoleptic episodes. Motivated by the high efficiency of reboxetine in this experiment, we further examined the role of adrenergic α1 receptors. Orexin-deficient mice were therefore treated with the α1 receptor agonist cirazoline or the α1 receptor antagonist prazosin. For the latter, there is a case study published demonstrating pro-cataplectic effects (Aldrich and Rogers, 1989). Since potential effects of these compounds on locomotor activity are relevant for the interpretation of compounds' effects on narcoleptic episodes, we also performed an open-field experiment.

1.8.2. **Project 2**

Between species the main symptoms of narcolepsy (disturbed night sleep, exaggerated daytime sleepiness (EDS), cataplexy, early REM-onset and hypnagogic hallucinations) differ but in mammals

these five main symptoms are homologous. Especially cataplexy, which is triggered by emotional exciting moments and lapses in full consciousness, could be shown in dogs, mice and humans.

The aim of Project 2 for the present MD thesis was to create a rat model with a narcoleptic phenotype by stereotactic injection of an orexin-specific neurotoxin. This neurotoxin was applied to the lateral hypothalamus to target the orexin-producing cells. Rats are very benefical as an animal model for pharmacological treatment because of their homological metabolism and genetical proximity to humans (Gibbs et al., 2004). In addition, the organ size of the rat brain is suitable for stereotactic manipulation such as injections. Although the behavioral characteristics of these rodents differ in comparison to humans, many neural circuitries have the same mechanism and therefore provide a valuable model (Nestler et al., 2002).

A prior study used the neurotoxin saporin linked to OxB to specifically lesion orexin neurons by local injection into the lateral hypothalamus (Gerashchenko et al 2001). In the present project the coordinates for stereotaxic injection were adopted from the Gerashchenko publication. However, we used the neurotoxin saporin coupled to an anti-Orexin-2-receptor-antibody (anti-Ox2R-SAP). This new neurotoxin was kindly provided by Advanced Targeting Systems (San Diego, CA, USA). The antibody leads saporin to cells wearing the Ox2R on their outer surface. Saporin is a protein from the seeds of *Saponaria officinalis* which inactivates the biosynthesis of cell proteins and is a so-called ribosome inactivating protein (RIP). These RIPs block the ribosom by N-glycosidase activity of RNAs and result in cell apoptosis (Stirpe et al., 1983; Stirpe et al., 1992; Wiley, 1992). In the present experiment, the effects of these injections were compared to effects of non-targeted antibody against rabbit IgG with linked saporin (IgG-SAP) and control injections with saline.

Four weeks after the stereotactic manipulation a set of behavioral testing concerning narcoleptic symptoms such as EDS and narcoleptic episodes but also other behaviors was examined. These evaluations were repeated eight weeks after surgery. Afterwards, the twenty-week-old rats were sacrificed and the brain prepared for an immunohistochemical analysis. The extent of orexin neuron loss in the lateral hypothalamus was assessed. An immunohistochemical stain with orexin antibodies and as a control for nonspecific destruction with MCH antibodies was therefore conducted. A correlation of orexin neuron loss and behavior characteristics was performed to evaluate the effects the different injections. The following questions were raised in project 2:

- Do stereotactic injections of anti-Ox2R-SAP reduce the number of orexin neurons?
- Does the reduction of orexin neurons induce narcoleptic symptoms, influence other behaviors, or affect body weight?
- Is the rat model presented suitable as a model for narcolepsy?

2. Material and Methods - Project 1: Effects of escitalopram, reboxetine, prazosin and cirazoline on narcoleptic episodesin orexin-deficient mice

2.1. Animals

For the present experiments we used 35 homozygous orexin-deficient mice with an age of 12-14 weeks at the beginning of the experiment. These mice have a deletion of the prepre-orexin gene (B6.129S6-Hcrt^{tm1Ywa}/J), were originally purchased from the University of Texas, Dallas, USA (Chemelli et al., 1999) and backcrossed for more than 10 generations on C57BL/6 background. The mice were bred and reared at the local animal facility.

The animals were kept in groups of two to four under standard holding conditions (Macrolon type III cages, temperature- and humidity-controlled rooms (22±2 °C; 50-55%), 12 h – 12 h light-dark cycle with lights-off at 3 pm food pellets and water ad libitum, standard bedding material. All experiments were performed in compliance with international guidelines on the care and use of animals for scientific purposes (ARRIVE; 2010/63/EU) and approved by the local authorities (Landesverwaltungsamt Sachsen-Anhalt, Az. 42502-2-1173 Uni MD).

2.2. Substances

The following substances were used: cirazoline hydrochloride, reboxetine mesylate, escitalopram oxalate (Tocris Bioscience, Bristol, UK) and prazosin hydrochloride (Sigma-Aldrich, Steinheim, Germany). All substances were dissolved in saline and administered intraperitoneally (i.p.) at the beginning of an experimental session. The norepinephrine α1 receptor agonist cirazoline was applied at doses of 0.1, 0.3, and 1.0 mg·kg^{-1}, the norepinephrine α1 receptor antagonist prazosin at 0.3 and 1.0 mg·kg^{-1}, escitalopram (SSRI) at 1.0, 3.0 and 9.0 mg·kg^{-1} and reboxetine (NRI) at 0.05, 0.55, 1.66 and 5.0 mg·kg^{-1}. Doses were chosen based on literature data (Harkin et al., 2001; Cryan et al., 2005; Cunha et al., 2013; Młyniec and Nowak, 2013; Piotrowska et al., 2013; Młyniec et al., 2015).

2.3. Assessment of narcoleptic episodes (experiment 1)

2.3.1. Testing apparatus

Two black boxes (46 x 46 x 46 cm³) composed of polyvinylchloride were used. The boxes were equipped with bedding material (Abedd LAB & VET Service GmbH, Wien, Austria), two little wooden

gnawing bricks, two mouse tunnels, a running wheel and a nest box (see Figure 1 in (Leibiger and Fendt, 2014)). Pellets and water was available during the whole experimental session. The behavior of the mice was videotaped with an infrared camera including an infrared light source (VC-1026 Conrad electronics, Hirschau, Germany).

2.3.2. **Behavioral procedure**

Mice were always tested with all their cage mates. The animals had tail marks which could be easily recognized on the video tapes. Notably, the animals were once tested without any treatment before as well after the test trials with treatment. We used a repeated-measure design, i.e. each animal was tested with all doses of one drug in a pseudo-randomized order with a wash-out period of at least 3 days between the tests. For each test session, animals were put for acclimatization into the experimental boxes 2 h before light-off. Then, 5-10 min before lights-off, the injections were given. At lights-off, video-recording started for 4 h. The animals were put back to their homecage at the next morning.

2.3.3. **Behavioral scoring**

The video tapes were analyzed offline by an observer blind to the mice's treatment condition. Both the number and the duration of narcoleptic episodes were scored. Narcoleptic episodes were defined as periods of total inactivity outside the hiding box. Those inactivity periods lasted longer than 10 s and were preceded by activity longer than 40 s (Chemelli et al., 1999; Scammell et al., 2009; Leibiger and Fendt, 2014). This procedure was validated by Mang and Gee (Mang, 2010; Mang et al., 2012) by EEG/EMG recordings. Narcoleptic episodes with an abrupt onset, which occur to ca. 50% of the total number of episodes, match all criteria for cataplexy. The other half with a gradual onset were 'sleep attacks' with rapid transition into NREM sleep (Chemelli et al., 1999; Lin et al., 2008). Both of these narcoleptic symptoms are also found in human patients (Scammell, 2003; Nishino, 2007b).

2.4. Assessment of locomotor activity (experiment 2)

2.4.1. Testing apparatus

Testing took place in 4 identical, plain and empty boxes (45 x 45 x 30 cm^3; TSE System, Bad Homburg, Germany) serving as open-fields, composed of opaque polyvinyl chloride. The position of each animal was determined by infrared light sensors (14 mm distance between two sensors) and used to calculate the distance moved.

2.4.2. Behavioral procedure

The measurement of the locomotor activity was conducted at beginning of the lights-off period in complete darkness. The mice were tested in a repeated-measure design, i.e. every animal received in a pseudo-randomized order each of the four drugs as well as saline, with a wash-out period of at least 3 days between the tests. The following concentrations were used: 1.0 mg·kg-1 cirazoline, 1.0 mg·kg-1 prazosin, 9.0 mg·kg-1 escitalopram and 5.0 mg·kg-1 reboxetine. Following intraperitoneal drug injection, the mice were put into the open-fields and locomotor activity was recorded for 30 min.

2.4.3. Statistical analysis

For descriptive and statistical analysis, GraphPad Prism 6.0 (GraphPad Software, Inc., La Jolla, CA, USA) was used. Normally distributed data (locomotor activity) were analyzed by a two-way repeated-measures ANOVA followed by post-hoc Dunnett's test. For non-normally distributed data (number and duration of narcoleptic episodes), the Friedman test and post-hoc Dunn's tests were used. The pre- and post-tests without treatment served as a quality control and were not considered for statistical analysis.

3. Results - Project 1: Effects of escitalopram, reboxetine, prazosin and cirazoline on narcoleptic episodes in orexin-deficient mice

3.1. Experiment 1: Treatment effects on narcoleptic episodes

In figure 3, the number of narcoleptic episodes after escitalopram application is illustrated. Escitalopram dose-dependently decreased the number of narcoleptic episodes in orexin-deficient mice (Figure 3A; Friedman test: $Q = 21.77$, $P < 0.01$). Post-hoc comparison with the vehicle treatment revealed significant effects of the tested escitalopram doses (Dunn's tests: $P = 0.07$ for 1 mg·kg^{-1}, $P < 0.01$ for 3 mg·kg^{-1} and $P < 0.01$ for 9 mg·kg^{-1}). Besides the number of narcoleptic episodes, also the total duration spent in narcoleptic episodes is significantly reduced (Figure 3B; Friedman test: $Q = 19.45$, $P < 0.01$) with increasing doses of escitalopram. Post-hoc comparison with the vehicle treatment revealed significant effects of 3 and 9 mg·kg^{-1} escitalopram (Dunn's tests: $P = 0.18$ for 1 mg·kg^{-1}, $P = 0.02$ for 3 mg·kg^{-1} and $P = 0.01$ for 9 mg·kg^{-1}).

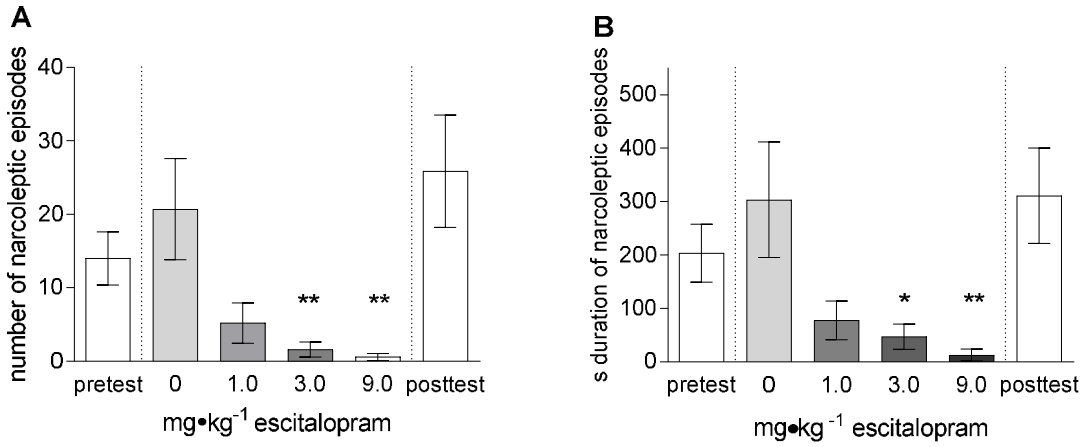

Fig. 3 Effects of escitalopram on mean number (A) and duration (B) of narcoleptic episodes (± SEM; 4h trial) in orexin-deficient mice (n = 10) [0, 1.0, 3.0, 9.0 mg·kg^{-1}]. Pretest and posttest were without injections. * P < 0.05; ** P < 0.01 (comparison with saline treatment).

Reboxetine treatment similarly affected mice behaviour and dose-dependently reduced the number of narcoleptic episodes in the orexin-deficient mice (Figure 4A; Friedman test: $Q = 30.20$; $P < 0.01$). In comparison to the vehicle treatment, reboxetine treatment of 0.55, 1.6 and 5.0 mg·kg^{-1} significantly suppressed the amount of narcoleptic episodes within the 4 hour test trial. The lowest dose of 0.05 mg·kg^{-1} reboxetine did not reveal any significant treatment effect (post-hoc comparison Dunn's tests: $P = 0.24$ for 0.05 mg·kg^{-1}, $P = 0.01$ for 0.55 mg·kg^{-1}, $P = 0.01$ for 1.66 mg·kg^{-1} and $P < 0.01$ for 5.00 mg·kg^{-1}).

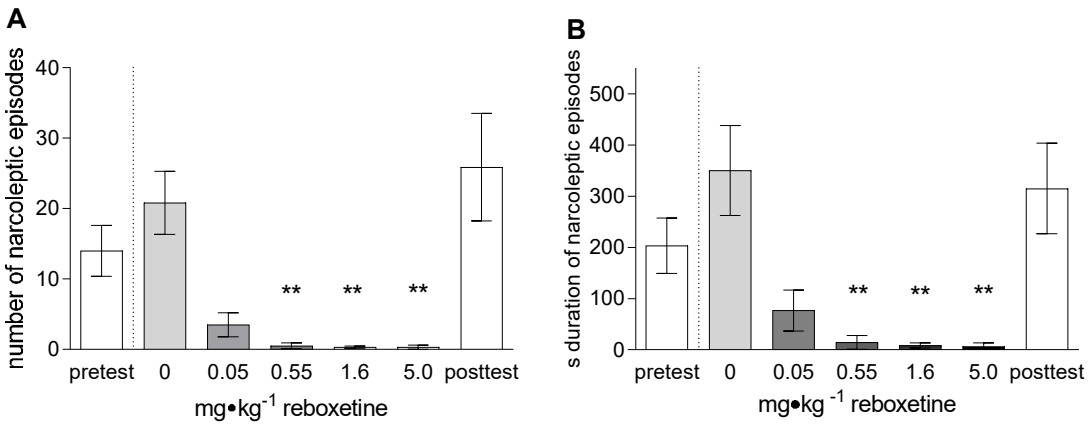

Fig. 4 Effects of reboxetine on mean number (A) and duration (B) of narcoleptic episodes (± SEM; 4h trial) in orexin-deficient mice (n = 10) [0, 0.05, 0.55, 1.66, 5.0 mg·kg^{-1}]. Pretest and posttest were without injections. * P < 0.05; ** P < 0.01 (comparison with saline treatment).

The same reboxetine doses also significantly decreased the total duration spent in narcoleptic episodes (Friedman test: Q = 31.20, P < 0.01, Dunn's tests: P = 0.20 for 0.05 mg·kg^{-1}, P = 0.01 for 0.55 mg·kg^{-1}, P < 0.01 for 1.66 mg·kg^{-1} and P < 0.01 for 5.00 mg·kg^{-1}). In contrast to the two reuptake inhibitors escitalopram and reboxetine, the two drugs acting on the adrenergic α1 receptors were ineffective.

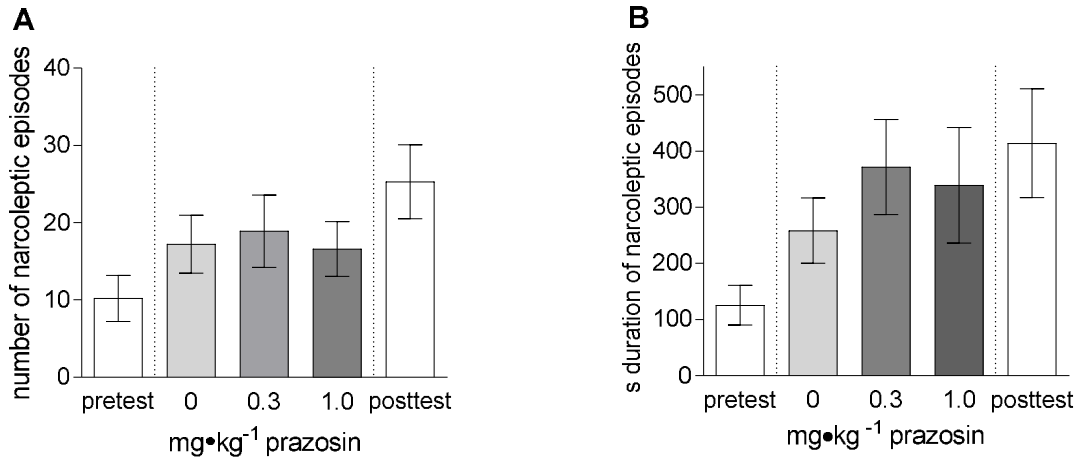

Fig. 5 Effects of prazosin on mean number (A) and duration (B) of narcoleptic episodes (± SEM; 4h trial) in orexin-deficient mice (n = 10) [0, 0.3, 1.0 mg·kg^{-1}]. Pretest and posttest were without injections. * P < 0.05; ** P < 0.01 (comparison with saline treatment).

The adrenergic α1 receptor antagonist prazosin neither significantly affected the number of narcoleptic episodes (Figure 5A; ANOVA: F(2,18) = 0.11; P = 0.89) nor the total duration spent in

narcoleptic episodes (Figure 5B; ANOVA: F(2,18) = 0.65; P = 0.52). The same was observed for the adrenergic α1 receptor agonist cirazoline: both, the number of narcoleptic episodes (Figure 6A; Friedman test: Q = 5.79, P = 0.12) and the total duration spent in narcoleptic episodes were not affected (Kruskal-Wallis test: H = 5.34, P = 0.15).

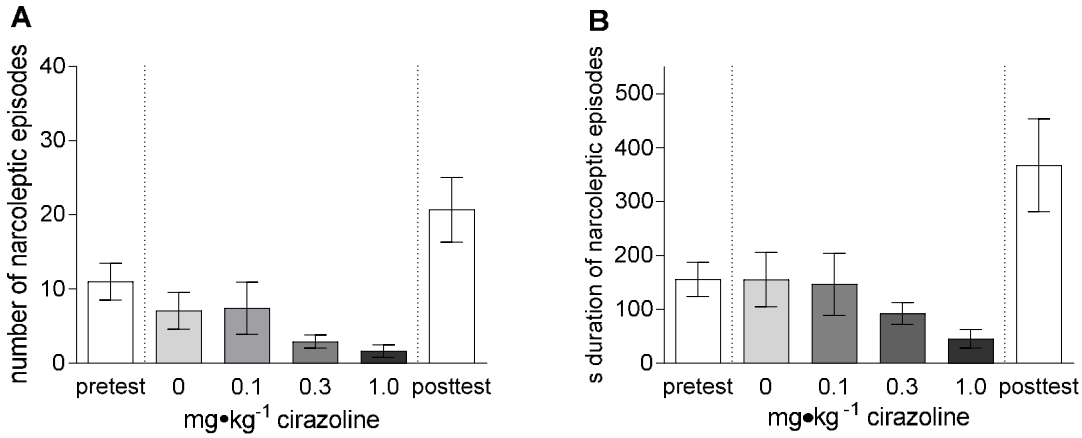

Fig. 6 Effects of cirazoline on mean number (A) and duration (B) of narcoleptic episodes (± SEM; 4h trial) in orexin-deficient mice (n = 14) [0, 0.3, 1.0 mg·kg^{-1}]. Pretest and posttest were without injections. * P < 0.05; ** P < 0.01 (comparison with saline treatment).

3.2. Experiment 2: Treatment effects on locomotor activity

Figure 7 shows the locomotor activity of orexin-deficient mice after intraperitoneal injection of saline, 9.0 mg·kg^{-1} escitalopram (SSRI), 5.0 mg·kg^{-1} reboxetine (NRI), 1.0 mg·kg^{-1} cirazoline, and 1.0 mg·kg^{-1} prazosin. For the sake of lucidity, the data with the reuptake inhibitors are shown in panel A, whereas the data with the drugs acting at the α1 receptor are shown in panel B. Statistical analysis of the obtained locomotor activity data, revealed a significant main effect of the factor treatment (F(4,48) = 5.33, P = 0.01) and time (F(5,240) = 9.50, P < 0.01) as well as a significant interaction of both factors (F(20,240) = 2.57, P < 0.01). Post-hoc Dunnett's multiple comparisons shows that locomotor activity was only affected after cirazoline treatment (t(5): P = 0.01; t(10): P = 0.03; t(15): P = 0.01) but not after treatment with the other drugs. The significant reduction in locomotor activity induced by cirazoline treatment was only obvious during the first 5 minute blocks.

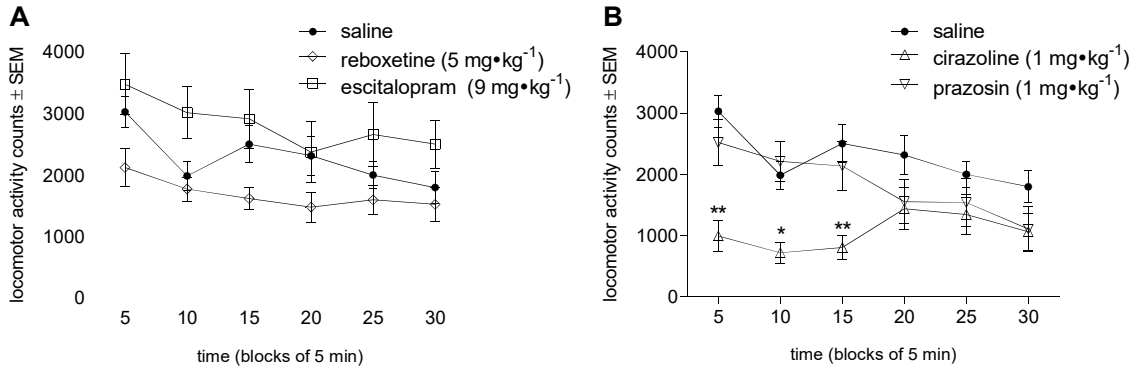

Fig. 7 Locomotor activity counts (means ± SEM) of orexin-deficient mice (n= 12) after treatment with saline, escitalopram (9.0 mg·kg^{-1}), reboxetine (5.0 mg·kg^{-1}), cirazoline (1 mg·kg^{-1}) and prazosin (1 mg·kg^{-1}) during a 30 min open-field test. * P < 0.05; ** P < 0.01 (comparison with saline treatment).

4. Material and Methods - Project 2: Local infusions of a putative orexin-specific saporin conjugate into the lateral hypothalamus of rats: Effects on orexin neurons and behavior

4.1. Animals

The present study was approved by the Landesverwaltungsamt Sachsen-Anhalt (Az. 42502-2-1173 Uni MD). The breeding took place in a certified animal facility at the Institute of Pharmacology and Toxicology of the Otto-von-Guericke University Magdeburg. The international guidelines of animal welfare were followed. The Sprague Dawley rats were kept under standard holding conditions in temperature- and humidity-controlled rooms (22±2 °C; 50-55%). The dark-light switch was set to a 12 h – 12 h cycle with lights-off at 6 pm.

Altogether 62 male Sprague-Dawley rats were tested in the experiments. They were kept in Macrolon type IV cages in groups of minimal three and maximal six animals. The tests were started when the animals obtained an age of eight weeks and gained a weight at least of 250 g. The food pellets (Sniff, Spezialdiäten GmbH, Soest, Germany) and water was available at all time besides the experiments. The cages were equipped with standard bedding material (Abedd LAB & VET Service GmbH, Wien, Austria).

4.2. Brain surgery with stereotaxic injection

The surgery was conducted either with intraperitoneal or inhalative anesthesia. In case of intraperitoneal anaesthesia flunixin as an analgetic drug was applied and the post-surgery monitoring was extended due to substance recede. The test animals were separated from the cage group and anaesthetized by an injection (3 ml·kg^{-1}) of a solution of ketamine (90 mg·kg^{-1}) and xylazine (10 mg·kg^{-1}) or isofurane (5 %(v/v), flow rate 2.2-2.4 l/min O_2) inhalation in an application chamber. The rats were fixed in the stereotaxic system by ear bars with continuous anesthesia via nose mask. After checking the blink and toe pinch reflex the skin was cut by scalpel. The sutures of the skull were exposed and the coordinates of bregma and lambda determined. It was aimed to have less than 0.2 mm difference on the dorsoventral coordinate between bregma and lambda. A 2 µl Hamilton syringe (23 gauge cannula, outer diameter, 0.642 mm, Hamilton 7102N, Hamilton Company USA, Reno, NV, USA) was attached to the stereotaxic apparatus to inject the test substances at the target coordinates. The coordinates for injection were taken according to (Paxinos and Watson, 1998).

In a pilot study an unilateral injection into the lateral hypothalamus were performed (rostrocaudal -3.3 mm, mediolateral +1.6 mm, dorsoventral -8.2 mm from dura mater). Later bilateral dual injections were aimed (rostrocaudal -3.2 and -3.4 mm, mediolateral ±0.6 and ±1.6 mm; dorsoventral -8.2 mm from dura mater). Target coordinates were calculated and the skull was opened up with an electric drill and a cavity burs (diameter 2.5 mm; Dentsply-Maillefer, Ballaigues, Switzerland). The ventral coordinates were obtained by placing the injection cannula on the dura mater. The patency of the hole was inspected with an injection cannula (B. Braun Melsungen AG, Melsungen, Germany). The injection syringe was filled with the substance and carefully lowered under stereotaxic control to its target coordinates. Different types of injections were made during the project. At the beginning single injection on one side, later on bilateral injection and furthermore two injections side on each hemisphere was bilaterally conducted.

The anti-Ox2R-SAP and as a control IgG-SAP or saline were injected at the coordinates described above. The substances were applied with a speed of 1.0 µl/5 min. With a latency of 2 min the cannula was retracted with a speed of 10 mm/3 min and later rinsed twice with saline to check for penetrability. The multiple injections were conducted clockwise in the same sequence (rostrocaudal/ mediolateral: -3.2 mm/ +0.6 mm, -3.2 mm/ -0.6 mm; -3.4 mm/ -1.6 mm, -3.4 mm/ +1.6 mm).

At the end of the surgery the scalp was sutured with an absorbable 4-0 Vicryl® filament (Ethicon Inc., Somerville, NJ, USA). The surgery was not conducted under totally aseptic condition, but in a clean, disinfected operating room and autoclaved instruments were used. For recovery the animals were kept separately from cage mates before joining them in their homecage.

4.3. Bodyweight

Beginning with the surgery the Sprague Dawley rats were weighted weekly by a laboratory balance (Mettler PM 3000, Mettler Toledo GmbH, Greifensee, Switzerland) at the same daytime. The results were joined in a table and evaluated.

4.4. Behavioral testing

Twice after surgery, after a four and an eight week period, the rats were placed in an enriched environment box (toys, food and water ad libitum) and video-taped for four hours in their dark period. The frequency and duration of behavioral arrests was analyzed by observer unknown to the treatment. This experiment was repeated four weeks later. Ensuing the animals was additionally assessed to different behavioral tasks which took place in the forenoon (light period). An open field test was followed by a light- dark box test, then tests on prepulse inhibition and fear-potentiated

startle were performed. Subsequently, a three day lasting monitoring of locomotion activity was performed. Finally at an age of at least 20 weeks all rats were sacrificed by an overdose of chloralhydrate.

4.4.1. **Test in behavior monitoring box**

The rats were observed four week past surgery for four hours in an enriched environment by video camera. The box (47 x 70 x 50 cm^3) was supplied with standard bedding material and a hiding box (24.5 x 17.5 x 12.5 cm^3). In addition locomotor activity of the animals were stimulated with a vertical running wheel and a transparent tube. Food and water was ad libidum available during the whole test. A few minutes before the usual light-off switch (6:00 pm) the animals were placed in the behavior monitoring box. An infrared camera combined to an infrared light source (VC-1026 Conrad Electronics, Hirschau, Germany) situated 1.4 m above the box recorded the animals for four hours. At 10:00 pm the rats placed back to their homecage and returned to the animal facility. The test in behavior monitoring box was repeated eight weeks after surgery. The number and duration of behavioral arrests were analyzed subsequent by an observer unknown to the treatment.

4.4.2. **Open field test**

The open field test was performed to evaluate locomotor behavior and risk assessment behavior of the rats in an entirely empty box. The locomotion activity of the test animals were tracked by ActiMot2 (TSE Phenomaster System). The rats were tested in a transparent box which was surrounded by a frame of infrared light-beams. Some were tested in a transparent box (50 x 50 x 40 cm, 32 infrared beams in 1.25 cm distance) or alternatively in a transparent box (45.5 x 45.5 x 31 cm, 16 infrared beams in 1.94 cm distance) under same conditions over 30 min in their light period.

No distractors such as food or water neither bedding material were contained in the box. The animals were placed single in each box. The locomotion was detected either with 16 or 32 infrared beams, which are situated in a frame around the box and mounted 6 cm above the ground. The raw data was collected and analyzed by TSE Phenomaster software. In addition to total distance (inactive, active) the time spent in the center of the box was analyzed. The center was defined as the 50% of box area in middle (median infrared beams 5-12 or 9-24).

4.4.3. **Light-dark box test**

The light-dark box was performed in the similar experimental set-up as the open field. The animals were placed in a semi-transparent box (50 x 50 x 40 cm^3, 32 infrared beams in 1.25 cm distance) and tracked by ActiMot2 (TSE Phenomaster System). In addition the box was divided in two compartments each half the size of the testing box. The light compartment was illuminated with usual room lights (100 lx) and the dark was dimmed using a black covered box with a transit door/opening (8 x 6 cm). At the beginning of the test, the rats were put in the dark chamber of the testing arena and the time spent in each compartment was analyzed by TSE Phenomaster software. Afterwards the rats were joined with their cage mates and return to the animal facility.

4.4.4. **Prepulse inhibition test**

The prepulse inhibition test was performed in a startle response system (SR-LAB, San Diego Instruments, USA), which includes eight testing chambers (35 x 35 x 35 cm), a stimulus generator, an amplifier, a software controller (SR-LAB-Software) with analysis unit and a floorplate with an accelerometer sensor. Inside of the testing chambers a transparent plexiglass tube (inner diameter 9 cm) was situated on a horizontal platform with the accelerometer sensor underneath. The tubes could be opened from both sides and were closed during testing with two stoppers. The movement of the animal in the case of a startle reaction was detected by the accelerometer. The peak-to-peak amplitude within 100 ms subsequent the startle response was amplified and recorded with a sample rate of 1 kHz by the SR-LAB software and later analyzed with an Excel macro.

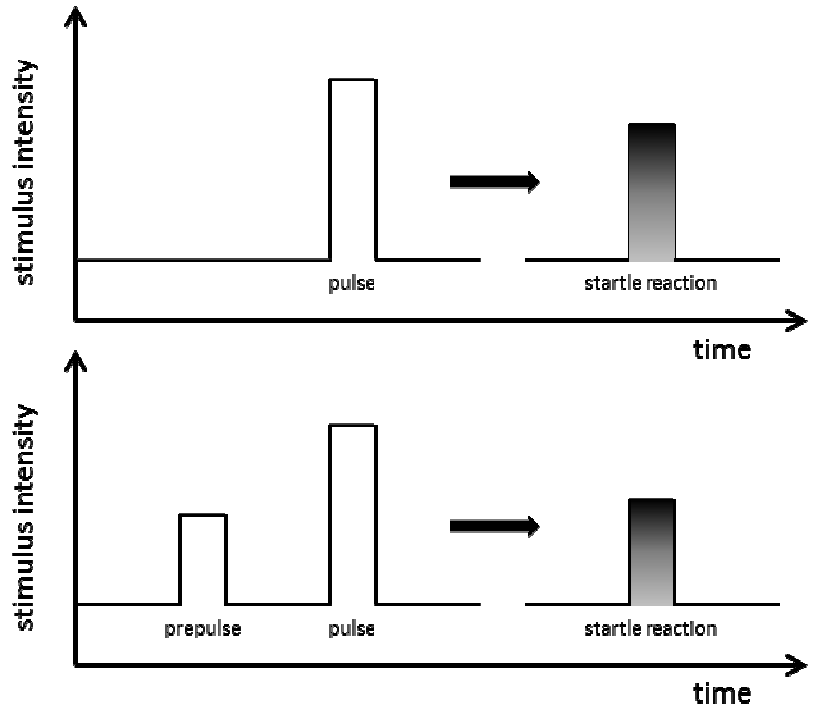

Fig. 8 Scheme of prepulse inhibition and startle response adapted by (Wikipedia, 2017)

The prepulse is a stimulus applied 100 ms prior to the startle stimulus (40 ms, 108 dB SPL) which inhibits the startle reaction. As a prepulse stimulus (white noise with a duration of 20 ms) of different intensities ranging from 2 – 16 dB SPL (2, 4, 8, 12 and 16 dB SPL above the 55 dB SPL background noise) were applied. The acoustic prepulse was generated by a loudspeaker inside of the testing chamber.

After a 5 min acclimatization period, 12 startle stimuli with an interstimulus interval of 20 s were presented. Then, six blocks consisting startle stimuli with or without the prepulses were applied in a pseudorandomized order. Each session of the test lasted 21 min.

4.4.5. **Fear-potentiated startle test**

The fear potentiated startle test was as well performed in a startle response system (SR-LAB, San Diego Instruments, USA) which was used for the prepulse inhibition test. The chamber was equipped with a loudspeaker (50 dB SPL background noise) and a light source (1000 lx). At the first day (conditioning session) the rats were placed in testing tube with the foot shock grid. For fear conditioning, 15 pairings of a light stimulus (1000 lux, 5 s) with a co-terminating aversive startle stimulus (foot shock intensity 0.8 mA, duration 0.5 s) were presented. On the second day, the effect of the now conditioned light stimulus on the startle response was measured. Therefore, the rats were again out into the testing tubes and 10 startle stimuli (40 ms, 96 dB) were presented to

habituate the startle response to a basal level. Then, 20 further startle stimuli were presented, 10 in the presence of the light CS, 10 without the light CS. These stimuli were presented in a pseudorandomized order with an interstimulus interval of 30 s. Subsequently the test animals were rejoined with their cage mates.

4.4.6. **Diurnal locomotor activity rhythm**

The diurnal locomotor activity and circadian sleep rhythm was assessed by a three day locomotion monitoring. The animals were singly placed in a transparent box (50 x 50 x 40 cm^3, 32 light barriers in 1.25 cm distance) and tracked with light barriers by ActiMot2 (TSE Systems GmbH, Bad Homburg Germany). The boxes were equipped with bedding material (Abedd LAB & VET Service GmbH, Wien, Austria) water and food (Sniff, Spezialdiäten GmbH, Soest, Germany) was accessible ad libidum. The room was illuminated in a 12-hour-cycle with lights-off at 6:00 pm. The locomotion was tracked with TSE Phenomaster and the active and inactive time was evaluated.

4.5. **Perfusion**

Following the behavioral testing the rats were sacrificed by an overdose of a saturated chloral hydrate solution. The chest of the anesthetized animal was opened with surgical instruments and a perfusion cannula was placed in the left heart ventricle. The intracardial cannula was connected to an electric pump (EP-1 Econo Pump, Bio-Rad Laboratories Ltd., Hemel Hempsted, UK) which conveyed phosphate buffer solution (PBS) in a velocity 13.0 ml/min to drain the blood of the animal. Surgical clamps were applied on the Vena cava inferior and Aorta abdominalis. Preventing the blood from clotting the 150 ml PBS drained the vast bulk of blood of the upper body. Afterwards about 300 ml fixation solution (see appendix Immunfixation) containing 15% picric acid and 4% paraformaldehyde were applied. The brains were removed and they were incubated two days in the perfusion solution. Finally the brains were transferred to a 20% sucrose paraformaldehyde solution prior cutting.

4.6. **Histology**

The rat brains were sliced by a cryostat (Leica CM 1950, Leica Biosystems Nussloch GmbH, Nussloch, Germany). The slices were dyed differently as Nissl stain and free-floating immunohistochemistry with orexin and MCH antibodies. The orexin and MCH antibodies were tagged with secondary antibody attached to a peroxidase which metabolized DAB (3,3'-Diaminobenzidine) and colored the slices with a brown hue.

4.6.1. **Brain preparation**

The brains were retrieved from the fixation solution (20% sucrose 4% paraformaldehyde solution). The appropriate object plates of the cryostat were coated with fast-freezing tissue (Jung® Tissue freezing medium, Leica Biosystems Nussloch GmbH, Nussloch, Germany) and places in a box of dry ice. A small solid platform of freezing tissue will be created in minutes and with a fresh drop of freezing tissue the brain was attached to the object plate. The brains on the object plate were kept in a freezer of -80°C. The sectioning of the slices itself was performed in an environment of -20 to -22°C depending on the ambient conditions. The brains were sliced in the regions of the lateral hypothalamus according to (Paxinos and Watson, 1998) and occasionally the locus coeruleus at a thickness of 40 µm. Every brain was sliced and split in four equal sections to obtain different staining of the same rat.

For Nissl staining the slices were gathered directly after cutting on a microscopy object plate and dried afterwards at room temperature. In case of immunohistochemistry the slices of the first section were captured in PBS, the following three were captured in freezing solution (glycerine, ethylen glycol, PBS). The microscopy object plates and containing tubes with brain slices were labeled as illustrated in the table below. Despite the first series all sections were kept in a freezer prior to staining.

4.6.2. **Nissl staining**

Occasionally the brain slices were captured directly on a microscopy object plate and they were stained without delay. But most of the slices were captured on microscopy object plates, washed three times in a row with PBS, dried at room temperature for 24 h prior staining.

For defatting the brain slices the object plates were incubated 2 min in EtOH of descending concentrations (70 %(v/v), 50 %(v/v). Afterwards they were incubated in the histological stain 1% cresyl violet solution for approximately 2 min. Dehydration steps with incubation in ascending EtOH concentrations (70 %(v/v), 96 %(v/v), 100 %(v/v)) followed. Finally the brain slices were dried a day room temperature and fixed with RotiHistol® (Carl Roth GmbH + Co. KG, Karlsruhe (Germany)) and covered with coverglas using Entelan® (Carl Roth GmbH + Co. KG, Karlsruhe, Germany). To analyze the slices they were photographed (Canon EOS 1100D, Canon Deutschland GmbH, Krefeld, Germany) under the microscope (Zeiss Axioscope, Carl Zeiss Microscopy GmbH, Jena, Germany) and processed with ImageJ (Public Domain, developed at the National Institutes of Health, Bethesda, MD, USA).

4.6.3. **Immunohistochemistry with orexin antibodies**

The slices were stained with a free-floating technic in little 6 well cell culture multidishes (TPP® tissue culture plates, Sigma-Aldrich Chemie GmbH München, Germany). The brain slices were placed in perfect fitting slice restrainers (inner diameter: 31 mm) and moved from one well to another. Every restrainer holds up to three to four slices to prevent them from clotting together. In addition the stain procedure was conducted on a laboratory shaker and with light protection. At the beginning of the staining and between almost every step a rinse cycle with three repeats each for 10 min was conducted. As washing solution of PBS added with Triton X-100® 0.3% (Sigma-Aldrich Chemie GmbH München, Germany) was applied and rinsed afterwards. After application of the ABC-Kit (Vector Laboratories, Burlingame, CA, USA) the washing solutions lack of Triton X-100® to reduce interactions with the detection reaction. Every added solution had a volume of 2 ml to diminish the disposal and to cover all of the 3-4 floating slices. As detection reaction a DAB (3,3'-Diaminobenzidine, Carl Roth GmbH + Co. KG, Karlsruhe, Germany) solution was quantitatively oxidized to a brown pigment by an antibody attached peroxidase.

First the frozen slices were thawed, distributed to the wells and washed by the rinse cycle as described above. The blockade of the endogenous peroxidase was conducted by a high concentrate hydrogen peroxide solution in which the slices were incubated for 10 min on a laboratory shaker. The polyclonal antibodies in the tissue were unspecific captured by normal goat serum (NGS; Vector Laboratory® LINARIS, Dossenheim, Germany) and bovine serum albumin (BSA; Sigma-Aldrich Chemie GmbH, München, Germany) applied afterwards. The brain tissue was incubated in this solution for 30 min and subsequently without a rinse cycle the primary antibody was added. For the staining of OxA and subsequently therefore the orexin-producing cells an anti-OxA-antibody (anti-OxA-rabbit, Calbiochem, San Diego, CA, (USA); concentration 1:500) was used. The brain slices were incubated in a solution with the anti-OxA-antibody (primary antibody) for 2 day at 4°C in a refrigerator. Subsequently the secondary antibody (biotinylated anti-rabbit IgG, Vector Laboratories, Burlingame, CA, USA) was added and incubated for 2 h by room temperature on the laboratory shaker. Afterwards the ABC peroxidase (Vectastain® Elite ABC-Kit, Vector Laboratories, Burlingame, CA, USA) was added for 1 h to connect to the secondary antibody and evoke the detection reaction. The next rinse cycle lacks of Triton X-100® to prevent interaction with the detection reaction.

Subsequently the slices were stained by DAB dyeing solution and incubated in respect of the brightness in between 4 to 12 min. The reaction was excited by adding a small quantity of hydrogen peroxide to the DAB dyeing solution shortly before application. The slices were again washed three times with rinse solution and afterwards incubated for one more night in PBS. The rinse solution and the dyeing solution were neutralized by adding hypochlorite to destroy the DAB. The following day the slices were taken up on object plates and dried at 37 °C. Two days later the object plates were

dipped in EtOH of ascending concentrations (70 %(v/v), 96 %(v/v), 100 %(v/v)) to avoid water from the brain slices. Afterwards the object plates were fixed with RotiHistol® and covered using Entellan® as glue for the cover plates. The evaluation of the staining was conducted under microscope and photographs analyzed by ImageJ. A standard operation procedure is linked in the appendix.

4.6.4. **Immunohistochemistry with MCH antibodies**

The melanin concentrating hormone (MCH) producing cells are located in the lateral hypothalamus like the orexin producing cells. As a control the anti-MCH staining was conducted to illustrate the selectivity of the anti-Ox2R-SAP neurotoxin used in the stereotaxic injections. Despite of another primary antibody (anti-MCH-rabbit, Phoenix pharmaceuticals Inc., Burlingame, CA, USA; concentration 1:2000) the staining of MCH cells was exactly carried out in similar procedures same as the anti-Orexin staining (see section 4.6.3 Immunohistochemistry with orexin antibodies).

4.6.5. **Analysis of the pictures**

The brain slices on the object plates were evaluated by microscope and photographed with a Canon EOS 1100 D. The region of interest, the lateral hypothalamus, was best captured at the 4x objective lens magnification and the light source set to 4. The sharpness was adjusted by using the focus and a digital 5x enlargement of the camera.

Afterwards the pictures were manually analyzed with ImageJ by using its plugin “Cell Counter”. The dark brown cells in the region of interest were counted, the marked picture was saved and the amount of cells collected in a table. The number of cells collected in one section was stated as OxA-positive or MCH-positive neurons/animal.

5. Results - Project 2: Local infusions of a putative orexin-specific saporin conjugate into the lateral hypothalamus of rats: Effects on orexin neurons and behavior

5.1. Effect of neurotoxin injections on the number of OxA-positive neurons

A total of 25 rats successfully received bilateral injections of either anti-Ox2r-SAP, IgG-SAP or saline. The different treatment groups, as shown in figure 9, varied in the number of OxA-positive neurons/animal. Statistical analysis confirmed a significant treatment effect ($F_{(2,22)} = 5.37$; $P = 0.01$). Post-hoc multiple comparisons revealed a significant lower amount of OxA-positive neurons in rats treated with anti-Ox2R-SAP or IgG-SAP in comparison to rats receiving injections of only saline (anti-Ox2R-SAP: $P = 0.01$; IgG-SAP: $P = 0.03$). There were no differences between animals treated with anti-Ox2R-SAP or IgG-SAP.

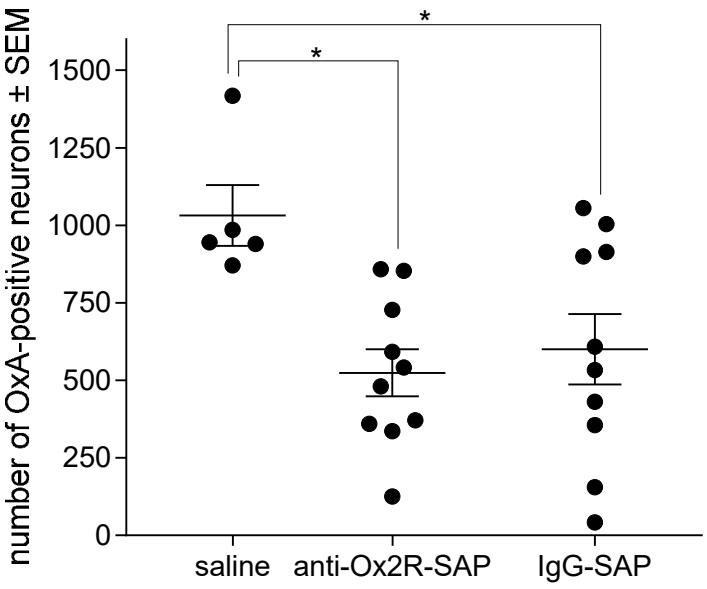

Fig. 9 Mean number of OxA-positive neurons/animal ± SEM of the LH brain slices after bilateral injections of saline (n = 5), anti-Ox2R-SAP (n = 10) or IgG-SAP (n = 10). Neurons were counted in brain slice in one of 4 sections. * P < 0.05; ** P < 0.01 (comparison with saline treatment).

The MCH neurons are situated adjacent to OxA neurons in the LH. Quantification of MCH-positive neurons helped to evaluate the selectivity of the neurotoxins (Gerashchenko et al., 2001). Brain slices of 20 rats were stained with an anti-MCH antibody and the results are demonstrated in figure 10. Throughout all treatment groups, the number of MCH-positive neurons was comparable ($F_{(2,17)} = 0.73$; $P = 0.50$).

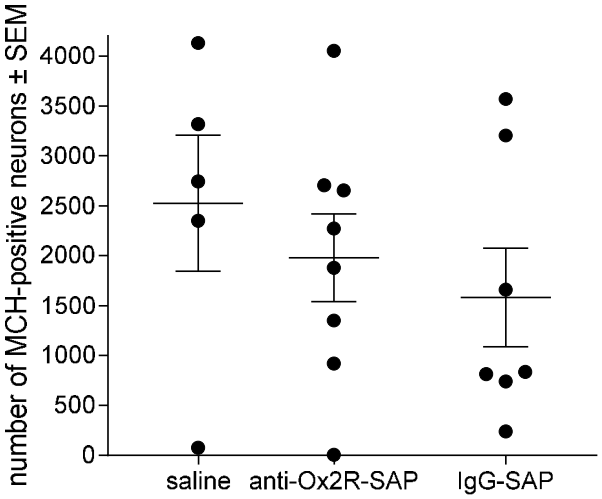

Fig. 10 Number of MCH-positive neurons/animal ± SEM of the LH brain slices after bilateral injections of saline, anti-Ox2R-SAP or IgG-SAP. The results according the treatment groups (saline: n = 5; anti-Ox2R-SAP: n = 8; IgG-SAP: n = 7) are illustrated.

Furthermore, it was analyzed whether the number of MCH-positive neurons were correlated with the number of OxA-positive neurons (figure 11). After saline injections, no correlation was observed (r^2 = 0.10; P = 0.58), whereas there were significant correlations after injections of anti-Ox2R-SAP (r^2 = 0.70; P = 0.01) and IgG-SAP (r^2 = 0.68; P = 0.02).

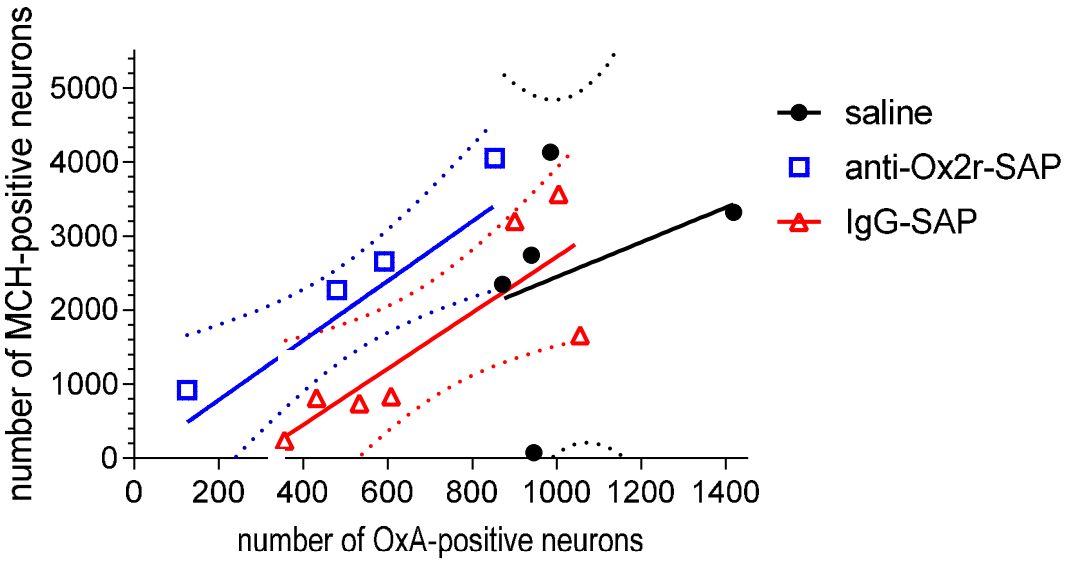

Fig. 11 Correlations of OxA-positive neurons and of MCH-positive neurons. The results according the treatment groups (saline: n = 5; anti-Ox2R-SAP: n = 8; IgG-SAP: n = 7) are illustrated.

The different regression lines are not different (≙ null hypothesis; P > 0.05) from each other ($F_{(1,11)}$ = 0.02; P = 0.88; same slope for anti-Ox2R-SAP and IgG-SAP), suggesting that the two neurotoxins equally affect both OxA- and MCH-positive neurons, i.e. the neurotoxic effects of both SAP conjugates are unspecific.

Based on the observation that there were no specific effects on OxA-positive neurons, the animals were re-grouped in group α and group β for further analysis. Group α consisted of animals with more than 750 OxA-positive neurons, i.e. a high number of orexin-positive neurons. All saline-treated rats, two anti-Ox2R-SAP-treated rats and four IgG-SAP-treated rats were combined in this group α (figure 12). Group α was considered as a control group. IgG-SAP group β consisted of animals injected with anti-Ox2R-SAP or IgG-SAP. In this group, a clear loss of OxA-positive neurons was observed (< 750 OxA-positive neurons/animal).

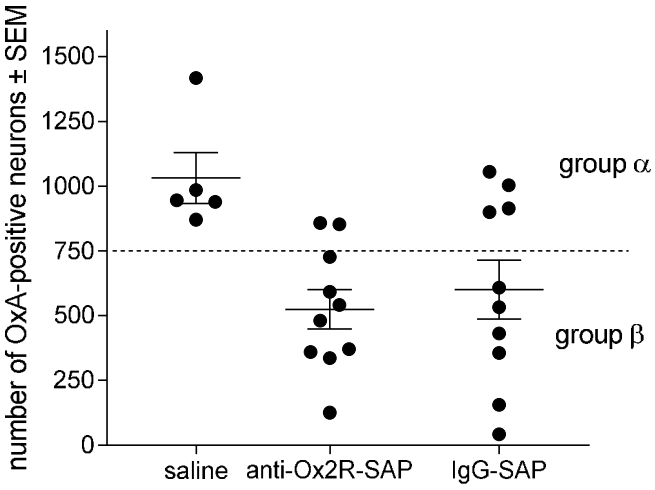

Fig. 12 Mean number of OxA-positive neurons/animal ± SEM of the LH brain slices after bilateral injections of saline (n = 5), anti-Ox2R-SAP (n = 10) or IgG-SAP (n = 10). Neurons were counted in brain slice in one of 4 sections. The figure shows the division in group α (> 750 OxA-positive neurons/animal)) and group β (< 750 OxA-positive neurons/animal).

Figure 12 depicts the number of OxA- and MCH-positive neurons in the new groups α and β. As intended, the groups clearly differed in the number of OxA-positive neurons and there was no overlap of group α and β. In group β, the number of OxA-positive neurons was approximately half of the number of OxA-positive neurons in group α (t = 7.82; df = 23; p < 0.01). Furthermore, the number of MCH-positive neurons were reduced in group β (t = 3.42; df = 18; p < 0.01). Most animals in group β (n = 10) had less than 2300 MCH-positive neurons while the animals in the control group α (n = 10) had mostly more than 2300 MCH-positive neurons.

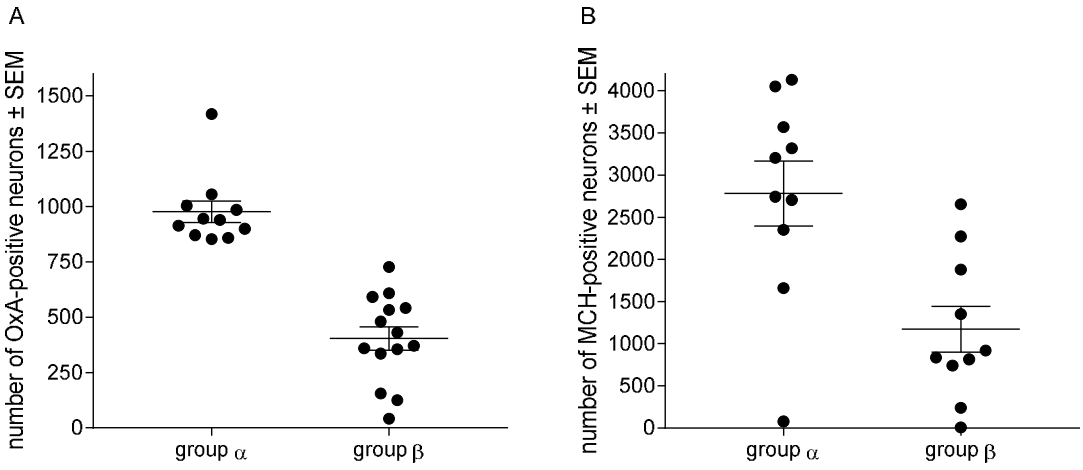

Fig. 13 Number of OxA-positive (Fig. 13A) and MCH-positive (Fig 13B) neurons/animal ± SEM in group α (n = 10) and group β (n = 10).

5.2. Effects on body weight

The body weight of all 25 rats was monitored prior and during the experiments on a weekly basis. Figure 14 illustrates the increase of body weight in control group α and group β over the duration of the experiment. The mean body weight ± SEM in all rats was above 300 g at the beginning of the experiments (injections of saline or neurotoxins in week 8) and did not differ between the groups(t = 0.79; df = 23; P = 0.44). At the end of the experiments, an increase of body weight of at least 100 g was observed in both groups. Starting with the 11th week, third week after application of neurotoxins, the animals in group α gained weight faster than rats in group β.

A two-way ANOVA revealed significant effects of time ($F_{(11,253)}$ = 165.6; P < 0.01) and treatment ($F_{(1,23)}$ = 4.37; P = 0.05), but no significant interaction effect ($F_{(11,253)}$ = 1.60; P = 0.10) was observed. Post-hoc multiple comparisons showed significant differences between both groups in the 11th to 18th week (Fisher's LSD test: P = 0.46 in week 8 prior stereotactic injections of saline or neurotoxins; P = 0.52 in week 9; P = 0.11 in week 10; P = 0.04 in week 11; P = 0.05 in week 12; P = 0.03 in week 13; P = 0.02 in week 14; P = 0.03 in week 15; P = 0.04 in week 16; P = 0.03 in week 17; P = 0.02 in week 18; P = 0.05 in week 19) whereby rats assigned to group α were significantly heavier than rats of group β.

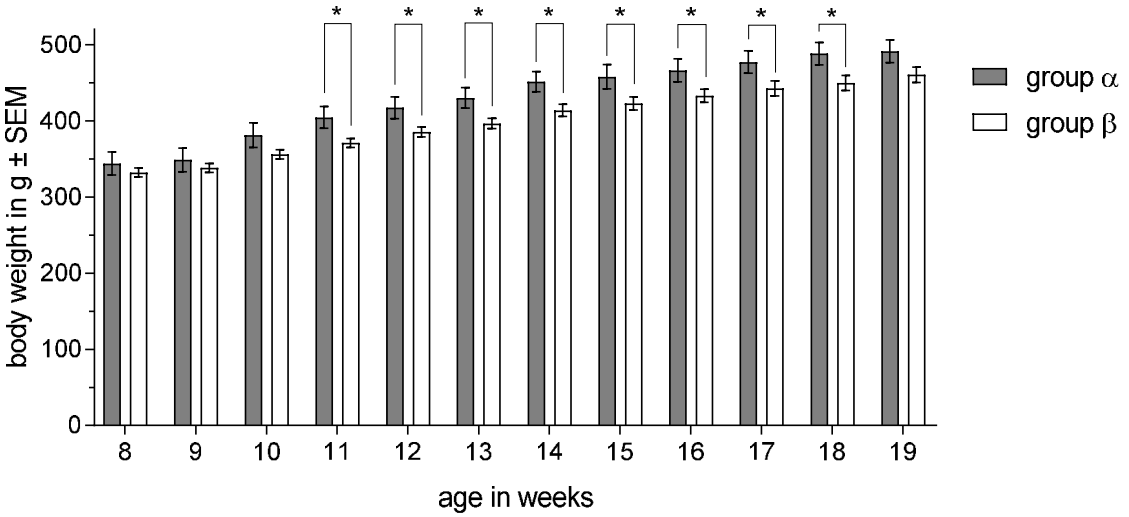

Fig. 14 Mean body weight in g ± SEM of rats from week 8 to 19 in group α (n = 11) and group β (n = 14). *P < 0.05 (post-hoc comparisons between group α and group β).

5.3. Effect on behavior

5.3.1. Test in behavior monitoring box

At week 12 and 16 the animals were exposed for 4 hours to an arena with enriched environment. The number and duration of behavioral arrests were manually scored for each individual animal. The results are illustrated in figure 15A and 15B. Testing 12 weeks old rats shows a significant effect of the factor time ($F_{(3,69)}$ = 4.09; P = 0.01) With increasing duration of the experiment animals in both groups had longer periods in behavioral arrest. However, there were no effects of group ($F_{(1,23)}$ = 0.34; P = 0.56) and no interaction between group and time ($F_{(3,69)}$ = 1.16; P = 0.33). A two-way ANOVA of the test in 16 weeks old rats again revealed a significant effect of the factor time ($F_{(3,69)}$ = 3.07; P = 0.03) but no effects of group ($F_{(1,23)}$ = 0.98; P = 0.33) and no interaction between group and time ($F_{(3,69)}$ = 0.99; P = 0.40).

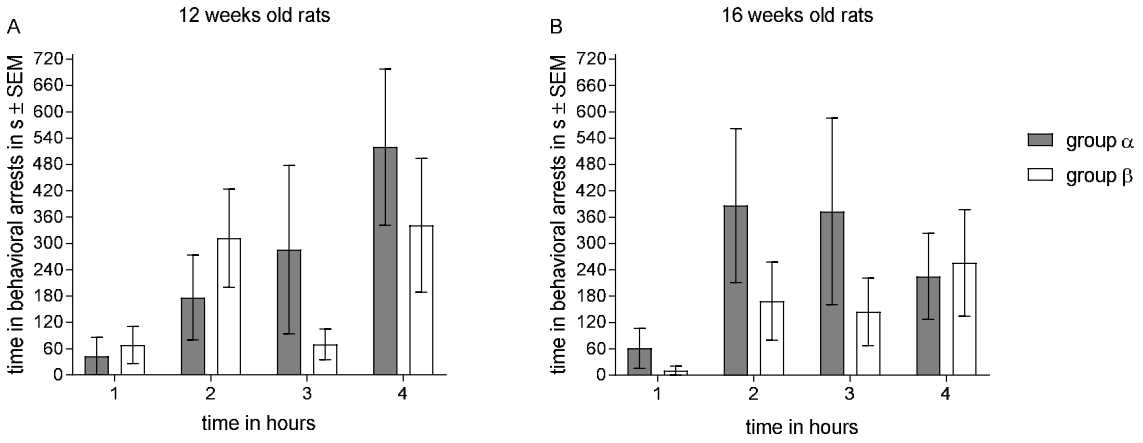

Fig. 15 Time spent in behavioural arrests (s ± SEM) of group α (n = 11) and group β (n = 14) during the 4 h monitoring tests in an enriched environment. Fig 15A illustrates the test in 12 and weeks old rats and fig 15B shows the test in 16 weeks old rats.

5.3.2. **Open field test**

In an open field test, the locomotor activity was assessed. Figure 16 illustrates the results in blocks of 5 minutes. The distance traveled in cm ± SEM is displayed in figure 16A while the percent time spent in the center of the open field ± SEM is shown in figure 16B.

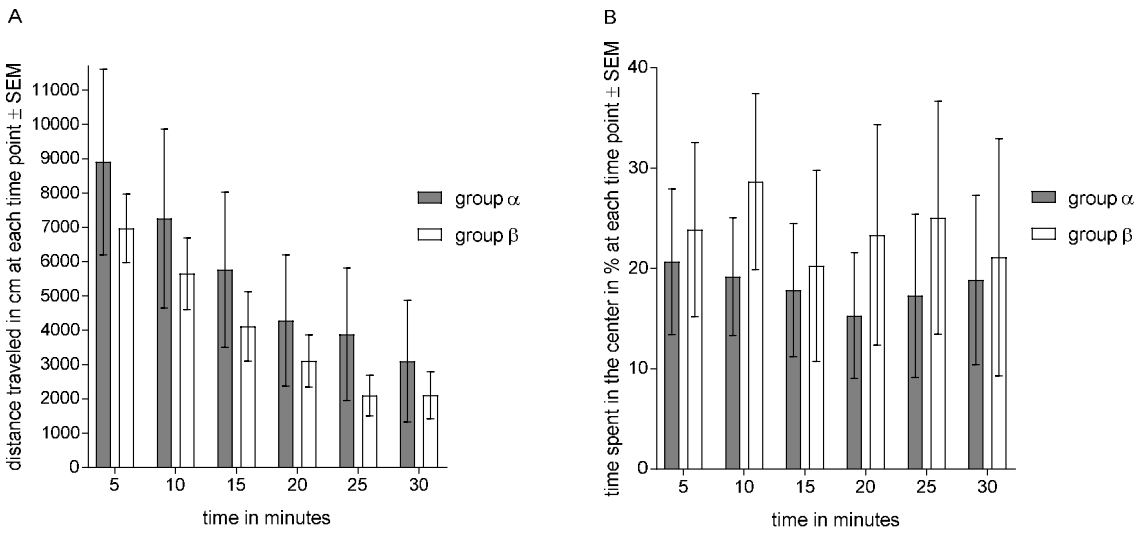

Fig. 16A Distance travelled in cm ± SEM of group α (n = 10) and group β (n = 14) during a 30 min open field test. Fig. 15B Percent time spent in the center ± SEM of group α (n = 11) and group β (n = 9) during 30 min open field test.

A two-way ANOVA of the distance traveled in the open field test revealed a significant effect of time ($F_{(5,110)} = 30.33$; $P < 0.01$). With increasing testing duration, animals of both groups travelled less. However, there were no significant effects of the group ($F_{(1,22)} = 0.57$; $P = 0.46$) and no interaction of group and time ($F_{(5,110)} = 0.23$; $P = 0.95$).A two-way ANOVA of percent time spent in the center in an

open field test revealed no effects of group ($F_{(1,18)} = 0.23$; $P = 0.64$) or of time ($F_{(5,90)} = 0.72$; $P = 0.61$) and there was no interaction ($F_{(5,90)} = 0.54$; $P = 0.75$).

5.3.3. **Light-dark box test**

In the light-dark box test, the percent time spent in the light compartment as well as the latency of entering into the light compartment was assessed. Figure 17 illustrates the results.

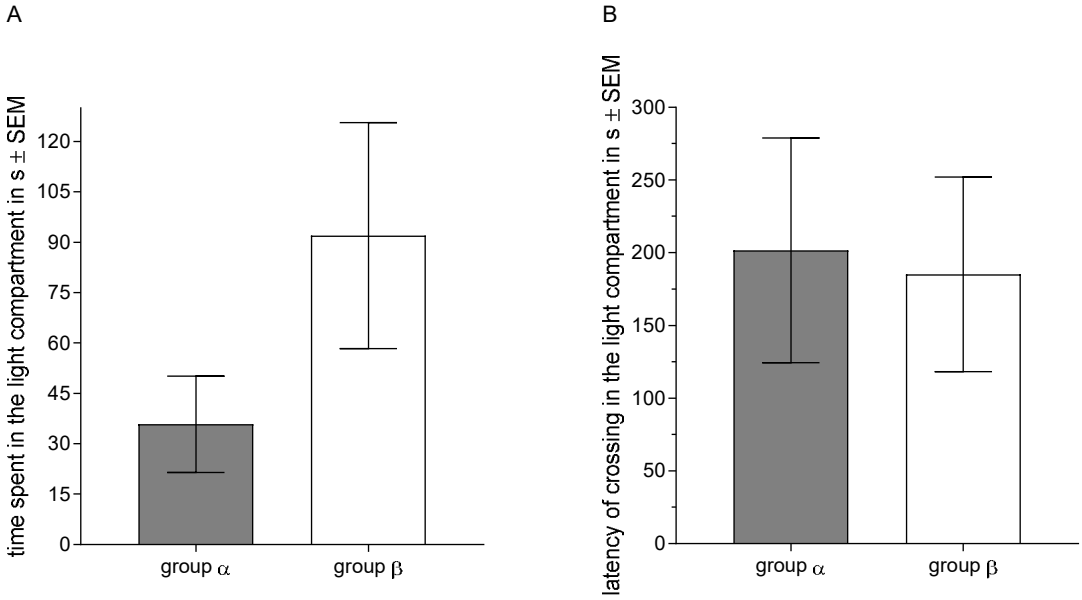

Fig. 17A Time spent in the light compartment in s ± SEM in group α (n = 10) and group β (n = 14) during the 5 min light-dark box test. Fig. 16B Latency of crossing in the light compartment in s ± SEM in group α (n = 10) and group β (n = 14) and during the 5 min light-dark box test.

A two tailed t-test analysis of the time spent in the light compartment revealed no significant difference between the control group α and group β during the 5 min light dark box test ($t = 1.40$; $df = 23$; $P = 0.18$). However the variance measured between both groups showed a significant difference (f-test: $P < 0.01$). A two tailed t-test analysis of the latency of crossing in the light compartment revealed no significant difference between group α and group β during the 5 min light dark box test ($t = 0.16$; $df = 23$; $P = 0.87$).

5.3.4. **Prepulse inhibition test**

The figure 18 shows the results of the prepulse inhibition test of 22 treated rats. Three animals had to be excluded from the analysis since they did not express any startle responses. A two tailed t-test analysis of the startle response revealed a trend for a lower startle magnitude in group β compared with group α ($t = 1.92$; $df = 20$; $P = 0.07$). A two-way ANOVA of the prepulse inhibition test showed a significant effect of the prepulse intensity on the startle magnitude ($F_{(4,80)} = 21.13$; $P < 0.01$), i.e. increasing prepulse intensities resulted in stronger prepulse inhibition. There were no effects of

groups ($F_{(1,20)}$ = 3.24; P = 0.09) and no interaction between group and prepulse intensity ($F_{(5,100)}$ = 2.32; P = 0.05).

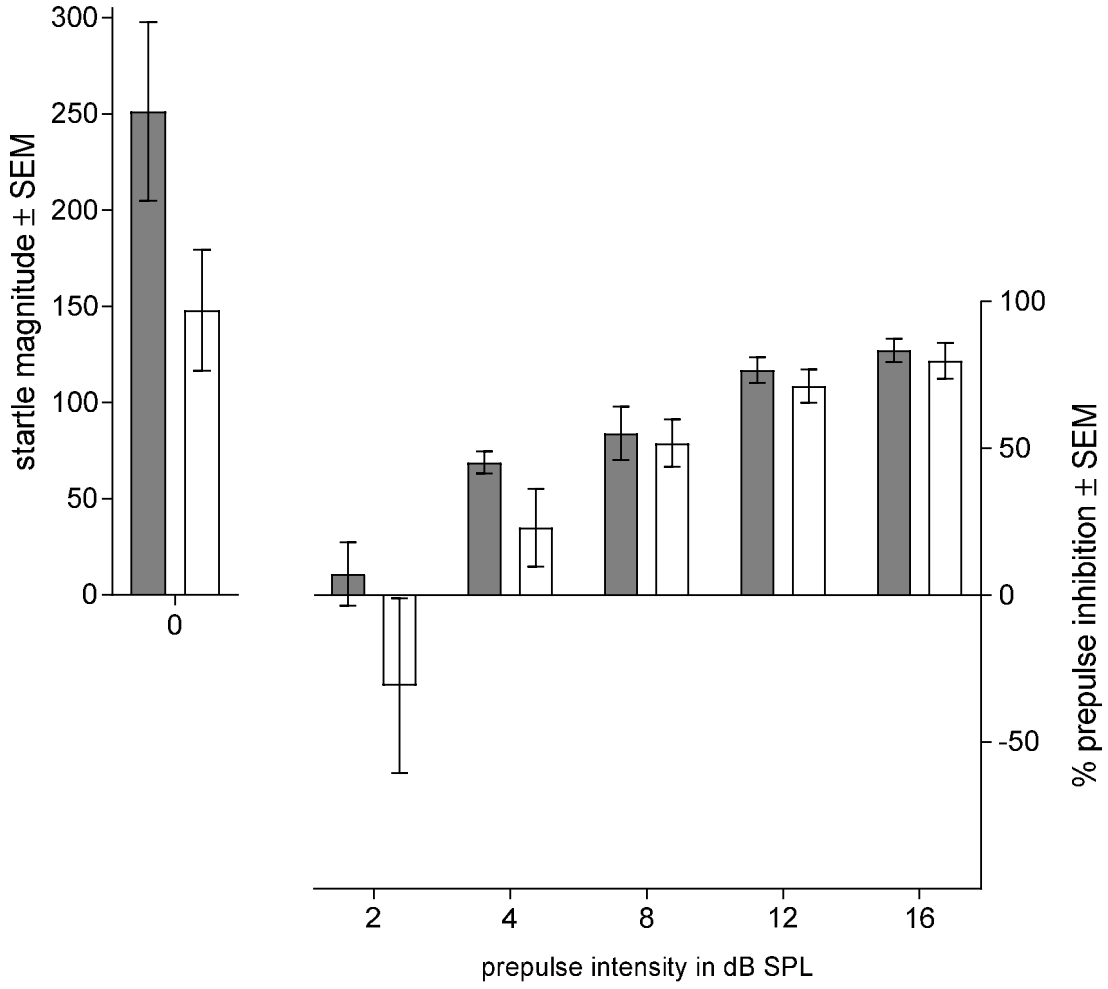

Fig. 18 Startle magnitude (left panel) and prepulse inhibition in % ± SEM (right panel) in group α (n = 9; grey bars) and group β (n = 13; white bars).

5.3.5. **Fear-potentiated startle test**

In a fear-potentiated startle test the behavior of control group α (n = 9) and group β (n = 13) were assessed. The results are displayed in figure 19. A two-way ANOVA of the fear-potentiated startle test did not result in a significant effect of groups ($F_{(1,20)}$ = 0.13; P = 0.72) and trial types (startle alone vs. CS-startle: ($F_{(1,20)}$ = 3.75; P = 0.06). Furthermore, there was no interaction ($F_{(2,40)}$ = 1.86; P = 0.17).

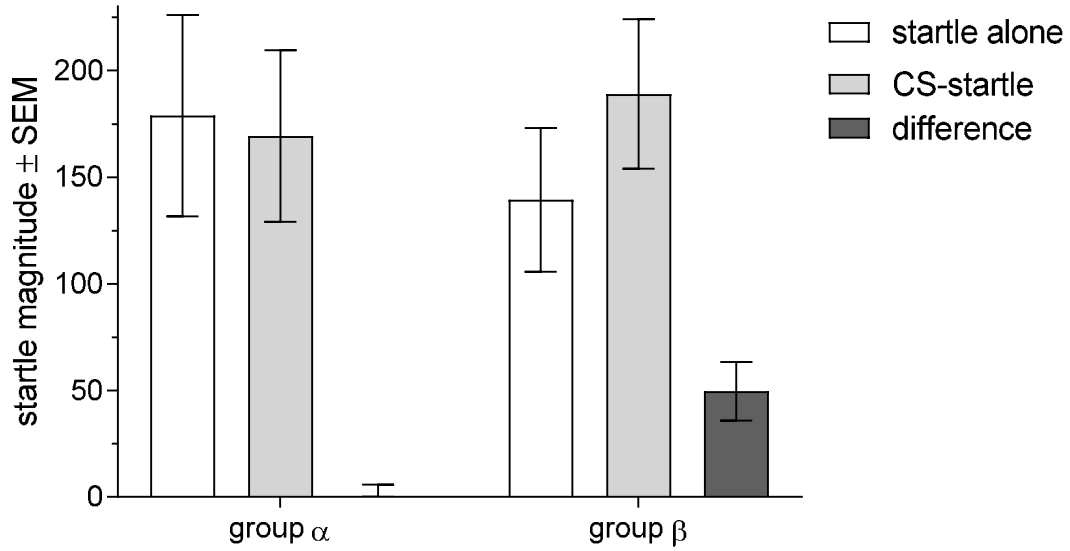

Fig. 19 Startle magnitude ± SEM during a fear-potentiated startle test in group α (n = 9) and group β (n = 13).

5.3.6. **Diurnal locomotor activity**

Lastly, the diurnals locomotor activity of 21 animals was tested for 72 h in a cage equipped with water and food ad libitum. The light was switched off at 6 pm and turned on at 6 am. The test started at 6 pm when the lights were switched off. The results of control group α (n = 11) and of group β (n = 10) are displayed in figure 20. A diurnal rhythm according to the illumination is shown in the figure.

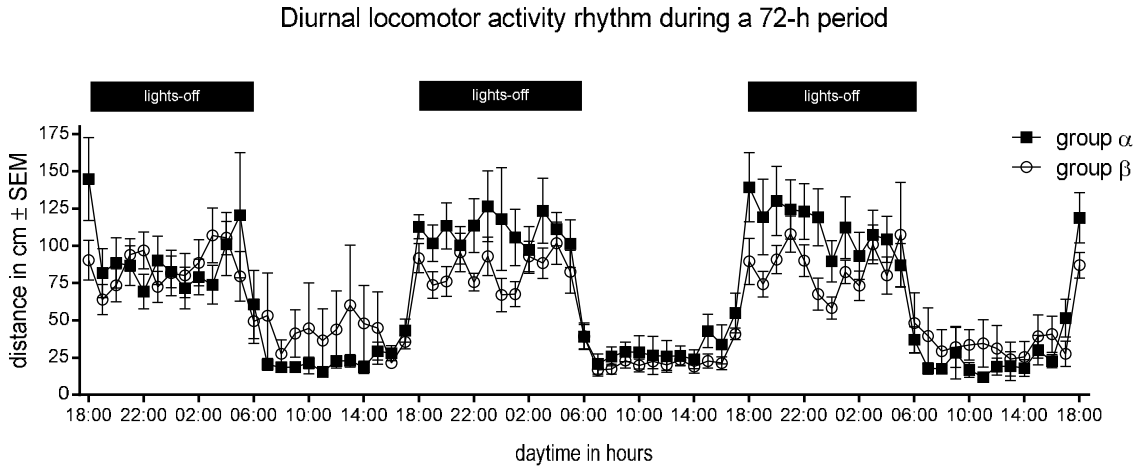

Fig. 20 Distance in cm ± SEM during a 72 h test of diurnal locomotor activity in group α (n = 11) and group β (n = 10). The rats were exposed to a 12 h light dark-cycle starting at 6 am and the test started in the lights-off period at 6 pm.

At the lights-off period, all rats regardless of group travelled longer distances than in the lights-on period. However, animals in group β seemed to move slightly more often in the lights-on periods and less often in the lights-off periods than group α rats.

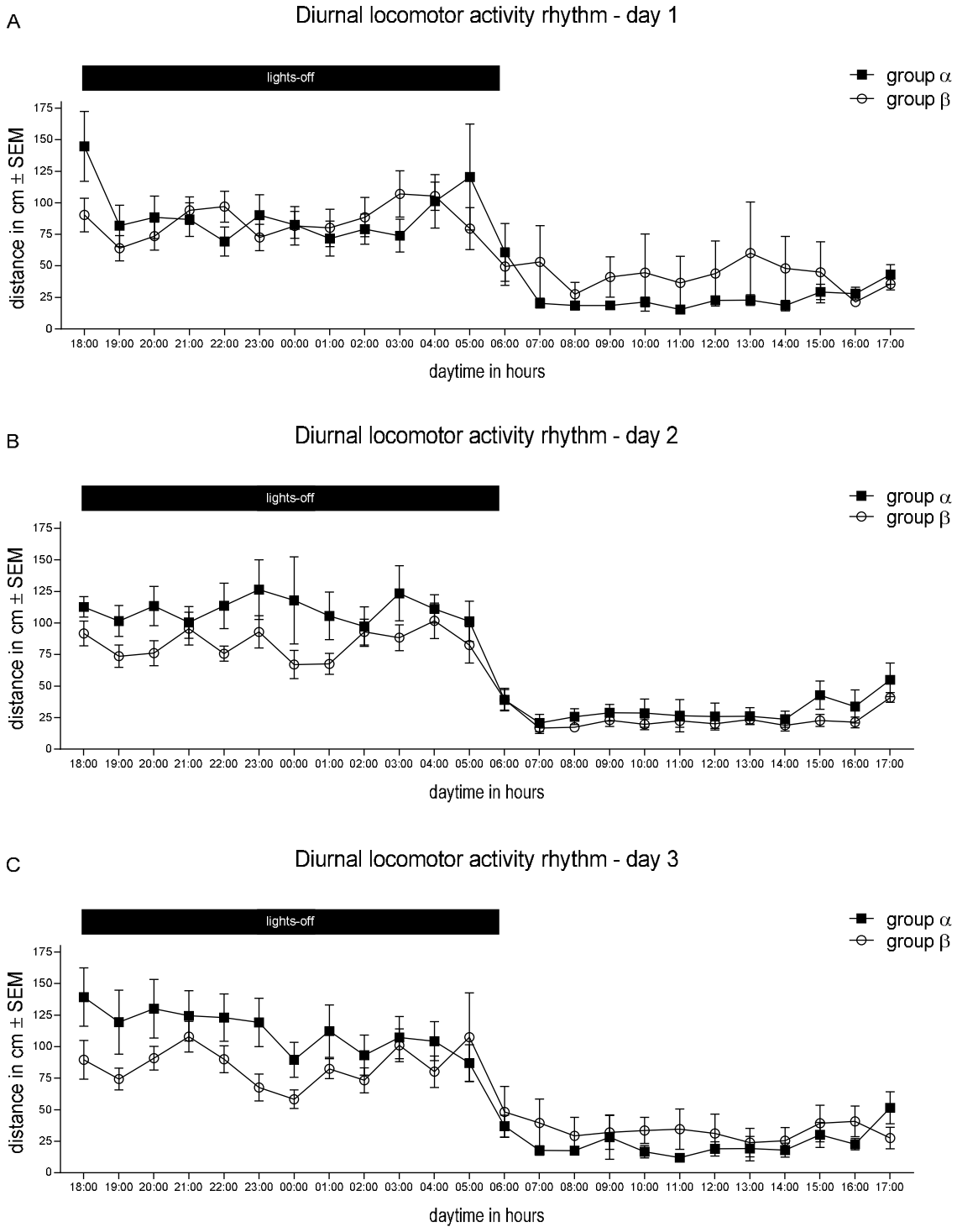

Fig. 21 Distance in cm ± SEM during a 72 h test of diurnal locomotor activity in group α (n = 11) and group β (n = 10). Figure 21A represents the results of day 1 (group α n = 11; group β n = 10), in figure 21B day 2 (group α n = 9; group β n = 9) and in figure 21C day 3 (group α n = 9; group β n = 9) are displayed. The rats were exposed to a 12 h light dark-cycle starting at 6 am and the test started in the lights-off period at 6 pm.

For better illustration, the overall data shown in figure 20 is divided in three 24 h parts which are displayed individually in figure 21. Furthermore, the mean distance travelled of each animal in the lights-off and in the lights-on period are compared separately in group α and group β and displayed in figure 22. A two way ANOVA with factors group and light period showed no significant effect of interaction ($F_{(1,38)}$ = 1.06; P = 0.31) or group ($F_{(1,38)}$ = 0.05; P = 0.83). Nevertheless a significant effect of light period ($F_{(1,38)}$ = 54.4; P < 0.01) is illustrated. The post-hoc multiple comparison highl sigificant effects in each group but no significant in between both group during the same light period.

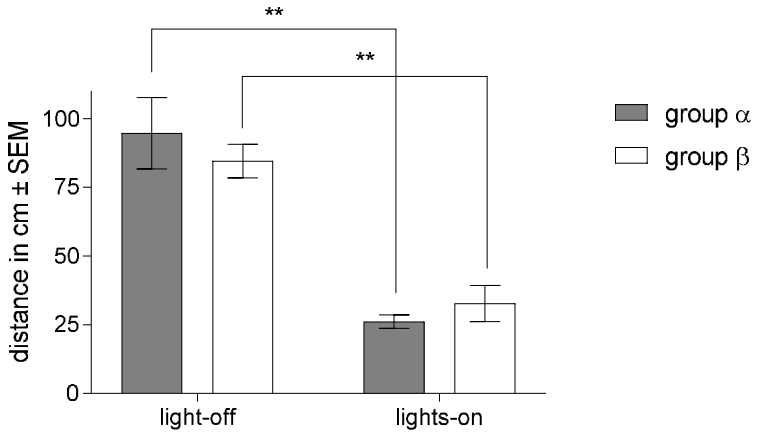

Fig. 22 Comparison of group α and β at different light periods. Distance traveled in cm ± SEM divided in light-off and lights-on period during a 72 h test of diurnal locomotor activity in group α (n = 11) and group β (n = 10).

6. Discussion and Outlook

6.1. Project 1: Effects of escitalopram, reboxetine, prazosin and cirazoline on narcoleptic episodesin orexin-deficient mice

The present study shows that application of the SSRI escitalopram and of the NRI reboxetine effectively suppressed the number of narcoleptic episodes in orexin-deficient mice. Reboxetine was efficient in lower doses than escitalopram. We further tested the effects of the α1 adrenoceptor antagonist prazosin and of the α1 adrenoceptor agonist cirazoline. The latter compounds had a tendency to increase (prazosin) and decrease (cirazoline), respectively, the number of narcoleptic episodes. However, in an open-field test, cirazoline but not all other used compounds had sedative-like effects on locomotor activity which may interfere with the measurement of narcoleptic episodes.

The working hypothesis of the present study was that a specific blockade of the norepinephrine reuptake is more efficient in treating narcoleptic episodes in mice than a blockade of the serotonin reuptake. This hypothesis was based on the observation that the affinity to the norepinephrine transporter predicts the efficiency of compounds in canine narcolepsy better than the affinity to the serotonin transporter (Mignot et al., 1993b; Nishino et al., 1993; Nishino, 2007a). Further support for this hypothesis comes from the observation that the norepinephrine brain system is more inhibited during cataplexy than the serotonin system (Wu et al., 1999).

During the early 1990s, specific NRIs were not available and in the experiments described above (Mignot et al., 1993b; Nishino et al., 1993) only SSRIs and tricyclic antidepressants were used. The latter are often not only serotonin and/or norepinephrine reuptake inhibitors but have also high affinities to the α1 adrenoceptors, histamine H1, muscarinic and 5-HT2A receptors (Gillman, 2007). Especially the antagonism at the histamine H1 receptor may interfere with potential anti-narcoleptic effects since beside orexin also histamine levels are decreased in narcolepsy (Nishino et al., 2009). Furthermore, tricyclic antidepressants suffer from several severe side effects, especially cardiovascular toxicity (Thanacoody and Thomas, 2005).

In the present study, we used the SSRI escitalopram and the NRI reboxetine. Escitalopram is regarded as the most selective SSRI, and reboxetine as the most selective NRI (Brunello and Racagni, 1998; Owens et al., 2001). Both compounds are clinically-validated medications for depression (Höschl and Svestka, 2008; Cipriani et al., 2009) and are used off-label for other neuropsychiatric diseases like panic disorders and attention deficit hyperactivity disorders (Andrisano et al., 2013; Ghanizadeh, 2015). Despite of off-label use of these two compounds for the treatment of human cataplexy in

narcoleptic patients (Mignot, 2012), no controlled clinical trials on narcolepsy were performed so far. However, clinical experience shows that both escitalopram and reboxetine are effective in treating cataplexy (Larrosa et al., 2001; Sonka et al., 2006). According to the hypothesis that NRIs should be more effective than SSRIs, reboxetine should be more effective than escitalopram. However, the two compounds were never directly compared in clinical studies.

Here we directly compared the effects of escitalopram and reboxetine on narcoleptic episodes in orexin-deficient mice. Beside the different canine models of narcolepsy (Cederberg et al., 1998; Lin et al., 1999), orexin-deficient mice are a well-established animal model for narcolepsy (Chemelli et al., 1999; Lin et al., 2008; Scammell et al., 2009; Leibiger and Fendt, 2014). Notably, we did not record EEG/EMG in the present study but measure so-called 'behavioral arrests'. About 50% of the behavioral arrests fulfill all criteria for cataplexy (Scammell et al., 2009) while the other half are sleep attacks (Chemelli et al., 1999). Both are main symptoms of human narcolepsy (Scammell, 2003; Nishino, 2007a) and behavioral arrests are therefore also called narcoleptic episodes (Leibiger and Fendt, 2014). Due to the waiver of EEG/EMG we cannot distinguish between treatment effects on cataplexy and sleep attacks but we were able to test mice in groups and in an enriched environment which both strongly increase the number of behavioral arrests (Chemelli et al., 1999; España et al., 2007; Burgess and Peever, 2013).

The present data clearly shows that both, escitalopram and reboxetine, dose-dependently reduces narcoleptic episodes in orexin-deficient mice. The minimum effective dose for escitalopram was 3 $mg \cdot kg^{-1}$ and for reboxetine 0.55 $mg \cdot kg^{-1}$ indicating that reboxetine is more efficient than escitalopram. Both compounds have similar pharmacokinetic properties in mice (Dostert et al., 1997; Jacobsen et al., 2014) as well as similar affinities to the respective transporter (Wong et al., 2000; Owens et al., 2001; Sánchez et al., 2003). Escitalopram has a more than 1000 fold selectivity for the serotonin transporter against the norepinephrine transporter (Owens et al., 2001), while reboxetine has about 100 fold selectivity for the norepinephrine transporter (Wong et al., 2000). This means that at the tested doses, reboxetine only inhibited the norepinephrine transporter and escitalopram only the serotonin transporter. In conclusion, our findings support the hypothesis that inhibiting norepinephrine reuptake is more efficient in treating cataplexy than inhibiting serotonin reuptake.

There is a clinical case report that the α1 adrenoceptor antagonist prazosin that was used as a treatment for hypertension induced an exacerbation of cataplexy in a narcolepsy patient (Aldrich and Rogers, 1989). This is strongly supported by a recent study in orexin-deficient mice showing that administration of the α1 adrenoceptor agonist phenylephrine decreases the number of cataplexy episodes whereas the α1 adrenoceptor antagonist terazosin increases them (Burgess and Peever, 2013). Blockade of norepinephrine reuptake increases norepinephrine concentrations in the synaptic

cleft and thereby induces a stronger and/ or prolonged action of norepinephrine (Hajós et al., 2004). This leads to the question of whether the activation of postsynaptic norepinephrine receptors also has anti-narcoleptic effects. Conversely, the blockade of these norepinephrine receptors could support narcoleptic episodes.

In our experiment, we could only observe a slight (non-significant) decrease in the number and total duration of narcoleptic episodes after application of the α1 adrenoceptor agonist cirazoline. However, locomotor activity was also reduced. This decrease in activity also reduced the probability of having narcoleptic events. The lack of a clear cirazoline effect in the present study stands in contrast to the pronounced cataplexy-decreasing effects of phenylephrine (Burgess and Peever, 2013). However, the pharmacokinetic properties of these two α1 adrenoceptor agonists are different. Cirazoline has a good blood-brain-barrier penetration, whereas phenylephrine is considered to be a compound mainly acting in the periphery (Alsene et al., 2006). Phenylephrine was effective at a relatively high dose (10 $mg \cdot kg^{-1}$). However, since in our study 1 $mg \cdot kg^{-1}$ cirazoline already had effects on locomotor activity, we did not test higher doses.

As mentioned above, administration of α1 adrenoceptor antagonists increases cataplexy in a narcoleptic patient and orexin-deficient mice (Aldrich and Rogers, 1989; Burgess and Peever, 2013). In the present experiment, the number of narcoleptic events was not affected by prazosin but the total duration of narcoleptic episodes was slightly increased, which somehow mirrors the published effects. However, in the present study both compounds acting directly on the α1 adrenoceptor failed to have significant effects (whereas the reuptake inhibitors had strong effects) suggesting that this pharmacological mechanism is not the optimal one for treating narcolepsy.

Taken together, the present study in a murine narcolepsy model strongly supports the hypothesis that specific blockade of the norepinephrine transporter, here by reboxetine administration, is a very powerful pharmacological mechanism to treat symptoms of narcolepsy. Blockade of the serotonin transporter, here by escitalopram administration, was also effective in our study. However, it should be noted that metabolites of escitalopram are not selective anymore and also block the norepinephrine transporter (Mignot, 2012), i.e. the escitalopram effects could also be explained by a blockade of the norepinephrine transporter.

The results of project 1 were published in Behavioural Brain Research. Christian Schmidt and Judith Leibiger performed the research and analyzed the data. Christian Schmidt and Markus Fendt designed the study and wrote the manuscript.

6.2. Project 2: Local infusions of a putative orexin-specific saporin conjugate into the lateral hypothalamus of rats: Effects on orexin neurons and behavior

6.2.1. Effect of neurotoxin injections on the number of OxA-positive neurons

The aim of project 2 was to establish and evaluate a rat model of narcolepsy utilizing stereotaxic injections of an orexin-2-receptor targeting saporin conjugate (anti-Ox2R-SAP). In addition an unselective neurotoxin, IgG-SAP, and saline as a control substance were injected into the lateral hypothalamus.

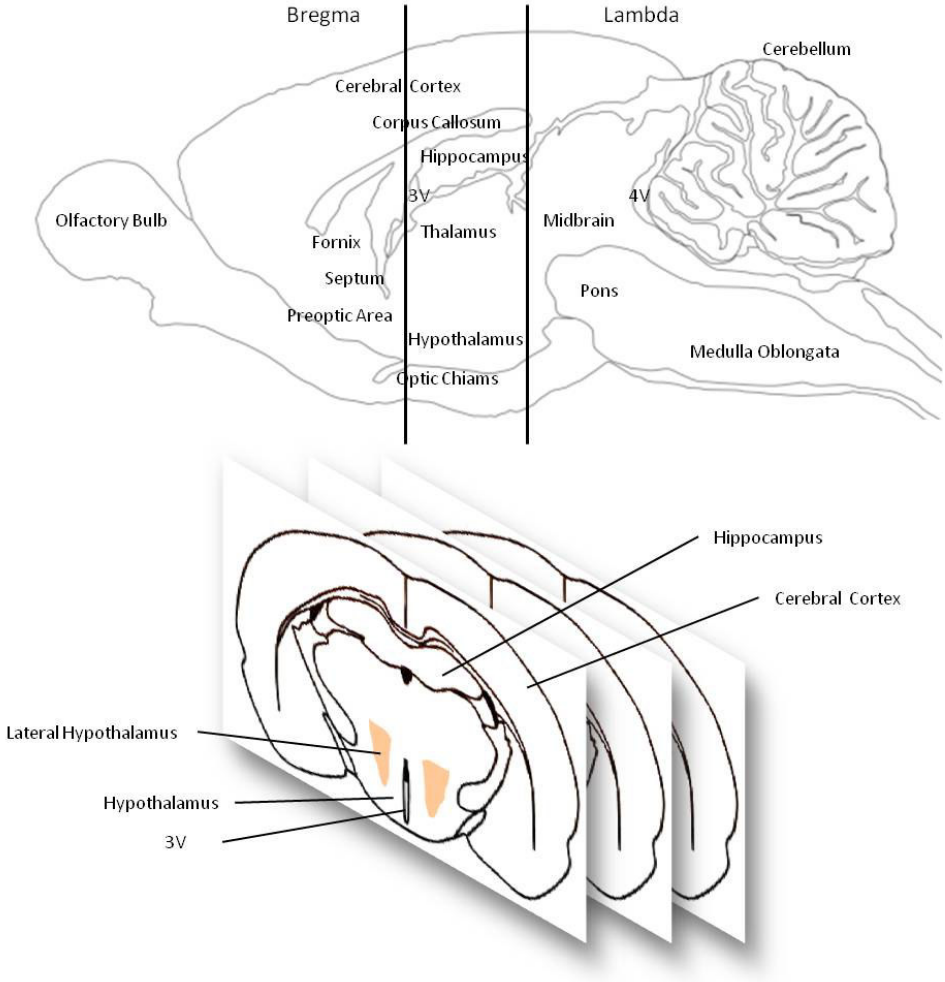

Fig. 23 Schematic drawing of the rat brain with marked lateral hypothalamus situated rostrocaudal -1.30 mm to -4.80 mm and rat brain slices at rostrocaudal -3.20 mm with a highlighted lateral hypothalamus.

In the Paxinos & Watson "The rat brain – in stereotaxic coordinates" fourth edition 1998 the lateral hypothalamus is situated rostrocaudal -1.30 mm to -4.80 mm with an expansion of 3.50 mm (Paxinos and Watson, 1998).

In the present study macroscopic lesions were detected in the above described region. In figure 24 the extent of lesions and injections traces are demonstrated prior to slicing and after immunohistochemistry. The lesions shown in figure 24A are generated after injection of saline. The

microscopic extent of these lesions is illustrated in figure 24B for the same animal and compared to an animal after injection of the anti-Ox2R-SAP neurotoxin (Fig. 24C).

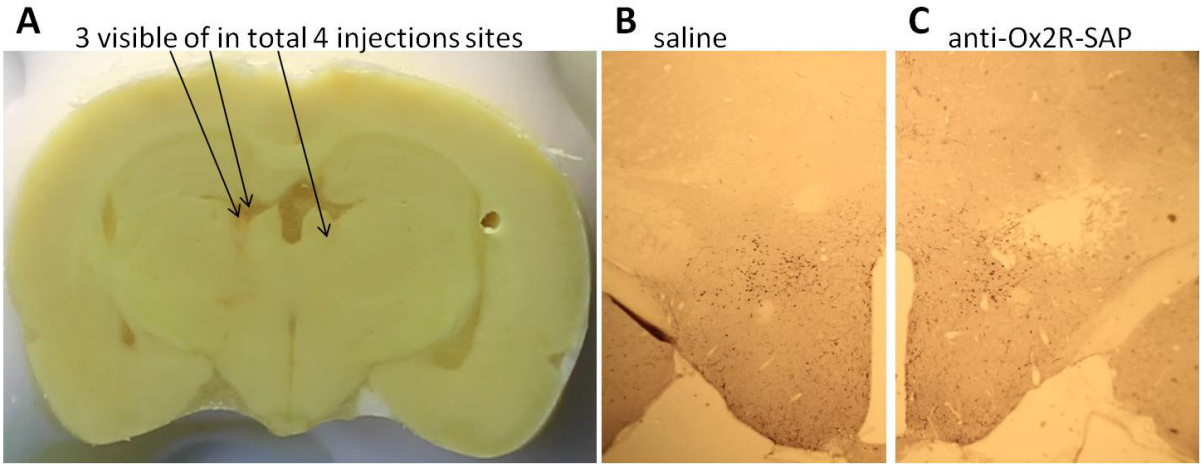

Fig. 24A Macroscopic picture of rat brain injections sites after saline injection(R041). Fig. 24B Microscopic picture of OxA positive neurons after saline injection (R041). Fig. 24C Microscopic picture of OxA positive neurons after anti-Ox2R-SAP injection (R044).

But in case of injections with IgG-SAP the brain tissue lesion was even larger. Factors such as intrasurgeon variability together with the diameter of needles (23 gauge; outer diameter 0.642 mm) may mask differences and contribute to these non-specific lesions. Even though there are a few reports on selective cell-specific lesions by injections of saporin-attached antibodies (Abdala et al., 2006; Carvalho et al., 2010), a huge personal impact on success is obvious. Minimizing intrasurgeon variability, the sequence and duration of injections as well as surgical implementation had to be developed during the study and were logged.

In order to develope a narcolepsy rat model the injection site should be in the medial part of the lateral hypothalamus and a selective acting neurotoxin is needed. Gerashchenko describes that injections of an orexin-A-SAP neurotoxin more lateral to the LH in the TMN had no effect on sleep, although the TMN contains neurons with orexin receptors (Gerashchenko et al., 2001). Other published reports describes that lesions of ~30% of the orexinergic neurons in the dorsomedial (DMH) and posterior (PH) hypothalamus have no effect of the sleepiness and circadian cycle (Chou et al., 2001). These findings support the hypothesis that the remaining cells below a benchmark of 40% compensate the effects of neuron loss, a phenomenon which is also discussed for neurodegenerative diseases like Parkinson or Lewy body dementia (Fearnley and Lees, 1991). In the study by Gerashenko a group with an OxA-positive neurons loss of less than 30% and more than 60% were formed. An increase of SOREMP frequency, an important symptom of narcolepsy, is attributed to lesions higher than 60% of OxA-positive neuron. Nevertheless, the authors determine that MCH-positive neurons are likewise significantly decreaesd after injection of the OxB-SAP neurotoxin (Gerashchenko et al., 2001).

If the loss of neurons hits a certain threshold, the system deregulates and first symptoms are retained. For further experiments, it is recommended that the OxB-SAP neurotoxin be administered and the number of lesioned animals raised to create a robust rat model of narcolepsy (Nirogi et al., 2019). Modern techniques like optogenetic silencing of orexin neurons are a promising approach for development of a narcolepsy rat model (Carter, 2011).

In previous experiments by Gerashchenko, a saporin was attached to OxB to target orexin neurons in the LH (Gerashchenko et al., 2001). OxB-SAP binding was proven in vitro in Chinese hamster ovary cells containing Ox1R or Ox2R. In the present study, similar coordinates (rostrocaudal -3.20 mm and -3.40 mm) were used, but in contrast to Gerashchenko et al. an anti-Ox2R-SAP was administered. The "Advanced Targeting Systems, Inc." company offered this anti-Ox2R-SAP instead of OxB-SAP due to lack of reproducibility and validitiy. Likewise, in the Gerashchenko et al. study the selectivity of the neurotoxin was evaluated by immunohistochemistry. In the LH, orexin neurons are situated adjacent to MCH neurons. Series of brain slices were stained either with anti-OxA or anti-MCH (Gerashchenko et al., 2001).

In the present study, the number of OxA-positive neurons after local injections of anti-Ox2R-SAP or IgG-SAP into the lateral hypothalamus were similar. The suggested specific acting on OxA-positive neurons of anti-Ox2R-SAP could not therefore be confirmed. Nevertheless, the injections of neurotoxins (anti-Ox2R-SAP or IgG-SAP) reduced the number of OxA-positive neurons significantly. In order to evaluate the effects of the orexin neuron loss on the behavior of the rats, two groups were formed according the number of OxA-positive neurons. Group α consisted of animals with more than 750 OxA-positive neurons, i.e. a normal number of orexin-positive neurons. All saline-treated rats, two anti-Ox2R-SAP-treated rats and four IgG-SAP-treated rats were combined in this group α. As demonstrated, the number of OxA-positive and most of the MCH-positive neurons in group α is higher than in group β. Group β is therefore considered as the successfully lesioned animals where as group α served as a control group.

6.2.2. **Effect of neuron loss on body weight and food intake**

The neurotransmitter orexin was named after the greek word orexis which can be translated as appetite. The close link between the neuropeptides effect and the designation by one of its discoverers was already made at birth (Sakurai et al., 1998). The animals in the experiments were wieghed because there is evidence that the orexin brain system has an impact on feeding behavior (Barson, 2018). Lesions of the orexin neurons could therefore affect the weight gain of the animals during the study. The rats were weighed prior the surgery at the age of eight weeks and at weekly intervals until scarification at the age of 19 weeks. Both groups gained weight during the study.

However, the animals in group β gained less weight three weeks after the surgery compared to rats in group α. These findings support evidence that neurons in the lateral hypothalamus have an impact on food intake. However, the changes of body weight may be the result of an unselective LH lesion, which besides OxA-positive neurons decrease also includes a MCH-positive neuron reduction. A study by Edwards suggests an essential effect of orexin on food intake. The application of orexin, NPY, and MCH by ICV injections was compared in terms of weight gain. It was revealed that NPY has the strongest impact on body weight while OxA and OxB is as potent as MCH (Edwards et al., 1999). In the present study, the group β animals exhibit an overall neuron loss, irrespectively MCH- or OxA-positive neurons. Further research is required to evaluate the cell-specific effect of the different LH neurons.

One of the influencing compensational mechanisms of weight gain which has not been adressed in the present experiment may be consequent behavior of arousal such as locomotion activity (Kotz et al., 2012). Even though there was no significant effect between the groups in the open field test, light dark box test and diurnal locootor activity, reduced locomotor activity of group β was observed in all three tests. This hypothesis is supported by published data that ICV application of OxA induces weight loss in rats (Novak and Levine, 2009). OxA increases locomotion in lean and obese rats to similar extent. A selective orexin receptor 1 antagonist (SORA-1R), SB-334867-A, and a research dopamine 1 receptor antagonist both diminish locomotor action in obesity-resistant rats which are stimulated by OxA (Teske et al., 2014). SB-334867-A reduces the OxA stimulated energy rate augmentation (Kiwaki et al., 2004). Notably, a study in narcoleptic children reveals that sleepiness and cataplexy starts earlier in obese kids, although non-obese or obese children had no difference in sleepiness quality (Inocente et al., 2013).

6.2.3. **Effect of orexin neuron loss on behavior**

The behavior monitoring box test was designed to assess behavioural arrests as a surrogate parameter for narcolepsy-like behaviour and was applied in project 1 in the mouse model (Schmidt et al., 2016). In the present study, both groups displayed a similar amount of behavioral arrests four and eight weeks after surgery. In a previous study with orexin-deficient mice, a variety of behavioral characteristics was reported and compiled as narcoleptic episodes (Leibiger and Fendt, 2014). Similar findings such as significant effects on total REM sleep time or episode duration in the lights-off period are published by (Chemelli et al., 1999). Different from that, in project 2, behavioral arrests were observed to the same extent in group α and group β. Presumably the observed behavioral arrests in the control group do not correspond to narcoleptic episodes and are presumably different behavior. A recent publication hypothesizes that behavioral arrests in wild-caught rats may

correspond to non-convulsive seizures with loss of conciousness (Taylor et al., 2019). Behavioral arrests in mice are therefore not transferable to rats.

Comparing the first and the subsequent hours, behavioral arrests increased over time in both groups. Narcoleptic episodes seen in orexin-deficient mice occurred predominantly in the last two hours of the experiment. Besides the unselective lesion of neurons in the LH, interspecies variances are a possible explanation for the findings in the present study. In contrast, in a previous published paper by Gerashchenko SOREMPs, sudden falling of the rat with irregular respiration and -phasic motor twitches were observed (Gerashchenko et al., 2001). Additionally the narcoleptic episodes are behavioral arrests triggered by emotions. But the emotional triggers of narcoleptic episodes seem to differ between the species, in dogs running and playful, in sheep fearful and in mice foraging behavior (Willie et al., 2003; Wu et al., 2011). Further experiments are needed to identify the emotional triggers for rats.

In a narcoleptic sheep cataplexy is more reliable triggered by sounds of barking dogs or being knocked over than by joyful or eating behavior. Some authors see this as a hint for a higher efficiency of anxiety-eliciting stimuli in triggering cataplexy (Blouin et al., 2013). The triggers for narcoleptic symptoms in rats are still unclear. An open field test was therefore performed with the unspecifically lesioned rats. The animals in both groups show higher locomotion activity in the first and second 5 minutes of the test compared to the last 5 minutes. The exploration of a novel environment does not seem to impact rats in either control group α or group β. Previous studies with rats in an open field paradigm report that a hippocampal lesion magnifies the diurnal fluctuation in locomotor activity while neocortical lesion abolishes these diurnal fluctuations (Iuvone and van Hartesveldt, 1977). Although, it appears that LH lesions showed no alterations in the open field activity further analysis with selective orexin-targeted lesions is needed for the evaluation.

In the light dark box test, innate anxiety is assessed (Bourin and Hascoët, 2003). In the present study, the group β animals had the tendency to stay longer in the light compartment than the control group, which could be explained as a diminished innate fear. But this effect did not reach statistical significance and additionally the latency to enter the light compartment did not differ to the control group. Orexin is considered as a modulator of arousal and motivation behavior may also promote the exploration of a novel environment (Li et al., 2018). In mice disinhibition of the amygdala excited the orexin producing neurons in the LH (Zhang et al., 2009). In a fearful situation, orexin neurons are activated to evoke a defense reaction, by projections to the LC (Soya and Sakurai, 2018). Even though the effect is not significant the tendency towards a decreased innate fear in unselectively lesioned rats is demonstrated in the present study. Instead of the avoidance of fearful situations the rats stay longer in the light compartment.

A startle reaction can be inhibited by a preceding stimulus which is assessed in the prepulse inhibition test. In psychiatric disorders like schizophrenia prepulse inhibition is decreased, which is explained by a dysfunction in sensorimotor gating (Geyer et al., 1990). Since several authors suggested a link between orexin and schizophrenia (Deutch and Bubser, 2007; Borgland and Labouèbe, 2010), prepulse inhibition was assessed in the two groups. In the present study, there is no difference in the prepulse inhibition test between control group α and group β. A prepulse inhibition deficit was found in human narcoleptics (Frauscher et al., 2012) and in the orexin-deficient mouse model (unpublished data by Fendt et al.). Lammers et al. published that narcoleptic patients have an excessive startle reflex (Lammers et al., 2000). Additionally there are reports showing that prepulse inhibition is reduced in narcolepsy patients (Frauscher et al., 2012). According to the limitations of the stereotactic injections it is suggested that the prepulse inhibition tests be reproduced with a selective orexin-deficient animal model.

Conditioned fear can be assessed in a fear-potentiated startle paradigm and is crucially linked to amygdala processing. Sensory information received from the thalamus is integrated into the amygdala and projected to the hypothalamus (Davis et al., 1997). The influence of arousal maintenance by orexin in rats is accessed in the present study with a fear-potentiated startle test. We did not observe any significant differences in either group, although there are hints that strong emotions like fear may activate orexin neurons in the LH (Furlong et al., 2009). Predominantly emotional signs activate the orexin neurons which modulate the norepinephrine signaling from the LC to the lateral amygdala. An inhibition of the orexinergic effects by optogenetic and pharmacological treatment lowers the conditioned fear response. The effect of OxR1 inhibition has been conducted by pharmacological agents (Soya et al., 2017). In humans levels of orexin are augmented during social interaction e.g. anger or positive emotions and are not detectable during pain and sleep (Blouin et al., 2013). The results of the present study tend more towards the latter findings. Nevertheless, it is noteworthy that the methodological limitations in the present experiments restrict continuative conclusions.

Orexin neurons are known to discharge frequently in arousal situations and waking periods. The release of orexin in NREM sleep is not detectable, but occasional bursts appear in REM sleep (Lee et al., 2005; Mileykovskiy et al., 2005). In the present diurnal locomotor activity rhythm test, the rats in control group α have the tendency to show more locomotor activity in the lights-off period compared to animals with a lower number of orexin neurons. Nevertheless this result corresponds to a variety of published papers. In animals with decreased daytime sleepiness, REM sleep occurs more frequently in night sleep (Mistlberger et al., 1987). SOREMPs are identified by Gerashchenko with rocking behaviour of the rats at the beginning and a long-term effect on sleep architecture was

suggested by the authors (Gerashchenko et al., 2001). The criteria of SOREMPs by Carskadon consists of behavioral observation and electrophysiological monitoring. They are defined as REM sleep onset in 15 min and apply to humans, but not to animals (Carskadon et al., 1986). According to Shiromani, young Sprague Dawley rats have a fast sleep wake cycle with small wake periods ~1.9 min at daytime and at night ~6.3 min (Shiromani et al., 2000). The rats have therefore been awakened and dozed off in the 15 min of the human definition of SOREMPs. Gerashchenko modified the definition for rats. REM sleep onset in between above 2 min and less than 2 min of slow wave sleep. During the day the rats with orexin deficient cells had no increased sleep but in the night the slow wave sleep and REM sleep increases to twice that of non-lesioned animals (Gerashchenko et al., 2001).

In a paper by Adie a disturbance of the sleep wake cycle in narcoleptic patients was suggested (Adie, 1926). The circadian rhythm is determined by the suprachiasmatic nucleus (SCN) and influences the sleep onset as well as awakening. Approximately 26% of the SCN projections aim to the orexinergic neurons and 38% to the MCH producing neurons in the rat hypothalamus (Abrahamson et al., 2001). Destruction of the SCN shows that REM sleep duration and frequency are altered although the total quantity of sleep stages is not affected (Coindet et al., 1975). Rats with lesions of the dorsomedial hypothalamus (DMH) exhibit a decrease of REM sleep, NREM sleep and locomotor activity (Choue et al., 2003). The molecular background of the internal pacemaker in the SCN was discovered much later (Wijnen and Young, 2006). Although the early REM onset is a symptom of narcolepsy it is not affected by the SCN. The circadian clock in narcoleptic patients is unaltered (Dantz et al., 1994). In the present study, there are diffent locomotor activity levels in the lights-off and lights-on period. The switch is accompanied with the beginn of either lights-on or lights-off and equal in control group α and group β. This finding may be considered as the duration of total sleep. Even though the frequency of sleep attacks is increased in narcoleptics, the overall sleep duration does not differ from non-narcoleptic humans (Aldrich, 1991).

6.3. General discussion and future directions

An underlying receptor deficiency of the Ox2R and the link to canine narcolepsy has been investigated by Lin (Lin et al., 1999). For further investigation of narcolpesy a mice model with a prepro-orexin knockout has been established (Chemelli et al., 1999). This mouse model was applied in project 1, although there are limitations of the etiology compared to human narcolepsy which has been characterized by an absence of orexin producing neurons (Nishino et al., 2000). Nevertheless the application of NRI has been proven to be beneficial in reducing narcoleptic episodes more effectively than SSRI application. This result of project 1 matches with various papers which emphasize the importance of orexin transmission for LC activity and to the characteristic muscle tone

loss. In the first case of norepinephrine application to narcoleptics an exacerbation of cataplexy has been proven (Aldrich and Rogers, 1989). Short after the discovery of orexin, the strongest projection of orexin neurons in the LH to the norepinephrine center in the LC has been investigated (Peyron et al., 1998). Finally, the first explanation models of interrupted norepinephrine signaling to cause cataplexy has been published (Burgess and Peever, 2013).

Confirming these principles and considering the adverse effects of NRI are indispensable for further applications as anti-cataplectic drugs. For instance the influence of REM sleep and effects of memory consolidation by reboxetine are discussed. The increase of REM sleep latency in rats subsequent to reboxetine administration has been published (Wong et al., 2000). On the other hand, narcolepsy is characterized as a REM sleep behavior disorder with SOREMPs and occurrence of REM sleep periods at any daytime (Scammell, 2015).

Orexin neurons excite REM-off neurons population in the extended part of the ventrolateral preoptic nucleus (eVLPO), which projects to the cholinergic mesopontine tegmentum including the ventrolateral part of the periaqueductal grey matter (vlPAG) and laterodorsal pontine tegmentum (LDT). The VLPO neurons are most active during sleep and promote sleep via GABAergic projections to wake-promoting regions such as the histaminergic tuberomammillary nucleus (TMN) and other monoaminergic nuclei (Sherin et al., 1996; Sherin et al., 1998). A lesion of vlPAG or LDT results in increased frequency and duration of REM sleep at light periods and decreased REM sleep in dark periods. A single lesion of the LDT, but not vlPAG, increases the frequency of REM sleep at light periods and facilitates SOREMPs. REM-on neurons consisted of projections to sublaterodorsal nucleus (SLD) and pre-locus coeruleus (pre-LC) (Lu et al., 2006). According to a published hypothesis REM sleep suppression by reboxetine does not affect the memory consolidation of procedural motor-skill (Rasch et al., 2009). Further investigations especially in human-similar animal models like a rat model are needed to combine the knowledge of REM sleep symptoms like cataplexy with the emotional triggers.

In project 2 we evaluated a rat model, which was intended to suppress orexin signaling. Besides the limitations we demonstrated that with stereotactic injections of Ox2R-SAP in the LH, the suppression was reproducible and verified by immunohistochemistry. The close link between narcoleptic REM sleep-like symptoms with emotional triggers is yet to be determined. An essential connection regarding the emotional triggers of narcoleptic episodes is the close interplay of the hypothalamus and other parts of the limbic system (Dudas, 2013). Emotions but most commonly cheerful behavior is linked as a trigger for cataplexy (Blouin et al., 2013). Unlike in frightening situations orexin neurons discharge less frequently (Beuckmann et al., 2004). In a study with orexin-deficient mice, it is reported that innate fear is augmented in the open field and light dark box test (Khalil and Fendt,

2017). Recent studies revealed that depression is slightly more frequent in narcoleptic humans (Mahoney et al., 2019). Additionally, orexin signalling is proposed to prevent depression-like behavior (Ji et al., 2019). Ultimately the linkage between these trigger emotions and cataplectic attacks is still an enthralling domain of future research.

The present rat model of narcolepsy in project 2 has a variety of limitations. The immunohistochemical evaluation of the lesions was determined after time-consuming preparation with inherent possible disturbances. Orexin-specific lesions are a necessity to develope a robust animal model of narcolepsy. Conversely, the results of the behavioral tests in project 2 reflect the well-known linkage of the excitatory role of orexin characterized by glutaminergic secretion (Abrahamson et al., 2001) and the dysfunction of the orexin system which has been identified in human narcolepsy (Thannickal et al., 2000). Based on this cell-specific paradigm, the model additionally has to be reproducible, which favors a cross over design with a molecular switch to show or supress disease manifestations. Delay-free evaluation by optogenetic engineering might be a powerful tool to deepen the underlying pathomechanisms of cataplexy.

As stated above many hypotheses are currently referring to an autoimmune disease as a possible etiology (Cvetkovic-Lopes et al., 2010). Moreove,r the linkage between vaccination and acquired type 1 narcolepsy suggests itself (Luo et al., 2018). Future investigations in cell-models are needed to address the mechanisms of autoimmunologic pathways in narcolepsy.

The diagnosis of narcolepsy is often reserved to special care units due to its rare occurrence in the general population. This delay of diagnosis interferes extraordinarily with the quality of life in non-treated or wrongly-treated narcoleptic individuals (Kryger et al., 2002; Thorpy and Krieger, 2014). Even though the investigation of neuropathology and treatment has advanced in the recent years further efforts are indispensable. Like most published papers, the present studies focused on narcolepsy with cataplexy as the type with the highest occurrence in the general population. Nevertheless, there are still gaps in the comprehension of type 2 narcolepsy or even unknown subtypes. In diseases with low frequency of occurrence machine intelligence could be the means to identify helpful diagnosis criteria and ameliorate the diagnosis and treatment of patients (Zhang et al., 2018).

7. Summary and Conclusion

The neurodegenerative disease of narcolepsy is characterized by the loss of orexin/ hypocretin-producing neurons. In addition to increased daytime sleepiness, cataplexy is the disease-specific classifying symptom. Cataplexy is a loss of muscle tone lasting several minutes, which may be triggered by strong emotional excitement, such as joy or a fright reaction. Often these narcoleptic episodes are associated with falls.

In project 1, laboratory mice with a genetically-induced orexin deficiency (prepro-orexin-KO mice) and a described narcoleptic phenotype were used. Different pharmacological substances were administered to these mice to test their effects on narcoleptic episodes. The lack of orexin affects the activity of serotonergic, histaminergic and adrenergic centers in the mice brain. In a crossover design, different doses of serotonergic and adrenergic substances were injected intraperitoneally. Subsequently, the laboratory mice were observed for four hours in a test kit with toys and food and their behavior recorded on video.

In vehicle-treated mice, there was an increase of narcoleptic episodes over the four hours. The use of escitalopram, a selective serotonin reuptake inhibitor and reboxetin, a selective norepinephrine reuptake inhibitor, significantly suppressed the narcoleptic episodes. The administration of prazosin, an α1 adrenergic receptor blocker, and cirazolin, a selective α1 adrenergic agonist had no significant effect. However, cirazoline also significantly reduced spontaneous locomotion immediately after administration.

For further investigation in project 2 a rat model by stereotactic injection was developed. The neurotoxin saporin coupled to antibodies against Orexin-2 receptors was therefore injected into the lateral hypothalamus in which the orexin neurons are located. Immunohistological analysis revealed that both Ox2R-SAP and IgG-SAP successfully induced lesions in the lateral hypothalamus. There were significantly less orexin-positive neurons after the injections. However, the number of MCH neurons was also reduced with Ox2R-SAP, i.e. the effects were not orexin-specific. Animals without lesions and animals with clear lesions were pooled for further analyses. Lesions had only a minor impact on body weight of the animals or different behavioral paradigm, in which effects of orexin neuron loss were expected.

In summary, narcoleptic episodes can be more efficiently suppressed by adrenergic stimulation (reboxetine) then by serotonergic stimulation (escitalopram). Treatment with a selective α1 adrenergic agonist (cirazolin) induced sedation, which superimposes a potential decrease of narcoleptic episodes.

III. References

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IV. List of Figures

V. List of Tables

VI. Acknowledgments and Conflicts of Interest

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VII. Declaration

I declare in accordance with the doctoral regulations of the Medical Faculty of the Otto von Guericke University Magdeburg (§5(2)/Anlage 6).

Ich erkläre, dass ich die der Medizinischen Fakultät der Otto-von-Guericke Universität zur Promotion eingereichte Dissertation mit dem Titel

Dr. med.

im Institut für Pharmakologie und Toxikologie
mit Unterstützung durch die Arbeitsgruppe von Prof. Dr. rer. nat. Markus Fendt
ohne sonstige Hilfe durchgeführt und bei der Abfassung der Dissertation keine anderen als die dort aufgeführten Hilfsmittel benutzt habe.

Bei der Abfassung der Dissertation sind Rechte Dritter nicht verletzt worden.

Ich habe diese Dissertation bisher an keiner in- oder ausländischen Hochschule zur Promotion eingereicht. Ich übertrage der Medizinischen Fakultät das Recht, weitere Kopien meiner Dissertation herzustellen und zu vertreiben.

Magdeburg, den 29.02.2020

Unterschrift

VIII. Papers & Oral Presentations

Peer-review publications:

Schmidt C., Leibiger J., Fendt M. (2016) The norepinephrine reuptake inhibitor reboxetine is more potent in treating murine narcoleptic episodes than the serotonin reuptake inhibitor escitalopram. Behavioural Brain Research 308: 205-210

Christian Schmidt and Judith Leibiger: data acquisition
Christian Schmidt and Markus Fendt: drafting/revising the manuscript; and analysis or interpretation of data, study conceptor design, statistical analysis
Markus Fendt: study supervision

Stengel H., Vural A., Brunder A-M, Heinius A., Appeltshauser L., Fiebig B., Giese F., Dresel C., Papagianni A., Birklein F., Weis J., Huchtemann T., Schmidt C., Kortvelyessy P., Villmann C., Meinl E., Sommer C., Leypoldt F., Doppler K. (2019) Anti–pan-neurofascin IgG3 as a marker offulminant autoimmune neuropathy. Neurology: Neuroimmunology & Neuroinflammation 6, 5: 1-11

Christian Schmidt: data acquisition

Submitted: K-C. Broscheid, T. Behrendt, D. Hamacher, S. Böker, T. Gagelmann, C. Schmidt, C. Caspari, K. Meiler, A. Napiontek, J. Franke, L. Schega (2020) Effect of a multimodal movement intervention in patients with neurogenic claudication based on Lumbar Spinal Stenosis and/or degenerative Spondylolisthesis – A pilot study

Christian Schmidt: study design, study execution

Non-peer-reviewed publication:

Schmidt C., Exner L., Stichtenoth D. (2014) ATIS informiert Wechselwirkung zwischen Pantoprazol und Methotrexat? Niedersächsisches Ärzteblatt 87: 40-41

Oral presentations:

Schmidt C., Puhlmann E. (2010) Kaliuretische Arzneimittel. Qualitätszirkel derApothekerkammer Berlin; Berlin

Schmidt C., Leibiger J., Fendt M. (2015) Targeting the serotonergic and noradrenergic brain system to treat narcolepsy in a mouse model. 11th Göttingen Meeting of the German Neuroscience Society; Göttingen

Schmidt C., Leibiger J., Fendt M. (2016) Noradrenergic and serotonergic compounds to target narcoleptic episodes in a mouse model. Annual Meeting of the German Pharmaceutical Society – DPhG 2016; Munich

Christian Schmidt received traveling grants from the Commission for the Promotion of Young Scientists of the Medical Faculty University Hospital Magdeburg.

IX. Curriculum Vitae

Due to data protection the Curriculum vitae is not included in this version.

Der Lebenslauf ist in der Version aus Datenschutzgründen nicht enthalten.

X. Supplements

Material Projekt 1 & 2

Stereotaxic Alignment System	David Kopf Instruments, Tujunga, CA (USA)
Zeiss Axioscope (microscope)	Carl Zeiss Microscopy GmbH, Jena (Germany)
isoflurane vaporizer	Rothacker & Partner, Bern (Switzerland)
Canon EOS 1100D	Canon Deutschland GmbH, Krefeld (Germany)
Nouvag NM 3000 (electric drill)	Nouvag AG, Goldach, (Germany)
Leica CM 1950 (cryostat)	Leica Biosystems Nussloch GmbH, Nussloch, (Germany)
Schott KL-1500 electronic (surgery light source)	Lighting and Imaging SCHOTT AG, Mainz (Germany)
Mettler PM 3000 (balance rats)	Mettler-Toledo GmbH, Greifensee (Switzerland)
Sartorius BP3100P (balance mice)	Sartorius AG, Göttingen (Germany)
Temperature Controller CMA/150 Microdialysis	CMA/Microdialysis, Stockholm (Sweden)
Startle system	SR-LAB, San Diego Instruments, San Diego, CA (USA)
EthoVision XT	Noldus Information Technology, Wageningen, (Netherlands)
Melag 75 (hot air sterilizer)	MELAG Medizintechnik, Berlin (Germany)
ActiMot2 (measurement of locomotor activity with infrared light-beam)	TSE Systems GmbH, Bad Homburg (Germany)
EP-1 Econo Pump (electric pump)	Bio-Rad Laboratories Ltd., Hemel Hempsted (UK)

Tab. 2 Testing apparatus and instruments used in the experiments

GraphPad Prism 7	GraphPad Software, Inc.,La Jolla, CA (USA)
ImageJ	Public Domain, developed at the National Institutes of Health, Bethesda, MD (USA)
Microsoft Office Professional Plus 2010	Microsoft Corporation, Redmond, WA (USA)
Reference Manager	Adept Scientific, Göteborg (Sweden)
SR-LAB Software	SR-LAB, San Diego Instruments, San Diego, CA (USA)
Citavi 6	Swiss Academic Software GmbH, Wädenswil (Switzerland)
TSE Phenomaster	TSE Systems GmbH, Bad Homburg (Germany)
Windows Media Player	Microsoft Corporation, Redmond, WA (USA)

Tab. 3 Software used in the experiments

Bedding material	Abedd LAB & VET Service GmbH, Wien (Austria)
Food pellets	Sniff, Spezialdiäten GmbH, Soest, (Germany)
TPP® tissue culture plates	Sigma-Aldrich Chemie GmbH München (Germany)
Syringe	Hamilton Bonaduz AG, Bonaduz (Switzerland)
Needles	B. Braun Melsungen AG, Melsungen (Germany)
4-0 Vicryl filament	Johnson & Johnson Medical GmbH, Ethicon Deutschland, Norderstedt (Germany)

Tab. 4 Consumable supplies and expendables used in the experiments

3,3'-Diaminobenzidine (DAB)	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
IgG-SAP (Rabbit IgG antibody attached to saporin)	Advanced Targeting systems, San Diego, CA (USA)
Anti-Ox2R-SAP (Orexin-2 receptor antibody attached to saporin)	Advanced Targeting systems, San Diego, CA (USA)
Bovine serum albumin (BSA)	Sigma-Aldrich Chemie GmbH München (Germany)
Cresyl violet	See appendix
Entellan®	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
Isoflurane	Baxter Germany GmbH, Unterschleißheim (Germany)
anti-Orexin A-rabbit (primary antibody)	Calbiochem, San Diego, CA (USA)
anti-MCH-rabbit (primary antibody)	Phoenix pharmaceuticals Inc., Burlingame, CA (USA)
bioytinylated anti-rabbit IgG (secondary antibody)	Vector Laboratories, Burlingame, CA, (USA)
Vectastain® Elite ABC-Kit	Vector Laboratories, Burlingame, CA, (USA)
Jung®Tissue freezing medium	Leica Biosystems Nussloch GmbH, Nussloch (Germany)
Normal goat serum (NGS)	Vector Laboratory® LINARIS, Dossenheim, (Germany)
Picric acid (2,4,6-trinitrophenol)	See appendix
Phosphate buffer (PBS)	See appendix
Roti-Histol®	Carl Roth GmbH + Co. KG, Karlsruhe (Germany)
Triton X-100®, Octoxinol 9	Sigma-Aldrich Chemie GmbH München (Germany)

Tab. 5 Compounds used in the experiments

Standard operating procedures (German)

Nissl-Färbung mit Cresylviolett

1. Geräte: beschichtete Deckgläser mit Gehirnschnitten, Pinzette
2. Herstellen der 1%ige Cresylviolett-Lösung (2,5 g·250 mL^{-1} = 12 Tropfen CH_3COOH), evtl. Filtrieren und Rühren vor der Benutzung; Ethanol (70%, 50%, 96%, 100%); Roti®-Histol, Entellan
3. Einfärben der Objektträger:
   a. 70% EtOH ca. 2 h
   b. 50% EtOH 15 min
   c. Cresylviolett 4 – 20 min, blau; Färbung kontrollieren
   d. 70% EtOH ca. 2 min
   e. 96% EtOH 3 – 15 min; je nach Intensität der Färbung
   f. 100% EtOH-Lsg 1 ca. 2 min
   g. 100% EtOH-Lsg 2 ca. 2 min
   h. Roti®-Histol-Lsg 1 3 – 10 min kanzerogen → Abzug!
   i. Roti®-Histol-Lsg 2 3 – 10 min
   j. Entellan® 1 – 4 Tropfen
4. die Deckgläser werden im Winkel von 45° aufgesetzt und mit einer Pinzette die verbreibenden Luftblasen aus dem Präparat gedrückt
5. die Objektträger, sowie die verwendeten Utensilien verbleiben zumindest über 24h unter dem Abzug um das Verdunsten vom Roti®-Histol zu ermöglichen
6. die Objektträger möglichst einige Wochen horizontal stehen lassen (nicht sofort vertikal in einen Aufbewahrungskasten setzen)

Protokoll Immunfixation

	100	300	1000	1500	2000	Ml
4% Paraformaldehyd	4	12	40	60	80	G
Aqua bidest.	35	105	350	525	700	Ml
0,2 mol/l PBS	50	150	500	750	1000	Ml
15% gesättigte Pikrinsäure	15	45	150	225	300	Ml

- Paraformaldehyd und PBS auf max. 75°C erhitzen (bei Temp. Darüber fällt die Substanz aus) bis das Gemisch klar wird
- Aqua dest. U. Pikrinsäure dazugeben
- Abkühlen und filtrieren

Kryoprotektive Lösung

Ethylenglycol 30%ige Lsg (Aufbewahrung für Kryo-Schnitte bei -20 °C

(30%) Ethylenglykol	150 ml
(25%) Glycerin	125 ml
0,05 M PB	225 ml
	500 ml

Phosphatpuffer 0,2 M

Lösung 1		Lösung 2	
Na_2HPO_4 * $2H_2O$	Aqua dest.	Na_2HPO_4 * H_2O	Aqua dest.
35,6 g	1000 ml	8,28 g	300 ml
53,4 g	1500 ml	12,42 g	450 ml
71,2 g	2000 ml	16,56 g	600 ml
89,0 g	2500 ml	17,94 g	650 ml
106,8 g	3000 ml	20,70 g	750 ml
		23,46 g	850 ml

pH 7,35 – 7,4 (7,39)

Lösung 2 wird zu Lösung 1 gegeben bis der pH-Wert eingestellt ist

Na_2HPO_4 * $12H_2O$	Aqua dest.
71,63 g	1000 ml
107,45 g	1500 ml

DAB-Lösung (3,3‘-Diaminobenzidin-tetrahydrochlorid)

Herstellung: 1 g/100 ml Aqua dest. (10 mg/ml in Tubes; 100 mg/10 ml in Röhrchen)

1. Rühren
2. Ultraschall 2min
3. Rühren
4. Nicht filtrieren
5. Aliquotieren
6. Tiefkühlen Lagerung bei -20 °C

DAB-Lösung:	Abstoppen der DAB-Lösung mit
1 ml des DAB in 20 ml PBS	Hypochlorit 12%

Lagerung der Gehirnschnitte

Schritt	**Anweisung 1**	**Anweisung 2**	**Dauer**
1	Im Anschluss der Perfusion Gehirne in Immunfix für 1 Nacht einlegen		
2	Die Gehirne in 20% Saccharose PB inkubieren bis sie auf den Boden sinken		1 -2 d
3	zum Schockfrieren werden die Gehirne vorsichtig betupft	Flüssigkeit aus den Ventrikeln drücken, um Gefrierschäden zu vermeiden	
4	in -48 °C bis -60 °C kalten 2-Methylbutan werden die Gehirne schockgefroren	bei -80 °C lagert	
5	Schnitte mit einer Dicke von 40 µm anfertigen	in 4 x 12 Well-Platten gefüllt mit 0,1 M PB sammeln	
6	Schnitte in Kryo-Lösung bei -20 °C lagern		
7	Block-pAK ansetzen	BSA schlecht löslich	
8	AK-Verdünnung wählen	Volumen bestimmen (ca 2 ml je Serie)	

Immunhistochemie

AK: Anti-Orexin A (purified rabbit polyclonal antibody, Calbiochem®); Immunogen: synthetisches Peptid (CRLYELLHGAGNHAAGILTL) terminaler Abschnitt von Orexin A [14.- 33. AS]

Schritt	**Anweisung 1**	**Anweisung 2**	**Dauer**
1	Gehirne aus -20 °C (Kryo-Lösung) auftauen		
2	Spülen in 0,1 M PB + 0,3% Triton		3 x 10 min
3	Block-POX: Blockierung der endogenen Peroxidase		10 min
4	Spülen in 0,1 M PB + 0,3% Triton		3 x 10 min
5	Block-pAK: Blockierung für polyklonale AK		30 min
6	1. AK-Verdünnung		2 Tage, 4 °C KS, Schüttler
7	Spülen in 0,1 M PB + 0,3% Triton		3 x 10 min
8	2. AK-Verdünnung		2 h
9	Spülen in 0,1 M PB		3 x 10 min
10	AB-C Elite (Avidin-Biotin-Complex)		1 h
11	Spülen in 0,1 M PB		3 x 10 min
12	DAB (toxisch, Handschuhe) Vortexen + 2 min US-Bad Nach 12- 15 min abstoppen	(mit Verstärkung: $CoCl + NH_4NiSO_4$) nach 8 – 10 min abstoppen	Vortexen; 2 min im US-Bad
13	Färbung unter dem Mikroskop beurteilen		
14	Abstoppen von DAB mit 12% Hypochlorit-Lsg.	nach 8-10 min ohne Verstärkung nach 12-15 min mit Verstärkung	
15	Spülen in 0,1 M PB		3 x 10 min
16	Aufziehen, über Nacht bei 37 °C trocknen		1 Nacht
17	Entwässern in EtOH-Reihe und Eindecken mit Entellan®		

Immunofix	**Kryo-Lösung**	**NGS**	**Block-POX**
4 g Paraformaldehyd (4%)	150 ml Ethylenglykol (30%)	Normal goat serum	9,5 ml PB (0,1 M)
35 ml Aqua bidest.	125 ml Glycerin (25%)	Unverdünnt von Vector Laboratory®	500 µl MeOH
50 ml PB (0,2 M)	225 ml PB (0,05 M)		100 µl 30%H_2O_2
15 ml Pikrinsäure (15% gesättigt)			

Block-pAK	1. AK-Verdünnung **1:250**	1. AK-Verdünnung **1:500**	1. AK-Verdünnung **1:1000**
9 ml PB (0,1 M)	986 µl PB (0,1 M + 0,3% Triton)	988 µl PB (0,1 M + 0,3% Triton)	989 µl PB (0,1 M + 0,3% Triton)
1 g BSA	10 µl NGS	10 µl NGS	10 µl NGS
1 ml NGS (Normalserum)	4 µl AK	2 µl AK	1 µl AK

2. AK-Verdünnung	**Vectastain AB-C Elite**	**DAB**	**DAB-Verstärkung**
1 ml PB (0,1 M)	1 ml PB (0,1 M)	20 ml PB (0,1 M)	500 µl Co-Chlorid
4,4 µl bio anti (rabbit)	8,8 µl A	1 ml DAB	400 µl Ammonium-nickelsulfat
GOAT ANTI-Rabbit IgG (H+L): Biotin	8,8 µl B	Mischen → steril filtrieren	
CatNo: BA-1000	CatNo: PK-6100-PK-6106	220 µl 0,3% H_2O_2 Kurz vorher zugeben	

Permission for the submission of an English-language dissertation (German)

Sehr geehrter Herr Schmidt,

10. April erhielt Herr Prof. Dr. Mawrin, Vorsitzender der Promotionskommission, Ihren Antrag auf Erlaubnis zur Einreichung einer englischsprachigen Dissertation zur Verleihung des akademischen Grades „Dr. med." (doctor medicinae).

Im Auftrag von Herrn Prof. Mawrin möchte ich Ihnen mitteilen, dass Sie das Einverständnis haben, Ihre Dissertation in englischer Sprache einzureichen. Wie in der Promotionsordnung der Medizinischen Fakultät der Otto-von-Guericke Universität Magdeburg nach § 6 Abs. 4 gefordert,
soll die Dissertation zusätzlich eine deutschsprachige Zusammenfassung beinhalten. Die Verteidigung sollte in deutscher Sprache erfolgen.

Für weitere Rückfragen stehen wir Ihnen gern zur Verfügung.

Mit freundlichen Grüßen
Kerstin Schumacher
Mitarbeiterin für akademische Angelegenheiten

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Permission for animal experiments (German)

ANHALT 800 1212-2012

SACHSEN-ANHALT

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Über
den Tierschutzbeauftragten
Herrn Dr. Matthias

Halle, 08. Nov. 2012

Ihr Antrag vom 15.07.2012
Mein Zeichen: 203.h-42502-2-1173UniMD

Bearbeitet von: Hr. Dr. Schneider

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Tierschutz: Genehmigung von Versuchen an lebenden Tieren
Ihr Antrag vom 15.07.2012, Ergänzung vom 18.10.2012

Sehr geehrter Herr Professor Fendt,

aufgrund des § 8 Tierschutzgesetz in der geltenden Fassung erteile ich Ihnen **ab 19.11.2012** unter dem Vorbehalt des jederzeitigen Widerrufs die Genehmigung zur Durchführung von Versuchen an lebenden Tieren **bis zum 18.11.2015**.

1.Bezeichnung des Versuchsvorhabens:
„Narkoleptische Episoden bei Labornagern: neuronale Grundlagen und mögliche Behandlungsstrategien“,
unser Aktenzeichen: 42502-2-1173 Uni MD.

2. Leiter des Versuchsvorhabens:
Herr Prof. Dr. rer. nat. Markus Fendt

2.1. Stellvertretender Leiter des Versuchsvorhabens:
Herr Prof. Dr. rer. nat. Axel Becker

3. Bezeichnung und Anzahl der Versuchstiere:
Maus, 180; Ratte 150

4. Versuchsstandort:
Zentrales Tierlabor (ZTL im ZENIT Haus 65 oder Haus 30) und Institut für Pharmakologie und Toxikologie (IPT Haus 20), der Medizinischen Fakultät

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5. Versuchsdurchführung:

Bei der Durchführung des Versuchsvorhabens sind die Bestimmungen des Tierschutzgesetzes, insbesondere der §§ 9 und 9a zu beachten.

6. Änderungen:

Gemäß §8 Abs.7 Satz 2 des Tierschutzgesetzes sind Änderungen anzeigepflichtig, wenn

⇒ der Zweck des Versuchsvorhabens beibehalten wird;

⇒ bei den Versuchstieren keine stärkeren Schmerzen, Leiden oder Schäden entstehen;

⇒ die Zahl der Versuchstiere nicht wesentlich erhöht wird und

⇒ diese Änderungen vorher der zuständigen Behörde angezeigt worden sind.

Wechselt der Leiter eines Versuchsvorhabens oder sein Stellvertreter, so ist diese Änderung unverzüglich gem. § 8 Abs.4 Tierschutzgesetz anzuzeigen.

Alle anderen Änderungen genehmigungspflichtiger Tierversuche sind genehmigungspflichtig.

7. Auflage:

Nach Abschluss der Versuche bittet die Kommission bis Februar 2016 um eine Rückinformation über

- die Anzahl der tatsächlich im Versuchsvorhaben eingesetzten Tiere;
- die tatsächliche Belastung der Tiere,
- den Versuchsverlauf und ob sich die Versuchsergebnisse wie erwartet, gestaltet haben,
- die Eignung des gewählten Tiermodells;
- den geplanten Eingang der Versuchsergebnisse in den wissenschaftlichen Fortschritt (Publikation).

Rechtsbehelfsbelehrung

Gegen diesen Bescheid kann innerhalb eines Monats nach Bekanntgabe Klage beim Verwaltungsgericht Magdeburg, Breiter Weg 203-206, 39104 Magdeburg, schriftlich, in elektronischer Form oder zur Niederschrift des Urkundsbeamten der Geschäftsstelle dieses Gerichts erhoben werden. Die Klage muss den Kläger, den Beklagten und den Gegenstand des Klagebegehrens bezeichnen. Sie soll einen bestimmten Antrag enthalten. Die zur Begründung dienenden Tatsachen und Beweismittel sollen angegeben werden. Falls die Klage schriftlich oder zur Niederschrift erhoben wird, sollen der Klage nebst Anlagen so viele Abschriften beigefügt werden, dass alle Beteiligten eine Ausfertigung erhalten können. Falls die Klage in elektronischer Form erhoben wird, sind die elektronischen Dokumente mit einer qualifizierten elektronischen Signatur nach dem Signaturgesetz zu versehen. Sie ist bei der elektronischen Poststelle des Verwaltungsgerichts Magdeburg über die auf der Internetseite www.justiz.sachsen-anhalt.de/erv bezeichneten Kom-

231)

munikationswege einzureichen. Die rechtlichen Grundlagen hierfür sowie die weiteren technischen Anforderungen sind unter der vorgenannten Internetseite abrufbar.

Mit freundlichen Grüßen
Im Auftrag

Dr. Schneider

SACHSEN-ANHALT

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Über
den Tierschutzbeauftragten
Hr. Dr. Matthias

Tierschutz – Durchführung von Tierversuchen
Ausnahmegenehmigung nach § 9 (1) Satz 4 Tierschutzgesetz
Ihr Antrag vom 07.01.2013

Halle, 16. Jan. 2013

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Sehr geehrter Herr Professor Fendt,

aufgrund des § 9 des Tierschutzgesetzes in der geltenden Fassung erhält

Herr Christian Schmidt

unter dem Vorbehalt des jederzeitigen Widerrufes ab dem 16.01.2013 die Ausnahmegenehmigung zur Durchführung von Manipulationen an lebenden Tieren nach Anweisung.

1. Art der Eingriffe und Behandlungen:
   - Applikation von Substanzen (intracranial, oral, s.c., i.p., i.v.) ohne und unter Narkose mit Wiedererwachen
   - Stereotaktische Operationen unter Narkose mit Wiedererwachen (z.B. Implantation von Führungskanülen)
   - transkardiale Hirnperfusion unter Narkose ohne Wiedererwachen

2. Bezeichnung der Versuchstiere (Tierart)
   - Maus
   - Ratte

3. Auflage
   Herr Christian Schmidt in die speziellen Behandlungen einzuweisen.
   Er hat den nächsten universitären Kurs für Versuchstierkunde zu besuchen.

Rechtsbehelfsbelehrung

Gegen diesen Bescheid kann innerhalb eines Monats nach Bekanntgabe Klage beim

☐ Verwaltungsgericht Halle, Thüringer Str. 16 in 06112 Halle/Saale

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Mit freundlichen Grüßen
Im Auftrag

Dr. Schneider