*Alma Mater Studiorum – Università di Bologna*

DOTTORATO DI RICERCA IN

Scienze Biomediche
Progetto Formativo:
Fisiologia Applicata e Fisiopatologia

Ciclo XXIV

**Settore Concorsuale di afferenza: 05/D1-Fisiologia**

**Settore Scientifico disciplinare: BIO/09**

# EFFECTS OF AMBIENT TEMPERATURE ON CARDIOVASCULAR REGULATION DURING SLEEP IN HYPOCRETIN-DEFICIENT NARCOLEPTIC MICE

**Presentata da:** **Viviana Carmen Lo Martire**

**Coordinatore Dottorato** **Relatore**

**Chiar.mo Prof.** **Chiar.ma Prof.ssa**

**Lucio Cocco** **Giovanna Zoccoli**

**Esame finale anno 2012**


PRISM
Physiological Regulation In Sleeping Mice

# Index

# *Abstract*

Hypocretin 1 and 2 (HCRT, also called Orexin A and B) are neuropeptides released by neurons in the lateral hypothalamus. HCRT neurons widely project to the entire neuroaxis, excluding cerebellum (Peyron 1998). HCRT have been reported to participate in, or have an influence on, various physiological processes including cardiovascular functions (Shirasaka 1999), behavior (Ida 1999), wake-sleep cycle (Sakurai 2007), and they may also influence metabolic rate (Lubkin 1998) and the regulation of body temperature (Balaskò 1999). These physiological processes are strongly interrelated and the hypothalamus plays a critical role in the control of them.

HCRT neurons are lost in narcolepsy, a rare (prevalence 1:2000), debilitating neurological disorder, characterized by excessive daytime sleepiness, cataplexy, sleep fragmentation and occurrence of sleep-onset rapid-eye movement episodes (SOREMPs), (Bassetti 2005).

In narcoleptic patients, thermoregulatory cardiovascular control is impaired (Fronczek 2006), and it has been suggested that the related core body temperature and skin temperature alterations might causally affect vigilance and sleepiness in these patients (Fronczek 2008a). Furthermore, loss of HCRT neurons is often accompanied by defective energy and metabolic homeostasis, including a high risk for obesity (Kok 2003; Hara 2005) and the potential for altered thermoregulation (Plazzi 2011).

I investigated whether HCRT neurons mediate the sleep-dependent cardiovascular adaptations to changes in ambient temperature (Ta). Cardiovascular regulation is affected by Ta especially in small animals such as rodents (Swoap 2004). Ambient temperature also influences cardiovascular regulation in humans and it has been demonstrated that the exposure to cold environment is involved in coronary heart disease occurrence (Danet 1999).

Narcoleptic mice with genetic ablation of HCRT neurons (n=11) and wild-type controls (n=12), both with mixed genetic background, were implanted with a telemetric blood pressure transducer and electrodes to discriminate wake-sleep behavior. HCRT-ataxin-3 transgenic mice, with a mixed genetic background (75%

C57Bl/6J-25% DBA/2J) are more sensitive to the development of metabolic alterations than narcoleptic mice with a pure genetic background (Hara 2005). Thus, in order to better identify imbalances in metabolic homeostasis, experiments were performed on hybrid mice. Recordings were performed in each mouse at ambient temperatures of 25°C (acclimation temperature), 30°C, and 20°C in random order. Mean blood pressure (MBP) and heart rate (HR) were computed in each wake-sleep behavior and analyzed with 3-way analysis of variance and t-tests (significance at $p < 0.05$). Results revealed a significance of the main effects of wake-sleep state and Ta, their interaction effect, and the wake-sleep state x mouse strain interaction effect on MBP and HR in both mouse strains, with MBP and HR increased with Ta decreasing. This effect of Ta on BP was significantly lower in rapid-eye-movement sleep (REMS) than either in non-rapid-eye-movement sleep (NREMS) or wakefulness (W) regardless of the mouse strain. MBP and HR were higher in W than either in NREMS or REMS. This effect of sleep on BP was significantly reduced in mice lacking HCRT neurons at each Ta, both in the dark and light periods, and particularly during REMS. These data suggest that HCRT neurons play a critical role in mediating the effects of sleep but not those of Ta on MBP and HR in mice. HCRT neurons may thus be part of the central neural pathways that mediate the phenomenon of blood pressure dipping on passing from wakefulness to sleep. Several studies have revealed an association between non-dipping BP profile and increased cardiovascular risk. This concept may have clinical implications in patients with narcolepsy with cataplexy, who lack HCRT neurons.

# *1. Hypocretin/orexin system*

# 1.1 Hypocretin/orexin system: anatomy and function

Hypocretin 1 and 2 (HCRT-1, HCRT-2), also called orexin A and B, are neuropeptides produced by neurons in the lateral and posterior hypothalamus (De Lecea 1998; Sakurai 1998).

In 1998 two research groups, using different techniques, independently discovered these neuropeptides and named them differently. Sakurai et al. called them orexins, whereas De Lecea et al. called them hypocretins.

Sakurai et al. undertook a systematic biochemical search for endogenous peptide ligands for multiple orphan G-protein-coupled cell surface receptors (GPCRs), using a cell-based reporter system. These screening experiments led to the identification of a novel family of neuropeptides that bind to two closely-related orphan GPCRs. These peptide ligands were called “orexins,” after the Greek word *orexis*, which means appetite. The mRNA for the precursor of these peptides was found to be abundantly and specifically expressed in the lateral hypothalamus and adjacent areas, a region classically implicated in the central regulation of feeding behavior and energy homeostasis. In fact, when administered centrally in rat brains these neuropeptides stimulated food consumption and their production was influenced by the nutritional state of the animal (Sakurai 1998).

de Lecea et al. identified Clone 35 among 38 c-DNA clones of m-RNA specifically expressed in the hypothalamus. This clone was expressed in the posterior hypothalamus and encoded the precursor of two putative peptides, the hypocretins, which shared substantial amino acid identities with each other and with the gut hormone secretin. The HCRT mRNA, which accumulated primarily after postnatal week 3 and was a product of a gene on chromosome 11 in mice, was restricted to neuronal cell bodies of the dorsal and lateral hypothalamus (De Lecea 1998).

To avoid confusion, the hypocretin nomenclature is used throughout this script, but it should be noted that the names “orexin” and “hypocretin” are currently used synonymously in many papers.

HCRT-1 is a 33-amino acid peptide of 3,562 Da with an amino (N)-terminal pyroglutamyl residue, two intra-chain disulphide bonds and carboxy (C)-terminal amidation. This structure is completely conserved among several mammalian species (human, rat, mouse, cow, sheep, dog and pig). HCRT-2 is a 28-amino acid C-terminally amidated linear peptide of 2,937 Da. The C-terminal half of HCRT-2 is very similar to that of HCRT-1, whereas the N-terminal half is more variable. HCRT-2 is 46% identical to HCRT-1 (Sakurai 2007). Human HCRT-2 has two amino acid substitutions compared to the rodent sequence within the 28-residue stretch. The HCRT-2 found in pigs and dogs has two amino acid substitutions compared to human and rodent sequences. HCRT-1 and 2 are derived from a common precursor peptide, prepro-hypocretin (prepro-HCRT) polypeptide. An mRNA encoding the same precursor peptide was independently isolated as a hypothalamus-specific transcript. It was predicted that the transcript encoded a polypeptide precursor which is cleaved to form two neuropeptides. The prepro-HCRT gene encodes for a 130-residue (rodent) or 131-residue (human) polypeptide (Sakurai 1999). Human prepro-HCRT gene is localized on chromosome 17q21, which corresponds to chromosome 11 in the mouse. The prepro-HCRT gene consists of two exons and one intron distributed over 1432 base pairs. The 143-base pair first exon includes the 5’-untranslated region and a small part of the coding region that encodes the first seven residues of the secretory signal sequence. The second exon contains the remaining portion of the open reading frame and 3’-untranslated region. The gene contains only one intron which is 818 bp long. A fragment of human prepro-HCRT gene, which contains a 3.15-kb 5’-flanking region and the whole length of the 5’-noncoding region of exon 1 is sufficient to direct the expression of the Escherichia coli β-galactosidase (lacZ) gene in HCRT neurons without ectopic expression in transgenic mice. The lacZ-positive neurons

were positively stained with anti-HCRT antibody suggesting that this genomic fragment contains all the necessary elements for appropriate expression of the gene (Ohno 2008).

This fragment of the human prepro-HCRT gene, functioning as a promoter, is useful for examining the consequences of expression of exogenous molecules in HCRT neurons of transgenic mice, thereby manipulating the cellular environment in vivo. For example, this promoter was used to establish several transgenic lines, including HCRT neuron-ablated mice and rats (Hara 2001) in which HCRT neurons specifically express green fluorescent protein (Yamanaka 2003) or calcium-sensitive fluorescent protein (yellow cameleon Yc2.1).

The actions of hypocretins are mediated by two receptors, named hypocretin 1 receptor (HCRT-1R) and hypocretin 2 receptor (HCRT-2R).

Among various classes of G protein-coupled receptors, HCRT-1R is structurally similar to certain neuropeptide receptors, most notably to the Y2 neuropeptide Y (NPY) receptor (26% similarity), followed by the thyrotropin-releasing hormone (TRH) receptor, cholecystokinin type-A receptor and NK2 neurokinin receptor (25%, 23%, and 20% similarity, respectively).

There is a 64% amino acid identity between the deduced full-length human HCRT-1R and HCRT-2R sequences. Thus, these receptors are much more similar to each other than they are to other GPCRs. The amino acid identity between the human and rat homologues of each of these receptors is 94% for HCRT-1R and 95% for HCRT-2R, indicating that both receptor genes are highly conserved between the species. Competitive radioligand binding assays suggested that HCRT-1 is a high-affinity agonist for HCRT-1R. The concentration of cold HCRT-1 required to displace 50% of specific radioligand binding (IC50) was 20 nM. Human HCRT-2 also acted as a specific agonist on HCRT-1R. However, human HCRT-2 has significantly lower affinity compared to human HCRT-1: the calculated IC50 in competitive binding

assay was 250 nM for human HCRT-2, an affinity which was two orders of magnitude lower, compared to HCRT-1.

On the other hand, binding experiments demonstrated that HCRT-2R is a high-affinity receptor for human HCRT-2 with IC50 of 20 nM. HCRT-1 also had high affinity for this receptor with IC50 of 20 nM, which is similar to the value for HCRT-2, suggesting that HCRT-2R is a non-selective receptor for both HCRT-1 and HCRT-2 (Ohno 2008).

Both HCRT-1R and HCRT-2R are G protein-coupled receptors, which transmit information into cells by activating heterotrimeric G proteins. Activation of the signaling pathways associated with distinct G proteins may contribute to the diverse physiological roles of HCRT in particular neurons.

Although many G protein-coupled neurotransmitter receptors are potentially capable of modulating both voltage-dependent calcium channels and G protein-gated inwardly rectifier potassium channels (GIRKs or Kir3 channels), there might be a substantial degree of selectivity in the coupling to one or another of these channels in neurons.

HCRT-1R is thought to transmit signals through the Gα11 class of G protein, which results in the activation of phospholipase C with subsequent triggering of the phosphatidylinositol cascade and influx of extracellular $Ca^{2+}$. HCRT-2R is thought to be coupled to both Gα11 and inhibitory Gi G proteins (Zhu 2003).

HCRT-1R and HCRT-2R mRNAs exhibit a markedly different and basically complementary distribution, indicating that these receptors have distinct physiological roles (Marcus 2001). HCRT-1R mRNA was observed in many brain regions including the prefrontal and infralimbic cortex, hippocampus, paraventricular thalamic nucleus, ventromedial hypothalamic nucleus, dorsal raphe nucleus, and locus coeruleus. HCRT-2R mRNA was prominent in a complementary distribution including the cerebral cortex, septal nuclei, hippocampus, medial thalamic groups, raphe nuclei, and many hypothalamic nuclei including the tuberomammillary nucleus, dorsomedial nucleus, paraventricular nucleus, and ventral premammillary

nucleus. The differential distribution of HCRT receptors is consistent with the proposed multifaceted roles of hypocretins (Marcus 2001).

HCRT neurons originate in the hypothalamus and are localized in the lateral and posterior hypothalamus (Peyron 1998). These neurons are variable in size (the cell body diameter ranges from 15–40 μm) and shape (spherical, fusiform or multipolar), and have been assumed to number around 3000 in the rat brain, or 7000 in the human brain (Peyron 1998; Nambu 1999). From these regions, HCRT neurons project widely to the entire neuroaxis, excluding the cerebellum.

Hypocretins were recognized as regulators of feeding behaviour, firstly because of their exclusive production in the lateral hypothalamic area (LHA), a region known as the feeding centre, and secondly owing to their pharmacological activity.

The finding that prepro-HCRT RNA and HCRT immunoreactive cells were localized in a sub-region of the dorsolateral hypothalamus and the co-localization of HCRT with melanin-concentrating hormone (MCH), which is considered an orexigenic peptide, suggested a possible role of HCRT in the regulation of feeding behavior. Intracerebroventricular (ICV) injection of HCRT during the light period induces feeding behaviour in rats and mice (Sakurai 1999).

HCRTs also have a crucial role in regulating sleep and wakefulness; accordingly, the deficiency of these hypothalamic neuropeptides causes narcolepsy in humans and animals. The possible involvement of the HCRT system in the regulation of wakefulness and sleep was first noted in experiments performed on animal models of HCRT deficiency.

Different animal models of narcolepsy, with HCRT transmission deficiency, have been studied. These models include prepro-HCRT gene or HCRT-2R gene knockout mice, double receptor-1 and receptor-2 knockout mice, and hypocretin-ataxin-3 transgene-induced loss of HCRT neurons in rats and mice (Hara 2001; Willie 2003).

In 1999, Lin et al. discovered a mutation in the gene coding for the HCRT-2R as the cause of familial canine narcolepsy (Lin 1999). In the same year, a study involving

behavioral and electroencephalographic criteria by Chemelli et al. reported that HCRT knockout mice exhibited a phenotype that was strikingly similar to human narcolepsy patients, as well as canarc-1 mutant dogs, the only known monogenic model of narcolepsy (Chemelli 1999).

Narcolepsy is a disabling sleep disorder characterized by excessive daytime sleepiness and abnormal rapid-eye-movement (REM) sleep manifestations including cataplexy (sudden loss of muscle tone triggered by strong emotions), sleep paralysis, and hypnagogic (sleep-onset) hallucinations and sleep onset REMS periods (Scammell 2003).

In the following paragraphs, I will sum up the role of HCRT in the regulation of physiological functions: wake-sleep behavior, cardiovascular regulation, regulation of energy metabolism and body temperature, and respiratory control.

### 1.1.1 Hypocretins and wake-sleep behavior

It has been shown that in rats ICV injection of HCRT-1 or 2 during the light (rest) period, the equivalent of night-time in humans, increases awake time and decreases REM and NREM sleep time (Bourgin 2000), (ref. *3.1.1* for more detailed information regarding sleep characteristics).

HCRT neurons project widely to the entire neuroaxis, excluding the cerebellum. The densest staining of HCRT-immunoreactive nerve endings in the brain is found in the paraventricular nucleus of the thalamus, the arcuate nucleus and, most notably, the locus coeruleus (LC, containing noradrenergic neurons), dorsal raphe (DR, which contains serotonergic neurons) and tuberomammillary nucleus (TMN, containing histaminergic neurons), (Nambu 1999; Peyron 2000). The distribution of mRNA for the HCRT receptors is consistent with these projection sites; within the brain, HCRT-1R mRNA is most abundantly expressed in the LC, whereas HCRT-2R mRNA is highly expressed in the TMN (Marcus 2001). Both regions are important for the maintenance of arousal. The DR and ventral tegmental area (VTA) contain both HCRT-1R and HCRT-2R mRNA (Marcus 2001). These observations indicate that

these monoaminergic regions are important effector sites of hypocretins, involved in increasing arousal and promoting wakefulness (Sakurai 2007). Consistent with this hypothesis, electrophysiological experiments using brain slice preparations or isolated cells have shown that cells of these nuclei are activated by hypocretins *in vitro*. Indeed, noradrenergic cells of the LC, dopaminergic cells of the VTA, serotonergic cells of the DR, and histaminergic cells of the TMN have all been shown to be activated by hypocretins (Liu 2002; Yamanaka 2002).

The activity of monoaminergic neurons in the TMN, LC and DR is known to be synchronized and strongly associated with sleep and wakefulness: the neurons fire tonically during wakefulness, less during NREMS, and stop firing during REMS**.** These observations indicate that HCRT-mediated arousal results from the activation of these wake-active monoaminergic neurons. Specifically, HCRT neurons, activated during wakefulness, exert an excitatory influence on these wake-active neurons, thereby sustaining their activity. HCRT neurons need to be switched off in order to maintain consolidated NREMS and the muscle atonia that accompanies REMS, but have to be activated during the awake period (Sakurai 2007). Lee et al. found that the HCRT neurons fired during active waking, decreased discharge during quiet waking, and virtually ceased firing during both REMS and NREMS. HCRT neurons increased their firing before the end of REMS and thereby heralded the return of the awake state by several seconds. Although the numbers of cells examined are too small to provide a complete picture of HCRT neurons activity across the sleep–wake cycle, these seminal studies provide the strongest evidence that these cells are activated during wakefulness, and inhibited during sleep (Lee 2005).

HCRT neurons interact with the neuronal system and these interactions provide a key to understanding the physiological roles of these neurons. They receive inputs from the limbic system (Sakurai 2007). The importance of this connection is readily apparent in the defense, or *"fight or flight",* response: mice tested in a resident-intruder paradigm show cardiovascular and locomotor responses to the emotional

stress evoked by this test, but these responses are diminished in prepro-HCRT knockout mice (Kayaba 2003).

The neural input from the limbic system to HCRT neurons might be implicated in the pathophysiology of cataplexy, because strong, generally positive emotional stimuli are known to trigger cataplexy in narcolepsy-cataplexy patients (Sakurai 2007).

HCRT neurons receive a lot of innervations from the suprachiasmatic nucleus (SCN) and the dorsomedial hypothalamus (DMH). This indicates that HCRT neurons might receive circadian influences indirectly from the SCN via these regions.

The preoptic area, especially the ventrolateral preoptic area (VLPO), seems to have a crucial role in NREMS initiation and maintenance. Neurons in the VLPO fire at a rapid rate during sleep, with attenuation of firing during wakefulness. GABA and galanin are the primary inhibitory neurotransmitters of the VLPO, which sends out multiple inhibitory projections to the LC, TMN and DR.

HCRT neurons are innervated by GABA-containing cells in the VLPO (Yoshida 2006), indicating that the VLPO might be a source of GABA-containing inhibitory projections to HCRT neurons. This pathway might be important for turning off HCRT neurons during sleep.

To explain how HCRT neurons stabilize sleep and wakefulness through the neural circuits discussed above, Saper et al. proposed that the HCRT system is part of a flip-flop system. A *"flip-flop switch"*, a circuit containing mutually inhibitory elements, sets up a self-reinforcing loop, where activity in one of the competing sides shuts down inhibitory inputs from the other side, and therefore disinhibits its own action (Saper 2005).

The "flip-flop switch" circuit is composed of the arousal system, which promotes wakefulness, and the VLPO, which promotes sleep.

The arousal system consists of two major branches: the first is an ascending pathway to the thalamus that activates the thalamic relay neurons which project to the cerebral cortex. The major source of upper brainstem input to the thalamic-relay nuclei, as well as to the reticular nucleus of the thalamus, is a pair of acetylcholine-producing

cell groups: the pedunculopontine and laterodorsal tegmental nuclei (PPT/LDT), (Hallanger 1987). The neurons in the PPT/LDT fire most rapidly during wakefulness and REMS. These cells are much less active during NREMS, when cortical activity is slow. The second branch of the ascending arousal system bypasses the thalamus, instead activating neurons in the lateral hypothalamic area, and throughout the cerebral cortex. This pathway originates from monoaminergic neurons in the upper brainstem and caudal hypothalamus, including the noradrenergic LC, serotoninergic DR and median raphe nuclei, dopaminergic ventral periaqueductal grey matter and histaminergic TMN (Saper 2001).

On the other side of the circuit there is the VLPO. The VLPO neurons are primarily active during sleep, and contain inhibitory neurotransmitters, galanin and GABA. Experiments showed that cell-specific lesions of the VLPO reduce both NREMS and REMS by more than 50% (Lu 2000).

The VLPO also receives inhibitory afferents from each of the major monoaminergic and histaminergic systems. Both noradrenaline and serotonin inhibit VLPO neurons. Therefore, the VLPO can be inhibited by the arousal systems that it inhibits during sleep (Saper 2005).

To summarize, during wakefulness, HCRT neurons send excitatory influences to monoaminergic neurons, which send inhibitory feedback projections to HCRT neurons. This system might maintain the activity of monoaminergic neurons. A slight decrease in input to the monoaminergic neurons results in decreased inhibitory influence to HCRT neurons. HCRT neurons, therefore, are disinhibited and increase excitatory influence to monoaminergic cells to maintain their activity. These monoaminergic cells send excitatory projections to the thalamus and cerebral cortex, and inhibitory projections to the VLPO sleep centre. These mechanisms maintain wakefulness. During sleep, VLPO sleep active neurons are activated and send inhibitory outputs to monoaminergic neurons and HCRT neurons in order to maintain sleep. In narcolepsy, when HCRT neurons are lacking, monoaminergic neurons and VLPO neurons set up a mutually inhibitory circuit, which can cause unwanted and

abrupt transitions between the states. Activity in one of the competing sides shuts down inhibitory inputs from the other side, and therefore disinhibits its own action. So, when either side begins to overcome the other, the switch abruptly turns into the alternative state (Sakurai 2007).

### 1.1.2 Hypocretin and cardiovascular regulation

Behavioral activities such as exercise, fighting and food-seeking must be faced by the organism with an integrated response. Such a response is based on coordinated hypothalamic activation of somatomotor, hormonal and autonomic pathways. Accordingly, cardiovascular adaptative responses are mediated by different hypothalamic neuropeptides. HCRT containing-neurons and their receptors are distributed in brain regions closely associated with the regulation of cardiovascular and autonomic function. HCRT receptor mRNA is expressed in the hypothalamic paraventricular nucleus (PVN), which is involved in the integration of the autonomic nervous and neuroendocrine systems.

The pre-autonomic parvocellular neurons of the PVN send long descending projections to several areas within the CNS that are known to be important in cardiovascular function. These regions include the nucleus of solitary tract (NTS), where baroreceptor and chemoreceptor afferents terminate, the dorsal vagal complex, which is present in the dorsomedial medulla and contains vagal preganglionic neurons, the rostral ventrolateral medulla (RVLM), which is probably a major site for the generation of sympathetic tone for the vasculature, and the intermediolateral column of the spinal cord, which is the site of sympathetic preganglionic motor neurons involved in the regulation of blood pressure (BP) and heart rate (HR) (Shirasaka 2003).

Through these pathways hypocretins may modulate cardiovascular adjustment to different motivated behavior, modulating sympathetic activity.

Functional studies have shown that these peptides evoke changes in cardiovascular and sympathetic responses. The in vivo and in vitro studies suggest that the peptide

acting on neurons in the hypothalamic PVN increases the cardiovascular responses (Shirasaka 2002). Shirasaka et al. used conscious, freely moving rats to investigate the effect of ICV-administered hypocretins on mean arterial pressure (MAP), heart rate (HR), and sympathetic nerve activity.

Intracerebroventricular administration of 0.3-nmol HCRT-1 and 2 significantly increased MAP but not HR in conscious rats (Shirasaka 1999). A higher dose (3.0 nmol) of HCRT-1 and 2 rapidly increased MAP and HR, which reached a peak value within 15 min after injection. However, increases in MAP and HR were also observed in anesthetized rats, indicating that these responses were not due to increases in locomotor activities, such as chewing and grooming. Intravenous (IV) administration of hypocretins (11 nmol/kg) did not affect blood pressure and heart rate. These results indicate that hypocretins produce pressor and tachycardic responses by acting centrally.

The high dose of HCRT-1 additionally resulted in an 60% increase in renal sympathetic nerve activity (RSNA) 10 min after injection, which persisted for 15 min. RSNA also increased transiently at a low dose (0.3 nmol) of HCRT-1 but this increase was significantly lower than at 0.3 nmol (Shirasaka 1999).

Central HCRT-2 also produced a significant increase in MAP, with a 10.8 ± 0.2 mmHg increase at the high dose, this response pattern being similar to what was observed for HCRT-1 administration. HR also rapidly increased and returned to the control level within 30 min at 3.0 nmol. In contrast with the results obtained for HCRT-1, RSNA did not increase significantly at any dose of HCRT-2 (Shirasaka 1999).

To examine systemic sympathetic outflow induced by central hypocretins, plasma catecholamines were measured. High doses of HCRT-1 and 2 increase plasma norepinephrine (NE), the effect being larger and longer-lasting with HCRT-1. Therefore, it is likely that the HCRT-induced increase in sympathetic nerve outflow leads to the increase in plasma NE, which in turn induces cardiovascular responses. ICV-administered HCRT-1 also significantly increases plasma epinephrine (EP)

levels 10 min after injection (Shirasaka 2003). Central HCRT-1 infusion induces face washing behavior and grooming (Ida 1999), which is known to be related to a stress response. Stress has been reported to cause an increase in sympathetic nerve activity. Shirasaka et al. reported increases produced by central HCRT-1 administration in RSNA and plasma catecholamines, compatible to stress response. Moreover they demonstrated that central HCRT-1 at lower doses selectively causes the activation of RSNA, thus suggesting that HCRT-1 may be involved in renal excretory function through RSNA. Therefore, hypocretins may be involved in the central control of multiple homeostatic functions regulating sympathetic nerve activity. The role of HCRT in stress response has been widely examined. The defense response is characterized by increases in BP, HR, respiratory frequency, and resistance in visceral vascular beds, and by decreases in resistance in the airways and the skeletal muscle vascular bed.

The defense response elicited by stimulation of the hypothalamic perifornical area (PFA) is attenuated in genetically engineered mouse models of hypocretin deficiency. These mice show an attenuated response to emotional stressors with blunted increases in BP, HR and locomotor activity (Kayaba 2003).

The contribution of HCRT to the multiple efferent pathways of the defense response was investigated by stimulating PFA of HCRT knockout mice and HCRT-ataxin-3 transgenic mice. The aim was to verify the involvement of HCRT in determining all the features of the defense response.

As expected, bicuculline stimulation of PFA, increases in BP, HR, respiratory frequency, and h-band power of electroencephalogram (an index of cortical arousal) were smaller and/or shorter-lasting in HCRT knockout mice than in WT littermates.

In a similar manner, increases in BP, HR, and respiratory minute volume and vascular dilatation in skeletal muscle were attenuated in HCRT-ataxin-3 transgenic mice (Zhang 2006). Therefore, HCRT-containing neurons in the PFA play a role as a master switch to activate multiple efferent pathways of the defense response.

HCRT but not co-transmitters (glutamate, dinorphin and galanin) in the neurons seemed to be important at least for the changes in BP, HR, and respiration in the defense response considering the similarity in the examined parameters between the two animal models included in the experiments (Zhang 2006).

This, however, was not true of co-transmitters (glutamate, dinorphin and galanin).

Data regarding the role of HCRT in cardiovascular regulation are still conflicting.

Recent studies suggest a bidirectional dose-dependent cardiovascular response to microinjection of HCRT in the NTS (Shih 2007).

In particular, at a lower dose (5 pmol), HCRT-1 and 2 decreased arterial pressure, HR, and power density of the vasomotor components of BP signals, an experimental index for sympathetic neurogenic vasomotor tone. At higher doses (>20 pmol), these two compounds elicited cardiovascular excitatory responses (Shih 2007). Hypocretins probably exert their effects through activation of the nitric oxide (NO) synthase. Depending on the amount, NO exerts differential cardiovascular responses. In the NTS low and high concentrations of NO entail differential modulation of glutamatergic and GABAergic synaptic transmission. At a low level, NO potentiates glutamatergic excitatory postsynaptic potentials whereas at a high level it augments monosynaptic GABAergic inhibitory postsynaptic potentials (Wang 2007). So at a low dose HCRT activates NO synthase to generate a low level of NO, thus leading to vasodepression, while at a high dose HCRT induces a greater production of NO, which activates the GABAergic neurotransmission and overrides cardiovascular inhibition to yield excitation. If HCRT receptor antagonists are administered, these bidirectional cardiovascular effects are abolished (Shih 2007).

These results were confirmed by other experiments performed in order to evaluate the dose-dependent effect of IV or ICV injection of various doses of HCRT-1 on RSNA and BP. Tanida et al. found that injection of a low dose of HCRT-1 (10 ng IV or 0.01 ng ICV) suppressed RSNA and BP significantly. Conversely, a high dose (1000 ng IV or 10 ng ICV) elevated both RSNA and BP (Tanida 2006).

Furthermore, bilateral lesions of the hypothalamic suprachiasmatic nucleus (SCN) abolished the effects of both low and high doses of HCRT-1 on RSNA and BP. These findings suggest that HCRT-1 affects RSNA and BP in a dose-dependent manner and that the SCN may modulate this effect. In rats, the hypothalamic SCN, in addition to functioning as a master circadian oscillator, is involved in the control of BP through autonomic nerves to sympathetic and parasympathetic neurons modulating BP. Through their projections to the NTS, RVLM, VMMR, DMN, and intermediolateral column of the spinal cord, HCRT neurons may modulate cardiovascular adjustments to different motivated behaviors (Tanida 2006). In conscious rabbits, ICV injection of HCRT-1 elicited a dose-dependent increase in mean BP and RSNA; it also increased plasma epinephrine and vasopressin concentrations (Matsumura 2001). The effect of microinjections of HCRT-1 into the rostral ventromedial medulla (RVMM) of rats elicited a dose-dependent increase in HR with little or no change in MAP, thus activating neuronal circuits that both inhibit vagal activity and increase sympathetic activity to the heart (Ciriello 2003).

Chronic ICV infusion of HCRT-1 (50 pmol/h) elicited a significant increase in systolic blood pressure on the third day (+15.6 ± 2.9 mmHg), and during the continuous ICV infusion of HCRT-1, BP returned to the baseline levels at day 14. These results suggest that central HCRT-1 participates in the short-term regulation of blood pressure; however, the contributions of central hypocretins to the long-term regulation of blood pressure, sympathetic nervous system, and appetite may be minor (Lin 2002).

Results obtained from studies performed in narcoleptic patients regarding the role of hypocretins in cardiovascular control are not in full agreement with those obtained in animals.

Firstly in 1982, Sachs and coll. described the presence of an impaired autonomic control in narcoleptic patients (Sachs 1982). This study evaluated autonomic cardiovascular responses of narcoleptic patients to specific tests: 1) heart rate and blood flow in the resting forearm during contralateral isometric handgrip, 2)

respiratory sinus arrhythmia, and 3) heart rate response to the Valsalva maneuver. They found that autonomic cardiovascular reflexes, such as the diving reflex (in which bradycardia is elicited by an increase in vagal activity) and the orthostatic test (in which an increase in BP depends mainly on sympathetic vasoconstriction) were similar in narcoleptic patients and controls, indicating intact peripheral nerves. On the contrary, the controlateral isometric handgrip test (in which an increase in blood flow is mediated by β-adrenergic vasodilatation and an increase in HR depends on the inhibition of vagal efferent) highlighted reduced responses in narcoleptic patients. Vagal activity was evaluated measuring the respiratory sinus arrhythmia (RSA) and HR responses to the Valsalva maneuver (in which patients maintain a constant expiratory rate by blowing through a mouthpiece that permits a slow air flow). The results demonstrated an attenuated reactivity in the vegetative control system of narcoleptic patients. These findings suggested a central origin of the autonomic imbalance (Sachs 1982). Unfortunately, these results were not confirmed later by other studies. In 1994, Hublin et al. measured BP and HR changes during the deep breathing test, Valsalva test and Orthostatic test. No significant differences between narcoleptic patients and control subjects were found (Hublin 1994). More recently, Grimaldi et al. found normal functioning of cardiovascular reflexes in narcoleptic patients, but an impairment of HR modulation at rest with enhanced sympathetic activity (Grimaldi 2010a).

In 1986, Baker et al. reported that narcoleptic patients tended to have higher BP values (Baker 1986), without taking into account the effect of sleep on BP. Instead, Guillemineault found that in narcoleptic patients there was no difference in the blood pressure measurements throughout the night compared with normal control subjects (Guilleminault 1986).

Ferini-Strambi et coll. excluded primary disturbances of the cardiac autonomic control in narcolepsy, finding a higher sympathovagal balance in patients compared to controls during wakefulness before sleep. They ascribed this result to an attenuated

activation of the parasympathetic system, probably due to the wake-sleep cycle impairment in narcoleptic patients (Ferini-Strambi 1997).

Fronczek et al. hypothesized a reduced sympathetic tone in patients, even if mean HR did not differ between the groups (Fronczek 2008b ). Conversely, when studying HR variability in the supine rest condition, a recent study showed significantly higher HR values in narcoleptic patients than in controls. Authors suggested that this may reflect an increased sympathetic drive, or alternatively a reduced parasympathetic drive on HR in narcoleptic patients (Grimaldi 2010b). The variable results of these studies emphasize the insufficiency of single assessments of the autonomic nervous system during wakefulness and probably reflect the insufficient standardization of test conditions.

In order to find a unifying position as to whether HCRT deficiency involves autonomic cardiovascular impairment in narcoleptic patients, it is important to take into account the abnormal wake-sleep behavior often associated with narcolepsy.

The nighttime sleep of narcoleptic patients is unstable, with frequent stage shifts, arousals and increased motor activity during sleep such as periodic leg movements in sleep (PLMS), (Dauvilliers 2007). PLMS consists of sudden jerking movements of the legs which occurring involuntarily during sleep, during which the affected individual may remain unaware. It may involve kicking, twitching, or extension of the legs. It tends to increase with age and often accompanies restless leg syndrome (RLS). PLMS during sleep are frequently associated with arousals and awakenings. Narcoleptic patients have a higher frequency of PLMS than controls, especially during REMS. Interestingly, it has been reported that in narcoleptic patients PLMS during sleep may be associated with huge increases in BP (Ali 1991).

### 1.1.3 Hypocretin: feeding behavior and energy homeostasis

Feeding behavior is dependent upon the integration of metabolic, autonomic, endocrine, and environmental factors coordinated with an appropriate state of cortical

arousal (wakefulness). Historically, it has been recognized that the hypothalamus plays a critical role in maintaining energy homeostasis by integrating these factors and coordinating behavioral, metabolic, and neuroendocrine responses (Willie 2001).
In mammals, the neurons of the lateral hypothalamic area (LHA) are particularly important for feeding and behavioral arousal. Animal models with lesions of the LHA exhibit hypophagia, an increased metabolic rate and decreased arousal that frequently leads to death by starvation. Therefore, the LHA has classically been regarded as the hypothalamic *"feeding center"* and an important component of the autonomic nervous system with extensive projections within the hypothalamus and throughout the entire neuroaxis (Willie 2001).
The striking localization of HCRT-containing neurons in the lateral hypothalamus and some of its adjacent areas suggests the possibility that the neuropeptide may be involved in the regulation of food intake and energy metabolism, and animal studies confirm the involvement of HCRT neurons in these kind of regulations.
Sakurai et al. administered HCRT acutely into the lateral ventricle of male rats. In rats, acute ICV HCRT-1 injection during the early light phase produces a dose-dependent manner increase in food consumption within 1 hr (6- and 10-fold increase for 3nmol and 30nmol, respectively), (Sakurai 1998). Consistent with the dense projections of HCRT neurons to the hypothalamic arcuate nucleus (ARC), (Peyron 1998) several studies have suggested that the increased food intake following HCRT-1 administration is at least in part mediated by the activation of neuropeptide Y (NPY) neurons in the ARC (Yamanaka 2000). The ARC is an important station of pathways that generate integrated responses to afferent information on fuel stores. Reciprocal connections between HCRT neurons and neurons located in the ARC, NPY/AgRP (Agouti related peptide), which are orexigenic peptides, and POMC (propiomelanocortin)/CART (cocaine-amphetamine-regulated transcript), which are anorexigenic peptides, provide an indirect regulation of HCRT neurons by leptin and ghrelin, indicators of the metabolic status. Leptin is produced by adipose tissue proportionally with the fat mass. High leptin levels suppress HCRT expression and

inhibit HCRT neuron firing. Ghrelin, a hormone secreted by the stomach prior to meals and during fasting, directly activates HCRT neurons. Finally, HCRT neurons may play an important role in sustaining the phenomenon of hypoglycemia awareness, in which low glucose levels trigger autonomic and behavioral activation and may cause awakening from sleep. Elevated glucose concentrations induce hyperpolarization and cessation of action potentials in HCRT neurons (Yamanaka 2003). Supporting the physiological relevance of HCRT in the control of feeding, ICV administration of an anti-HCRT antibody or an HCRT-1R-selective antagonist has been showed to reduce food intake, and prepro-HCRT knockout mice and transgenic mice lacking HCRT neurons ate less than control wild-type mice (Hara 2001).

The LHA is also important in controlling metabolic rate. Animal models with lesions in the LHA consistently become hypercatabolic and remain so, even after they reach a lower body weight set-point. Conversely, the finding of decreased food intake and normal body weight suggests that HCRT knockout mice are likely to be hypometabolic. This hypothesis is supported by experiments performed by Lubkin & Stricker-Kongrad (Lubkin 1998). Using indirect calorimetry they found that HCRT-1 injection during the light phase increases oxygen consumption and the respiratory quotient by an apparent increase in carbohydrate metabolism. A significant body of evidence has been accumulated for a role of hypocretins in the regulation of energy metabolism and autonomic function (Willie 2001). Besides increasing feeding behavior in a dose-dependent manner (Sakurai et al., 1998) and increasing oxygen consumption in mice indicating an increased metabolic rate (Lubkin 1998), hypocretins also have positive influences on the sympathetic outflow, as suggested by the increase in mean arterial BP and HR obtained in rats with HCRT administration into the lateral ventricle or ventrolateral medulla (Shirasaka 2002). These effects generally result in increased energy consumption.

The role of hypocretins in energy homeostasis is confirmed by studies in animal models of narcolepsy. HCRT-ataxin-3 mice show late onset obesity, consistent with

human reports. However, food consumption in HCRT-ataxin-3 mice is decreased by almost 30% at 8 to 10 weeks of age, before obesity becomes apparent. Thus, reduced food intake is unlikely to be a compensation for increased weight gain. Rather, reduced feeding and obesity may together reflect a reduction in energy expenditure due to decreased motor activity, a lower basal metabolic rate, or both. A decreased spontaneous motor activity in the dark (active) period was observed in HCRT-ataxin-3 mice. Decreased basal energy expenditure may result directly from the elimination of HCRT-containing neurons, which normally promote energy expenditure (Hara 2001).

The metabolic abnormality in HCRT-ataxin-3 mice seems more severe than that of prepro-HCRT knockout mice reported by Chemelli et al. (Chemelli 1999). These mice reportedly display normal growth rather than obesity, although they are also hypophagic. The observed metabolic differences between HCRT-ataxin-3 mice and prepro-HCRT knockout mice may stem from different genetic backgrounds and environmental effects. Loss of neuropeptides or modulatory factors expressed by HCRT-containing neurons may also contribute to phenotypic differences in these mice models. In particular, dynorphins, which influence feeding behavior, may play a role. Another intriguing possibility is that phenotypic differences result in part from fetal and/or early mouse postnatal development in the presence or absence of HCRT signaling in HCRT-ataxin-3 transgenic mice and knockout mice, respectively (Hara 2001).

Metabolic abnormalities in food intake and/or energy expenditure may affect narcoleptic patients. Narcoleptic patients have a decreased caloric intake but an increased body mass index, indicating that the abnormality that gives rise to narcolepsy has links to reduced energy expenditure or a low metabolic rate. This is suggested by increased frequencies of obesity and non-insulin-dependent (Type II) diabetes in narcoleptic patients compared with a control group (Schuld 2000).

Recently, it has been demonstrated that narcoleptic patients show a high prevalence of metabolic syndrome. Several metabolic alterations are present in narcoleptic

patients, even though they show a significant reduction in food intake. The dysmetabolism is independent of BMI, and BMI is inversely associated with food intake: these data support the notion that the lack of HCRT may directly influence some metabolic parameters (Poli 2009).

To summarize, the lack of HCRT leads to hypophagia, but also to late onset obesity, suggesting a separate effect of HCRT signaling on energy expenditure and feeding behavior. It has been shown that HCRT administration increases sympathetic outflow, with increased body temperature (Yoshimichi 2001), as well as increased BP and renal sympathetic nerve activity (Shirasaka 2002). Thus, lack of HCRT could result in reduced energy expenditure through decreased thermogenesis, and in obesity despite hypophagia.

### 1.1.4 Hypocretin and regulation of body temperature

Maintenance of energy homeostasis depends on long-term and short-term regulatory processes. In the long-term, metabolic requirements must be met by proportional food intake; unbalanced intake-consumption produces changes in body weight. The short-term balance between energy production (actual metabolic rate) and energy dissipation is a function of temperature regulation; any imbalance is manifested as change in body temperature (Székely 2002).

Loss of HCRT neurons is often accompanied by defective energy and metabolic homeostasis, including a high risk for obesity (Kok 2003) and the potential for altered thermoregulation (Plazzi 2011).

HCRT-containing fibers and HCRT receptors have been described in the rostral raphe pallidus region of the ventromedial medulla, the principal site of sympathetic and somatic premotor neurons for the regulation of thermal effectors (Morrison 2011). These neurons have a well-established role in the regulation of body temperature promoting brown adipose tissue (BAT) thermogenesis and cardiovascular adaptations to thermogenic needs (i.e., cutaneous vasoconstriction and increase in heart rate). Nanoinjection of orexin-A (12 pmol) into the rostral raphe pallidus, the site of BAT

sympathetic premotor neurons, produced large, sustained increases in BAT sympathetic outflow and in BAT thermogenesis (Tupone 2011).

Moreover, HCRT neurons receive afferent projections from the pre-optic area (POA), and express c- Fos when muscimol (GABA-A agonist) is injected into the POA (Rusyniak 2011). These data, suggest that GABAergic neurons in the POA provide tonic inhibitory tone to HCRT-synthesizing neurons and that removing this tone contributes to an increase in body temperature, HR and BP (Satoh 2004).

Many studies have explored the link between thermoregulation and HCRT. ICV infusion of HCRT-1 in rats causes an initial reduction of body temperature and metabolic rate, followed by a long-lasting hyperthermic period. On the contrary, HCRT-2 directly causes hyperthermia, without any metabolic changes (Székely 2002). After the ICV injection of HCRT-1, rats show an increase in the firing rate of the sympathetic nerves projecting to interscapular BAT, in BAT and abdominal temperatures, and in HR. Thus, HCRT may regulate both BAT energy expenditure and thermogenesis through sympathetic nerve activity. A decreased sympathetic tone in absence of HCRT signaling could explain the increased fat accumulation and reduced energy expenditure (lower thermogenesis) described in narcolepsy (Plazzi 2011).

In narcoleptic patients the normal pattern of the circadian and state-dependent core body temperature cycle has been found to be preserved (Grimaldi 2010a) or decreased (Fronczek 2008a). Fronczek et al., when studying skin temperature regulation in unmedicated narcoleptic patients throughout the day, reported an elevated distal skin temperature. In normal subjects, during daytime quiet wakefulness, distal skin areas are usually cooler than proximal ones, which is expressed as a negative distal- to-proximal gradient (DPG). The distal skin warms up when hypothalamic regulated sympathetic cutaneous vasoconstrictor tone is released, opening a dense network of arterio-venous anastomoses in the skin of the extremities. This vasodilatation seems to be correlated with sleep-onset. Experimentally-induced

changes in core body and skin temperature of narcoleptic patients seems to modify vigilance and sleepiness (Fronczek 2008a).

Mockizucki et al. studied core body temperature (Tb) in HCRT knockout mice. These authors hypothesized that HCRT knockout mice would have a lower Tb compared to controls, due to their lower sympathetic vasoconstrictor tone. Moreover, because HCRT is released during wakefulness, they hypothesized that HCRT knockout mice would have lower Tb while awake. Surprisingly, Tb was the same in HCRT knockout mice and wild-type littermates during sustained wakefulness, whereas Tb of HCRT knockout mice fell much less steeply than normal during sleep.

There are different possible explanations for these results. The first is that heat loss mechanisms might not be fully engaged in HCRT knockout mice thus accounting for the fact that Tb falls more slowly. It may also be that HCRT KO mice fall asleep without much activity in sleep-promoting preoptic neurons. Because these preoptic neurons also activate heat loss mechanisms, Tb falls less steeply during sleep.

A second explanation is that HCRT deficiency may directly impair mechanisms that reduce Tb. Stimulation of the caudal lateral hypothalamus triggers tail vasodilation in rats and the lateral hypothalamic HCRT neurons may contribute to this response.

Furthermore, the HCRT neurons project to many brain regions implicated in thermoregulation, including the preoptic area, dorsomedial hypothalamus, ventromedial hypothalamus, posterior hypothalamus, periaqueductal grey matter, medullary raphe, and intermediolateral cell column of the spinal cord (Peyron 1998; Marcus 2001; Berthoud 2005). These neurons work together to regulate Tb depending on physiological needs, and HCRT may be a key signal in the control or synchronization of the activity of these neurons.

Finally, a third possibility is that the reduced fall in Tb could be caused by sustained thermogenic activity during sleep. HCRT deficiency may reduce sympathetic tone because HCRT knockout mice have lower blood pressure. If HCRT deficiency reduces sympathetic activity and locomotor activity during wakefulness, then other heat-generating mechanisms may compensate in order to produce normal Tb during

wake. Thermogenic endocrine signals might be upregulated to maintain heat production, normalizing Tb during wake, but producing an inappropriately high Tb during sleep (Mochizuki 2006).

In conclusion, all these results indicate a potent modulation of body temperature by HCRT released from the terminals of HCRT neurons to the cerebral areas involved in thermoregulation and provide a potential mechanism contributing to the disrupted regulation of body temperature and energy metabolism as a consequence of HCRT signaling impairment.

### 1.1.5 Hypocretin and respiratory control

Because basal respiration and respiratory reflex regulations are different during wakefulness and sleep, HCRT may represent a link between vigilance state and vigilance state-dependent respiratory control (Nakamura 2007).

The involvement of the lateral hypothalamus in both cognitive arousal and breathing was recognized many decades ago. In relation to breathing, a key prediction of a stimulatory role for the lateral hypothalamus was made by Redgate and Gellhorn in the 1950s. These authors used high-frequency currents to produce localized lesions in the lateral hypothalamus of lightly anaesthetized cats, while monitoring the rate and depth of respiration (Redgate 1958). The lesions resulted in an immediate decrease in the rate and/or depth of respiration, and these effects increased with the size and number of lateral hypothalamic lesions. Chemical inhibition of lateral hypothalamic activity, caused by injection of barbiturates, also reduced respiratory activity. On the basis of these data, Redgate and Gellhorn concluded that “impulses from the lateral hypothalamus exert a tonic facilitatory action on the respiratory centre”. This is supported by recent data revealing an anatomical ‘hotspot’ for breathing stimulation very close to the hypothalamic area where HCRT neurons are found. HCRT neurons project to different areas involved in respiration: the rostral vetrolateral medulla (RVLM), the pre-Bötzinger region (part of the respiratory rhythm generator), the NTS and the hypoglossal and phrenic nuclei (Williams 2008).

Central administration of HCRT-1 in mice increased the tidal volume (i.e. the amount of air inhaled or exhaled during normal ventilation). Effects were present shortly (within a few seconds) after the administration and lasted 10–40 min depending on the dosage. The responses were dose-dependent. However, the minimal dose producing a statistically significant response was 0.03 nmol for respiratory frequency and tidal volume and 0.3 nmol for blood pressure and heart rate, indicating that respiratory responses were not secondary to the cardiovascular changes (Zhang 2005).

Further evidence linking the HCRT system and breathing comes from mice with genetic deletion of the gene encoding prepro-HCRT.

These mice showed an attenuated fight-or-flight response, including increases in respiration and BP, when exposed to stressors (Kayaba 2003), although basal ventilation was comparable to that in the control mice. This observation indicated that HCRT might be a master switch, which elicits integrated behavioral and autonomic outputs, modulating respiration only in a stress condition.

In a series of recent papers, several clear abnormalities in the respiration of HCRT-knockout mice were identified. When HCRT- knockout mice breathe normal room air, their respiratory frequency and tidal volume tend to be higher and lower, respectively, than those of control mice. Regardless the abnormal wake-sleep cycle of narcoleptic mice, minute ventilation results almost identical between strains during every behavioral state (Nakamura 2007). Therefore, HCRT does not appear to contribute to basal breathing when animals are at rest and under room air conditions.

The situation changes when air composition is altered. The magnitude of the hypercapnic (5% $CO_2$–21% $O_2$ and 10% $CO_2$–21% $O_2$) and hypoxic (15% $O_2$) ventilatory responses in both narcoleptic and control mice show a clear dependence on the behavioral state (Nakamura 2007).

During wakefulness, increases in breathing caused by increased hypercapnia are reduced by 50% in the HCRT knockout mice (Nakamura 2007). On the other hand, no difference is found between HCRT-knockout and control mice in the hypoxic

ventilatory responses during W and in the hypercapnic ventilator responses during NREMS and REMS.

These findings suggest that HCRT plays a crucial role both in $CO_2$ sensitivity during wakefulness and in preserving ventilation stability during sleep.

Considering that hypercapnic chemoreflex in prepro-HCRT knockout mice is attenuated during wake but not during the sleep period, Deng et al. administered an HCRT-1R antagonist (SB-334867) in wild-type controls before introducing a hypercapnic gas mixture in the plethysmographic chamber and showed that hypercapnic chemoreflex in those mice decreased without affecting their behavior. Supplementation of either HCRT-1 or 2 partially restored the hypercapnic chemoreflex in HCRT- knockout mice (Deng 2007).

These results indicate that HCRT-1R is necessary for chemoreflex control. However, the response of HCRT-knockout mice to hypoxia is not different from that of wild-type mice.

Selective inhibition of both HCRT receptors by Almorexant, a dual HCRT receptor antagonist administered per os, induces an attenuated $CO_2$ response by 26% only in wakefulness during the dark (active) period of the diurnal cycle, reaching the same level observed during NREMS in the light period in controls. Almorexant also decreases wakefulness and increases NREMS and REMS during the dark period, as previously reported, and unexpectedly decreases the number of sighs and post-sigh apneas during wakefulness in both the light and dark period and during both wakefulness and NREMS in the dark period (Kuwaki 2008).

All these studies indicate that the HCRT system plays a critical role in central chemoreception during wakefulness, and the sleep–wake difference in the $CO_2$ response can be in large part attributed to HCRT. Hypocretins may also be involved in the mechanisms of stabilization of breathing. Post-hypoxic long-term facilitation, a physiological feature that is presumed to stabilize the respiratory control system and reduce sleep apnea, is absent in HCRT-knockout mice. The result is that HCRT-

knockout mice also display a markedly increased frequency of spontaneous sleep apneas (Nakamura 2007).

Narcoleptic patients also have a higher frequency of sleep apneas compared to healthy controls; this has been considered a disorder of respiratory control (Chokroverty 1986).

There is evidence pointing to an impaired chemo-responsiveness in patients with narcolepsy-cataplexy. Sleep in narcoleptic patients is characterized by overwhelming excessive sleepiness occurring during the daytime and fragmented nocturnal sleep that is interrupted by numerous awakenings. A number of studies have demonstrated that one night of sleep deprivation significantly reduced both ventilatory chemo-sensitivity and arousal responses in healthy adults (Schiffman 1983). The long-term nocturnal sleep disturbance in narcoleptics may alter the chemo-responsiveness and mimic the change of sleep deprivation. The fragmented sleep and hypoxia induced by a high incidence of sleep apnea may further impair the ventilatory responsiveness of these patients.

Among sleep apneas, the most common in humans are the OSAs (Obstructive Sleep Apneas). These apneas are induced by the interruption of airflow in the upper airways, even if the respiratory muscles keep working. The apneic event causes a rise in BP and patients display a daytime hypertension and a higher cardiovascular risk (Davies 2000). In obese patients, the occurrence of OSAs is higher than in normal subjects. Narcoleptic patients are often obese, as a consequence of metabolic alterations and thus, develop a major risk for OSAs and cardiovascular morbidity.

# *2. Narcolepsy*

A post-mortem study of narcolepsy patients found that hypocretin peptides were undetectable in the cortex and pons, in which HCRT projections are normally found, and that there was an 80-100% reduction in the number of neurons containing detectable prepro-HCRT in the hypothalamus (Thannickal 2000). This was supported by other reports that HCRT-1 was undetectable in the cerebrospinal fluid of narcolepsy patients (Nishino 2000).

A recent finding showing concomitant loss of dynorphin, neuronal activity-regulated pentraxin and HCRT, which co-localize in HCRT- neurons, further indicates a loss of hypocretin neurons in narcolepsy-cataplexy (Crocker 2005). The cause of the specific loss or degradation of HCRT neurons in narcolepsy is unknown so far, but because of its strong association with specific human leukocyte antigen (HLA) alleles it has been hypothesized that narcolepsy results from selective immune-mediated degeneration of HCRT neurons, although no specific antibody against HCRT neurons has been found in the serum of affected subjects.

## 2.1 Epidemiology

Narcolepsy is a disabling sleep disorder characterized by excessive daytime sleepiness and abnormal REMS, manifestations including cataplexy (sudden loss of muscle tone triggered by strong emotions), sleep paralysis, and hypnagogic (sleep onset) hallucinations (Scammell 2003). Firstly described by Dr. Gelineau, narcolepsy is one of the most studied sleep disorders at a molecular level since it is a good model for studying REMS regulation and sleepiness in human beings, as well as the study of stimulant drugs. Studies performed in animal models showed that narcolepsy is produced by deficient HCRT transmission (Lin 1999). A striking decrease in HCRT-1 concentrations in the cerebrospinal fluid (CSF) and in the number of HCRT neurons in post-mortem brain tissue have been reported in narcoleptic patients (Thannickal 2000).

In USA, the prevalence of narcolepsy with cataplexy is estimated between 0.05% and 0.067%. The study of a cohort of 8000 twins from Finland, reported that 0.026% were affected by daily sleep attacks and at least one episode of muscular weakness per week. Similar data were also obtained from other studies performed in Europe, Hong Kong and MN, USA. The US study also reported an incidence of 0.74 per 100,000 inhabitants per year. The prevalence of narcolepsy is higher in Japan and lower in Israel than in Europe and North America. Most studies found a slight male predominance. The age of onset is extended from early childhood to the 50s, with a bimodal distribution, including a large peak around 15 years of age and a smaller peak around 36 years. More than one half of narcoleptic patients report important events in the days or weeks preceding the onset of symptoms (excessive daytime sleepiness, cataplexy, SOREMPs). The gap between the emergence of initial symptoms and diagnosis is usually more than 10 years (Dauvilliers 2007).

A tight association between narcolepsy and human leukocyte antigen markers HLA-DR2 and HLA-DQB1* 0602 has been reported, but the roles of HLA (also called major histocompatibility complex or MHC) in narcolepsy are still not yet known. This HLA haplotype is relatively frequent in the general population, and only one out of 600 HLA-DR2 and HLA-DQB1*0602 positive subjects develops the disease. Because many HLA-associated diseases are known to be autoimmune, the discovery of an association between narcolepsy and HLA-DR2 led to the hypothesis that narcolepsy may result from an autoimmune process. Nevertheless, it is important to note that up to 10% of narcoleptic-cataplectic patients are HLA negative (Nishino 2007). Therefore, environmental factors are also important for the development of the pathology, as substantiated by the fact that onset is not at birth but rather in adolescence, suggesting the existence of triggering factors.

# 2.2 Clinical features

- ✓ **Excessive daytime sleepiness**:

In most cases, daytime sleepiness is the first symptom to appear. It is also the most severe symptom and the most frequent cause for consultation (Scammell 2003). Daytime sleepiness occurs daily, recurring typically every 2 hours, although this can vary widely. Sleepiness is exacerbated when the patient is physically inactive. The sleep episodes have several characteristics: (1) they are often irresistible, despite the individual making desperate efforts to fight the urge to sleep; (2) they are usually short, although their length can vary with environmental factors (the duration can increase with passive activities such as watching television); (3) they are frequently associated with dreaming; and (4) they typically restore normal wakefulness for up to several hours. The refreshing value of short naps is of considerable diagnostic value, except in children who are frequently tired on awakening. Severe sleepiness can also lead to unconscious microsleep episodes or lapses (Dauvilliers 2007).

- ✓ **Cataplexy**

Cataplexy is specific to narcolepsy and is the best diagnostic marker of the disease. It is characterized by a sudden drop of muscle tone triggered by emotional factors, most often by positive emotions such as laughter, repartee, pleasant surprise, or by anger, but almost never by stress, fear, or physical effort. All striated muscles (but not the diaphragm) can be affected, causing the individual to collapse. Cataplectic attacks are sometimes limited to facial muscles or to the arms or legs, with dysarthria, facial flickering, jaw tremor, head or jaw dropping, dropping of objects, or unlocking of the knees. The patient might not necessarily see these symptoms as pathological, given that they remain fully conscious during the episode. The duration of cataplexy varies from a split second to several minutes. Its frequency varies from less than one episode per year to several episodes per day. Cataplexy worsens with poor sleep and fatigue. Patients can occasionally have a “status cataplecticus”, which comprises continual cataplectic episodes lasting several hours. This state can occur

spontaneously but is more often seen on withdrawal from anticataplectic antidepressant drugs. Many neurophysiological and pharmaceutical studies indicate that cataplexy shares common neurophysiological mechanisms with REM sleep atonia (Dauvilliers 2007).

Moreover, it has been shown that the dopaminergic system modulates cataplexy, sleep attacks and sleep-wake behavior in narcoleptic mice, with different mechanism. In particular, dopamine receptor modulation has powerful effects on sleep attacks and cataplexy. Activation and blockade of D1-like receptors has been shown to decrease and increase sleep attacks, respectively, without affecting cataplexy. Pharmacological activation of D2-like receptors increases cataplectic attacks and blockade of these receptors potently suppresses them (Burgess 2010).

Specifically, brain imaging studies show that narcoleptics have an increased D2-like receptor binding that is tightly correlated with cataplexy. D2 receptors are also associated with the sleep attacks experienced by Parkinson's patients, who like narcoleptics, have hypocretin cell loss (Thannickal 2007).

- ✓ **Other symptoms**

Narcolepsy includes sleep-related hallucinations and sleep paralysis. These symptoms are present in about 50% of those with the disease, but can also be seen in people who do not have narcolepsy. Hypnagogic or hypnopompic (on awakening) hallucinations can be auditory (the sound of a ringing phone, hearing someone walking on the stairs), visual (a threatening figure, passing shadows when driving, animals), or somesthetic (an out-of-body experience, where the person feels like someone is brushing against them). Even if patients are well aware retrospectively of the nature of these phenomena, some patients report difficulty in differentiating dreams from reality and might occasionally be misdiagnosed as schizophrenic (Dauvilliers 2007).

Sleep paralysis is an inability to move the limbs or the head or to speak or breathe normally either at sleep onset or on awakening (mainly from REMS) despite being mentally awake. It can be associated with hypnagogic hallucinations. Usually, sleep

paralysis does not last long (just a few seconds, but in rare instances can persist several minutes).

Nocturnal sleep is disrupted in a third of patients. Typically, patients fall asleep as soon as they get into bed but wake up several times during the night.

Narcoleptic patients also have higher body-mass indices than normal subjects. Some patients, especially children, gain weight after the onset of narcolepsy.

Narcolepsy is also associated with increased Body Mass Indices (BMIs), increased prevalence of non insulin-dependent diabetes and sleep apnea syndrome.

Periodic leg movements are also reported in narcoleptic patients, especially as they get older, but the contribution of these movements to daytime sleepiness remains controversial. Sleep-talking and REM sleep behavior disorders (in which patients physically enact their dreams) have been recorded in a third of patients. Depression has also been reported in 18-37% of cases. It is difficult to describe the general course of narcolepsy. In most patients, however, daytime sleepiness is the first symptom. Sleepiness and cataplexy persist throughout life although they often improve after retirement, probably due to better management of activities, daytime napping, and adjustment of nighttime sleep. By contrast, nocturnal sleep disturbances usually worsen with age (Dauvilliers 2007).

## 2.3 Diagnosis

The diagnosis of narcolepsy with cataplexy is based on excessive daytime sleepiness (occurring almost daily for at least 3 months) and on a history of cataplexy. The confirmation of clinical diagnosis can be carried out using with nighttime polysomnography, followed by a daytime multiple sleep latency test (MSLT).

The aim of the polysomnography is to rule out other causes of daytime sleepiness (mainly sleep apnea) and to verify whether the patient slept enough (at least 6 h) before the MSLT. In 40% of cases polysomnography reveals a shortened REMS latency (less than 15 minutes), a fragmentation in REMS with imperfect loss of

muscle tone, an increased proportion of stage 1 sleep, and a relative augmentation in slow-wave sleep at the end of the night.

The MSLT consists of five nap times, scheduled at 2-hourly intervals, starting at least 1,5 h after awakening (Carskadon 1986). Each test is stopped after 15 minutes of sleep or after 20 minutes if the patient does not fall asleep. A sleep-onset REM period (SOREMP) is defined as the occurrence of REM sleep within 15 minutes of sleep onset. Since typical patients with narcolepsy frequently have two to five SOREMPs during MSLT, two or more SOREMPs are needed for the diagnosis. In narcoleptic patients the mean sleep-onset latency is usually shorter than 8 minutes, although latency increases with age. Polysomnography and MSLT must be performed in patients who are not under therapy, since drugs can substantially alter sleep-onset latency and the occurrence of REM sleep.

Another diagnostic criteria commonly accepted by the American Academy of Sleep Medicine is the presence of the human leucocyte antigen HLA-DQB1*0602 genotype in patients, which is neither sensitive nor specific in narcolepsy, and can only support, not determine, diagnosis.

Whenever possible, the diagnosis should be confirmed by measurement of HCRT-1 levels in the cerebrospinal fluid (CSF), which must be less than or equal to 110 pg/ml or one third of mean control values in order to qualify. HCRT-1 is measurable in the CSF and levels can be assessed by radioimmunoassay. Many authors assume that CSF HCRT-1 levels reflect the quantity of HCRT-1-producing cells in the hypothalamus. In a rodent study, a decrease of 50% in the CSF HCRT-1 levels was obtained after losing 70% of the HCRT-1-containing neurons (Gerashchencko 2003).

Samples for the assessment of CSF HCRT-1 levels are obtained with a lumbar puncture during daytime. Some patients refuse this procedure, hindering a correct and definitive diagnosis of narcolepsy (Dauvilliers 2007).

## 2.4 Experimental animal models of narcolepsy

The first clues towards an involvement of hypocretins in narcolepsy came from animal models. Using forward and reverse genetics, two groups of researchers independently revealed the pathogenesis of narcolepsy. Either the lack of the hypothalamic neuropeptide HCRT (mouse gene knockout, KO; hypocretin-ataxin-3 transgenic mice, TG), (Chemelli 1999; Hara 2001) or mutations in one of the HCRT receptor (HCRT-R) genes (autosomal recessive canine narcolepsy in Dobermans and Labradors), (Lin 1999) result in the narcolepsy phenotype.

The first canine model of narcolepsy was identified in 1973. Narcoleptic dogs have cataplexy (mainly elicited by the presentation of food), sleepiness, and present SOREMPs. The disease is transmitted as a recessive autosomal trait with complete penetrance in Doberman Pinschers and Labrador Retrievers, whereas it is polygenic and determined by environmental factors (sporadic narcolepsy) in Poodles, Beagles, and other breeds. In 1999 a mutation in the gene coding for HCRT-2R was found as the cause of familial canine narcolepsy, resulting from decreased HCRT transmission (Lin 1999). This alteration consists in a single amino acid substitution (E54K) in the N-terminal region of the HCRT-2R. Results indicate a truncated HCRT-2R protein, an absence of proper membrane localization, and undetectable binding and signal transduction for exon-skipping mutated constructs (Lin 1999).

Cataplexy in dogs closely resembles human cataplexy. Canine cataplexy is typically elicited during play with other dogs or with human caretakers; catching or retrieving objects, and tugging on towels or sticks are particularly effective triggers. Copulation will often elicit cataplexy in male narcoleptic dogs. Cataplexy can also be readily elicited by eating, particularly if the food is especially appreciated. In fact, narcoleptic dogs usually have about 2 episodes of cataplexy/hour during the day, but during the Food Elicited Cataplexy Test, they can have 5 or more than 5 episodes in just 1 or 2 minutes (Scammell 2009).

Well-characterized mouse models of narcolepsy with cataplexy have been produced with deletion of the prepro-HCRT gene and with transgenic expression of a toxic protein that selectively kills the HCRT-producing neurons. These mice have cataplexy-like behavioral arrests, poor maintenance of wakefulness, and fragmented sleep (Chemelli 1999), with occasional direct transitions to REMS from wakefulness. double knockout mice for HCRT-1R and HCRT-2R also exhibit similar phenotypes that have strong parallels with the human condition.

HCRT knockout mice with congenital hypocretin deficiency were developed by Chemelli et al . in 1999. The researchers a targeting vector to replace exon-1 of pre-pro HCRT gene by homologous recombination. The result was a null mutation in prepro-HCRT gene and an autosomal recessive narcoleptic phenotype. Mice carrying the mutation were then crossed with C57Bl/6J mice of the parental strain (this strategy is called “backcrossing”), in order to fix the mutation on a pure C57Bl/6J genetic background. Thus, mice were homozygous for the mutation and fully congenic to C57Bl/6J (Chemelli 1999).

Hara et al. developed HCRT-ataxin-3 transgenic mice in 2001(ref. Chapter 5 for molecular details).

Since narcoleptic patients do not have mutations in the prepro-HCRT or HCRT receptor genes, decreased HCRT levels in these patients is a consequence of a dysfunction or loss of HCRT-expressing neurons. This was confirmed by the discovery by Thannickal et al., who in 2000 described a global loss of HCRT neurons and residual gliosis in the brain of four narcoleptic patients.

Thus, mice in which HCRT- containing neurons are specifically ablated would represent an useful model for human narcolepsy (Hara 2001).

Brains of HCRT-ataxin-3 mice showed a progressive loss of HCRT-containing neurons as determined by anti-HCRT immunostaining. At postnatal day 5 no differences in the number of HCRT-containing neurons in HCRT-ataxin-3 mice and their wild-type littermates were detected. However 56% ± 12% of HCRT-containing

neurons were lost by 2 weeks of age. Thereafter, 75% ± 5% and 90% ± 8% of HCRT-containing neurons were eliminated by 4 and 8 weeks of age, respectively. At 12 weeks of age, more than 99% of HCRT-containing neurons were already lost and at 15 weeks of age, nearly no HCRT-containing neurons could be found in the hypothalamic regions of all lines. These observations suggest that ablation of HCRT-containing neurons occurs postnatally, and most drastically within 2 weeks after birth (Hara 2001).

Phenotypically, prepro-HCRT knockout mice and HCRT-ataxin-3 transgenic mice are similar despite some differences. The first is that in prepro-HCRT knockout mice the congenital HCRT deficiency may lead to development of compensatory responses exerted by other neuronal circuits.

Moreover, the loss of neuropeptides or neuromodulators co-expressed by HCRT-containing neurons may also contribute to phenotypic differences in these models.

In the transition from active wake into cataplexy, the EEG changes from a typical waking pattern to one indistinguishable from REMS or the pre-REMS spindling often seen in mice. Once cataplexy is fully established, the EEG is dominated by high amplitude theta activity with an EEG power spectrum very similar to REMS. Bastianini et al. showed that short (1-2 s) high-amplitude bursts of pointed theta waves (7 Hz) occurred during either REMS or cataplexy in 80% of HCRT-deficient mice. Theta bursts were significantly more likely to occur during the dark period and in the last third of REMS episodes. Similar EEG events were detected in a significantly lower fraction (27%) of wild-type mice and with a significantly lower occurrence rate (0.8 vs. 5 per hour of REMS). These data demonstrate that occurrence of high amplitude theta bursts is facilitated during REMS and cataplexy in narcoleptic mice. The analysis of EEG frequency, and of daily and intra-episode patterns of event occurrence do not support the interpretation of theta bursts as temporally displaced pre-REMS spindles. Facilitation of high amplitude theta bursts may thus represent a novel neurophysiological abnormality associated with chronic HCRT deficiency (Bastianini 2011b).

Genetic factors and recording conditions may alter the frequency of cataplexy. Cataplexy occurs about four times more often in narcoleptic mice on a DBA/2J background compared to those on a C57BL/6J background (Hara 2005).

The “International Working Group on Rodent Models of Narcolepsy” established a consensus definition of the features of murine cataplexy, expressing the confidence that it resembles human cataplexy closely enough for the term “murine cataplexy” to be appropriate. Murine cataplexy is defined as an abrupt episode of nuchal atonia lasting at least 10 seconds, during which the mouse is immobile. Theta activity dominates the EEG during the episode and at least 40 seconds of wakefulness precede the episode (Scammell 2009).

# *3. Wake-sleep cycle, ambient temperature, and physiologic regulation*

# 3.1 Influence of wake-sleep cycle on thermoregulation and cardiovascular autonomic control

## 3.1.1 The wake-sleep cycle

The wake-sleep cycle consists of the single sequence of at least three behavioral states, which are called wakefulness (W), non-rapid eye movement sleep (NREMS) and rapid-eye movement sleep (REMS).

W is defined by a high electromyographic activity (EMG) and a desynchronized electroencephalographic activity with low-voltage rapid waves.

NREMS or slow wave sleep, is easily distinguishable from both wakefulness and REMS by high voltage and synchronous EEG rhythms, including sleep spindles, K-complexes and high-voltage slow waves, associated with low muscle tones and minimal physiological activity. In humans, NREMS is divided into four stages, corresponding with an increasing depth of sleep, as indicated by progressive dominance of the EEG by high-voltage, low frequency, “synchronized” wave activity (Rechtschaffen 1968). Such low-frequency waves dominate the deepest stages of NREMS (stages III and IV). The four NREMS stages (stages I, II, III, IV) roughly parallel a depth of sleep continuum, with arousal thresholds generally lowest in stage I and highest in stage IV sleep. NREMS is associated with fragmentary mental activity. REMS (or paradoxical sleep) requires the coincidence of specific activities in all three electrographic measures: “activated” or desynchronized EEG, bursts of rapid eye movements in the EOG and suppression of EMG activity. During REMS, the EEG pattern is characterized by a relatively low voltage, and mixed frequency (frequency in θ range is fairly common during REMS, particularly in proximity to eye movements). Activity in the α range (1-2 Hz slower than waking activity) may

also be seen in the REMS EEG. In REMS, muscles are atonic and dreaming is typical. Tonic and phasic events may occur during this state (Dement 2005).

## 3.1.2Homeostasis and physiologic regulation during sleep

The hypothalamus is a highly integrative part of the diencephalon, promoting specific behaviors by coordinating somatic, autonomic, and endocrine motor activity on the basis of external and internal sensory information (Zoccoli 2011).

The role of the hypothalamus in this complex integrative activity is critical for the maintenance of body *homeostasis*. The principle of homeostasis in physiology was defined by W.B. Cannon in 1929 as "the coordinated physiologic processes which maintain most of the steady states in the organism" (Cannon 1929). The homeostasis can be experimentally tested across the behavioral continuum by studying the stimulus-response relationship in physiological functions at different levels of integration. The physiological homeostasis is maintained by effectors that minimize the influences of internal and external disturbances on the stability of fundamental variables such as temperature, pH, water volume, osmolality and nutrients. On the contrary, *poikilostasis* represents a condition in which an impairment of the stability of the variables is produced by effector activity (Parmeggiani 2005). Homeostasis is the result of continuous adjustments of "instrumental" variables such as HR, stroke volume, BP, cardiac output, vascular resistance and ventilation, which are directly affected by the activity of somatic and visceral effectors.

Different physiological functions, such as thermoregulation, osmoregulation, regulation of energy balance and metabolism and autonomic cardiovascular control are closely linked to the regulation of wake-sleep cycle. Physiological variables change differently on passing from W to NREMS and from NREMS to REMS. These different behavioral states are characterized by either *homeostasis* (NREMS) or *poikilostasis* (REMS) of physiologic function. Physiological regulation is therefore organized at a hypothalamic level according to the different wake-sleep states. Thus,

wake-sleep states must be taken into account when physiological functions are studied (Parmeggiani 2005).

An important difference between NREMS and REMS is that NREMS is functionally similar in different species, while REMS varies within and between species. NREMS is characterized by the assumption of a thermoregulatory posture and a decrease in muscle activity; from an autonomic point of view in this state there is the functional prevalence of parasympathetic influences over sympathetic activity. These peculiarities indicate a closed loop mode of operation that automatically maintains homeostasis at a lower level of energy expenditure compared with that found in wakefulness; the leading neural structures are diencephalic. Conversely, REMS is characterized by muscle atonia, rapid eye movements and myoclonic twitches; moreover, there is great variability in sympathetic activity and phasic changes in tonic parasympathetic discharge (Parmeggiani 2005). The variability of somatic and visceral phenomena of REMS is the result of open-loop operations of central origin, which impair the homeostasis; the leading neural structures are in this case rhomboencephalic (Parmeggiani 1980).

This basic functional dichotomy may also be applied to the nervous control of body temperature and of circulatory and respiratory functions.

## 3.1.3Wake-sleep states and thermoregulatory control

The hypothalamus represents the most important integrative center for the regulation of the body temperature. In mammals, homeothermy is controlled by preoptic hypothalamic integrative mechanism which drive subordinate brainstem and spinal somatic and visceral mechanism, thus eliciting thermoregulatory effector responses (Parmeggiani 2005).

Thermoregulation and wake-sleep regulation strongly interact. During NREMS thermoregulatory mechanisms are operative, as they are in wakefulness, despite some state differences in the threshold and gain of effector responses to thermal loads and a down-regulation of body and hypothalamic temperatures, together with energy

expenditure. The thermoregulatory responses to ambient thermal loads are present during NREMS and absent or depressed during REMS. During NREMS in cat, for example, its posture clearly varies in relation to the ambient temperature, while the drop in postural muscle tone during REMS is unrelated to ambient temperature (Parmeggiani 1970). Warm temperature notwithstanding, tachypnea in cats and heat-exchange vasodilatation in cats, rabbits and rats disappear and sweating in humans decreases during REMS. In a similar way with a cold temperature, shivering and piloerection in cats, and heat-exchange vasoconstriction in cats, rabbits and rats are suppressed during REMS. Moreover, the cold-defense function of brown fat is altered in rats (Parmeggiani 2005). Crucial proof of the behavioral state-dependent changes in the function of the preoptic-hypothalamic thermostat has been provided by experiments of direct thermal stimulation of preoptic-hypothalamic thermosensitive neurons across behavioral states in cats and rats. The change in thermosensitivity of the majority of such neurons parallels that found in thermoregulatory responsiveness to central thermal loads during NREMS and REMS. Indirect proof of this can be seen in the disappearance of shivering during REMS in a cold ambient in particular in cats with pontine lesions which produce REMS without muscle atonia (Parmeggiani 2005).

In conclusion, experimental evidence shows that during NREMS, thermoregulatory mechanisms are active as they are in wakefulness, despite some state-dependent differences in the threshold and gain of effector responses to thermal loads. The other difference compared to wakefulness is that, in NREMS, body and hypothalamic temperatures are regulated together with energy expenditure.

Events in REMS are not only simply the result of state-dependent changes in threshold and gain of the different thermoregulatory responses; REMS is characterized by effector activity that is not only functionally inconsistent with the aim of temperature regulation but also lacks any proportional relationship with the intensity of the thermal stimulus. The conclusion is that the temperature of the body

changes according to its thermal inertia, as in a poikilothermic organism (Parmeggiani 2005).

### 3.1.4 Wake-sleep states and cardiovascular regulation

During a typical night's sleep, autonomic patterns change dramatically, providing both respite and stress to the cardiovascular system. NREMS is associated with relative autonomic stability and functional coordination between respiration, the pumping action of the heart, and maintenance of arterial blood pressure. During REMS, surges in cardiac sympathetic and parasympathetic activity provoke accelerations and pauses in heart rhythm; these phasic alterations of rhythm are a sign of a central nervous system activation (Verrier 2005).

The cardiovascular changes in NREMS are consistent with changes in ventilation and thermoregulation in a condition of postural and motor quiescence (Parmeggiani 2005).

NREMS is characterized by a down-regulation of cardiovascular activity of variable intensity, depending on the species and on the previous level of cardiovascular activity in wakefulness (Parmeggiani 2005), with vagal nerve dominance and heightened baroreceptor gain (Verrier 2005). Recent studies on sleep onset in human subjects have shown that a progressive fall in BP ensues during relaxed wakefulness with the lights off (Carrington 2005). Conversely, in animal models, a decrease (in mice and dogs), (Schneider 1997; Schaub 1998) or no significant change (in cats, rabbits), (Mancia 1971; Cianci 1991) in BP has been reported on passing from wakefulness to NREMS (Silvani 2008). In humans, BP may decrease during NREMS due to a reduction in HR and, hence, in cardiac output, without significant changes in stroke volume or total peripheral conductance. A reduction in HR is, in fact, a common finding during NREMS in humans, as well as in experimental animal models, in which a reduction of BP is also detected (Silvani 2008). In cats, the reduction in HR during NREMS is attributed to an increase in vagal tone, because it is inhibited by vagotomy but not changed by ablation of the stellate ganglia (Baust

1969). However, in humans an early report showed a decrease in BP during NREMS without consistent changes in cardiac output (Bristow 1969). Moreover, a decrease in sympathetic nerve activity (SNA) directed to the skeletal muscle vessels has been shown in the deep stages of NREMS compared to wakefulness during experiments on human subjects (Somers 1993). Finally, in rats, NREMS entails a decrease in renal SNA (Miki 2003) and an increase in renal vascular conductance (Yoshimoto 2004) compared to wakefulness in rats. These recent findings indicate that vascular conductance may increase during NREMS due to a decrease in sympathetic constrictor tone to the blood vessels in skeletal muscles and kidneys. The available evidence is too limited to determine the extent to which such an increase in conductance contributes to the reduction in BP during NREMS (Silvani 2008).

Different phenomena characterize REMS in both humans and animals. Endogenous brain activation, typical of this state, is accompanied by a prominent variability of HR and BP (Verrier 2005). This variability is an important feature of REMS and it is generally associated with bursts of rapid eye movements, myoclonic twitches and breathing irregularities.

During REMS in humans, mean BP increases compared to its values during NREMS. On the contrary, either a reduction or no substantial change in BP has been observed during REMS in different inbred mouse strains (Campen 2002). Early reports in cats showed a marked reduction in ABP during REMS, whereas an increase in BP has been reported during REMS of cats in more recent experiments, in which a longer postoperative recovery of the animals was allowed (Sei 1994). Thus, the reported changes in mean BP between NREMS and REMS are inconsistent both among and within species (Silvani 2008). Factors related to stress and thermoregulation may also be involved (Silvani 2008), and both prolonged postoperative recovery in cats and a shift of ambient temperature towards thermoneutrality in rats (Sei 1996) favour an increase in BP when passing from NREMS to REMS. It is worth noting that the impairment of thermoregulation during REMS also involves the hemodynamic adjustments driven by a thermal challenge in this state (Silvani 2008). During REMS,

an increase in mean BP was associated with an increase in HR in studies on humans (Khatri 1967). As far as HR changes during REMS are concerned, data obtained in rats are not consistent: an increase (Sei 1996), a decrease (Miki 2003) or no significant change (Zoccoli 2001) in HR have been reported during REMS. Similarly, in different inbred mouse strains, an increase, a decrease or no significant change in the mean value of HR has been observed during REMS without any apparent connection to changes in BP (Campen 2002). The changes in BP on passing from NREMS to REMS cannot be attributed to changes in cardiac output. The inconsistent relationship between changes in mean BP and those in HR and cardiac output suggest that differences in vascular conductance are involved in determining the hemodynamic pattern of REMS (Silvani 2008). Accordingly, in cats, early studies demonstrated an increased conductance of the splanchnic and renal vascular beds and a decreased renal SNA during REMS (Mancia 1971). An increased mesenteric vascular conductance (Miki 2004) and a decreased renal SNA (Miki 2003) have also recently been reported during REMS in rats.

Taken together, these data indicate a substantial repatterning of sympathetic activity directed to the different vascular beds during REMS. However, these data do not clarify the extent to which the changes in vascular conductance contribute to the changes in BP on passing from NREMS to REMS (Silvani 2008).

BP is controlled by two major nervous mechanisms, baroreflex and the central autonomic commands. They both act on the two branches of the autonomic nervous system (i.e. sympathetic and parasympathetic nervous system), eliciting different effects. The baroreflex operates a feedback control of BP in order to keep constant BP value in the face of perturbations. On the other hand, central autonomic commands may operate anticipatory feed-forward control of hemodynamics preparing the body for potentially dangerous situations such as the so-called fight-or-flight response. This response thus involves changes in BP. The baroreflex and the central autonomic commands are likely to interact continuously but their actions and their relationship change according to body needs and behavioral states.

During NREMS, the contribution of the baroreceptor reflex in controlling heart rhythm has been demonstrated to be stronger than during either wakefulness or REMS. This distinctiveness of NREMS may in part underlie the greater stability in blood pressure observed during this state. The modification in the baroreceptor reflex mechanism in NREMS may be the result of a baroreflex change in sensitivity or a resetting compared to in wakefulness. The latter phenomenon is more consistently supported by experimental evidence in animal models and humans (Silvani 2008). Taken together, these findings suggest that the cardiovascular changes on passing from wakefulness to NREMS involve a resetting of the arterial baroreflex, but result primarily from centrally driven changes in the autonomic outflow to the heart and vessel resistance. To summarize, NREM sleep with its autonomic stability, provides a relatively salutary neurohumoral background during which the heart has an opportunity for metabolic restoration (Silvani 2008).

During REMS central autonomic commands are thought to cause a repatterning of SNA directed to the different cardiovascular effectors (Silvani 2008). Central autonomic commands are also responsible for the phasic surges of BP described during REMS. The hemodynamic pattern of REMS is modulated by sinoaortic reflexes, but is not primarily caused by changes in their properties (Silvani 2008).

Conversely, some evidence suggests a central origin of the hemodynamic pattern of REMS. In cats subjected to a mid-collicular transection, periods akin to REMS entail a decrease in BP and HR, which are associated with a decrease in the SNA directed to the heart and to the cutaneous, splanchnic and renal vascular beds and an increase in the SNA directed to the skeletal muscles (Futuro-Neto 1982). There is a striking correspondence between these findings and those often reported during physiological REMS in intact animals and humans, thus suggesting that, during REMS, stereotyped central autonomic commands produce a repatterning of the SNA directed to the different cardiovascular effectors. The variety of hemodynamic patterns and changes in BP that are observed on passing from NREMS to REMS are thought to

result, therefore, from a variable reflex modulation of this basic central command (Silvani 2008).

There is growing evidence that nighttime BP values are prognostic for cardiovascular mortality. Ohkubo demonstrated that a diminished nocturnal decline in BP is predictable for cardiovascular risk and in particular, that each 5% decrease in the decline in the nocturnal BP was associated with a 20% increased risk for cardiovascular mortality (Ohkubo 2002). The physiological decline of BP on passing from wakefulness to sleep is usually defined as “non-dipping”. The mechanism of the action responsible for non-dipping is not clearly understood, but probably “non-dippers” have an increase in sympathetic and a decrease in parasympathetic nervous system activity (Kohara 1996). The close relationship between wake-sleep architecture and dipping in BP has been shown in humans (Loredo 2004) as well as in mice (Silvani 2009).

## 3.2 Influence of ambient temperature on wake-sleep cycle and cardiovascular autonomic control

### 3.2.1 Central neural pathways involved in the regulation of body temperature

Central neural circuits orchestrate a homeostatic repertoire for the maintenance of body temperature during environmental temperature challenges and for the alteration of body temperature during the inflammatory response. Morrison and Nakamura (Morrison 2011) recently summarized the functional organization of the neural pathways through which cutaneous thermal receptors control thermoregulatory effectors, i.e. cutaneous circulation for heat loss, brown adipose tissue, skeletal

muscle and the heart for thermogenesis, and species-dependent mechanisms (sweating, panting and saliva spreading) for evaporative heat loss.
Briefly, when the hypothalamic temperature centers detect that the body temperature value is far from the set-point value, they institute appropriate temperature-decreasing or -increasing procedures.

The temperature control system uses three mechanisms in order to reduce body temperature:

- Vasodilatation of skin blood vessels, by inhibiting the sympathetic centers in the posterior hypothalamus that are responsible for vasoconstriction;
- Sweating, which increases the rate of evaporative heat loss;
- Decrease in heat production, through the inhibition of thermogenesis pathways.

When body temperature is too cold, the temperature control system institutes exactly the opposite procedures (Guyton 2006).
Thermogenesis, the production of heat energy, is an essential component of the homeostatic repertoire for the maintenance of body temperature during the challenge of low environmental temperature. (Morrison 2008). CNS thermoregulatory networks can stimulate thermogenesis in response to a cold environment, to a fall in core body temperature or to the presence of pyrogenic cytokines primarily in three tissues: brown adipose tissue (BAT), heart and skeletal muscle.
Nonshivering thermogenesis in brown adipose tissue, shivering thermogenesis in skeletal muscles, thermoregulatory cardiac regulation, heat-loss regulation through cutaneous vasomotion and ACTH release (ACTH stimulates release of glucocorticoid, which then increases glucose availability and facilitates lipolysis to provide more fuel for thermogenesis) are involved in the regulation of body temperature. To defend thermal homeostasis from environmental thermal challenges, feed-forward thermosensory information on environmental temperature sensed by skin thermoreceptors ascends through the spinal cord and lateral parabrachial nucleus to the preoptic area (POA). The POA also receives feedback signals from local thermosensitive neurons, as well as pyrogenic signals of prostaglandin E2 produced

in response to infection. These afferent signals are integrated and affect the activity of GABAergic inhibitory projection neurons descending from the POA to the dorsomedial hypothalamus (DMH) or to the rostral medullary raphe region (rMR). Attenuation of the descending inhibition by cooling or pyrogenic signals leads to disinhibition of thermogenic neurons in the DMH and sympathetic and somatic premotor neurons in the rMR, which then drive spinal motor output mechanisms to elicit thermogenesis, tachycardia, and cutaneous vasoconstriction. Warming signals enhance the descending inhibition from the POA to inhibit the motor outputs, resulting in cutaneous vasodilation and inhibited thermogenesis. This central thermoregulatory mechanism also functions for metabolic regulation (Nakamura 2011).

During the rapid, repeated skeletal muscle contractions of shivering and during increases in heart rate, thermogenesis arises primarily from the inefficiency of energy utilization in cross-bridge cycling and calcium ion sequestration and, to a lesser degree, from mitochondrial membrane proton leaking in the course of ATP production from fuel substrate oxidation. The muscle contractions that characterize shivering result from rhythmic bursts of activity in the α-motoneurons, which innervate skeletal muscle fibres. The CNS network which generates the cold-evoked bursts of α-motoneuron activity is not well understood, but includes transmission of cutaneous cold afferent signals through the lateral parabrachial nucleus (LPB) (Nakamura 2008), integration of thermoregulatory signals in POA (Zhang 1995) and activation of fusimotor neurons that is dependent on neurons in the RVLM (Tanaka 2001).

The increases in heart rate in response to cold or during fever are sympathetically mediated and involve a neural network that parallels the one controlling the sympathetic activation of BAT thermogenesis (Morrison 2008).

BAT is involved in non-shivering or adaptive thermogenesis. The specific metabolic function of this tissue is the heat-generating capacity induced by a significant facilitated proton leak across the extensive mitochondrial membranes of the brown

adipocytes, which occurs because of the high expression of uncoupling protein 1 (UCP1) in BAT mitochondria. The level of BAT sympathetic nerve activity (SNA) and noradrenaline release and β3-adrenergic receptor binding to brown adipocytes determine the level of thermogenesis in BAT by regulating both the activity of lipases providing the immediate fuel molecules for BAT mitochondria and the level of expression of BAT mitochondrial UCP1. BAT has developed as an essential thermoregulatory effector in cold defense in rodents and other small mammals (Golozoubova et al. 2006), including infant humans. However, several recent observations, using positron emission tomographic scanning to assess tissue glucose uptake, have demonstrated a remarkable amount of BAT in adult humans, and the locations of BAT depots in adult humans bear a striking similarity to those in rodents: a large BAT pad in the vicinity of the scapulae and shoulders and individual pads atop each sympathetic ganglion and surround adrenal glands and kidneys. The curious localization of BAT pads, exemplified by those over each sympathetic ganglion, suggests that, in addition to the defense of core temperature in the cold, BAT may also serve to maintain optimal neuronal and synaptic function in specific locations in situations of reduced core body temperature. Similar to its function in smaller mammals, adult human BAT activity (assessed by glucose uptake) is highly responsive to β-adrenergic agonists and to environmental temperature (Morrison 2008).

Within the hierarchical organization of the central network controlling thermogenic thermoregulatory effectors, medullary neurons play key roles as premotor neurons, providing excitatory input to spinal motor neurons, for the circuits regulating sympathetic BAT thermogenesis, heart rate and somatic shivering thermogenesis (Morrison 2008). Physiologically, activation of neurons in the restricted region of the RVLM, including the rostral raphe pallidus nucleus (rRPa), whose activation leads to stimulation of cold-defense responses, increases BAT SNA, BAT thermogenesis and heart rate (Morrison 2008). Disinhibition of rRPa neurons following blockade of local GABA-A receptors with nanoinjections of bicuculline elicits a large and

sustained increase in BAT SNA and BAT temperature and a sympathetically mediated tachycardia in anaesthetized rats with external body temperature support (Cao 2003) and a marked increase in heart rate with no change in body temperature in awake rats (Zaretsky 2003). These data indicate that when normothermic body temperature is maintained with an external heat source, the rRPa neurons controlling BAT SNA, the sympathetic outflow to the cardiac sino-atrial node and the discharge of motoneurons for skeletal muscle receive a tonic, GABAergic inhibition. In fact, inhibition of the activity in neurons in the rRPa reverses the increases in BAT SNA, BAT heat production and heart rate elicited by every thermogenic stimulus that has been tested (Morrison 2008).

Thermoregulatory cardiac regulation is a very important component of the pathways facing ambient temperature changes. The cooling signals lead to disinhibition of thermogenic neurons in the DMH and sympathetic and somatic premotor neurons in the rMR, which then drive spinal motor output mechanisms to elicit thermogenesis, tachycardia, and cutaneous vasoconstriction. Warming signals enhance the descending inhibition from the POA, resulting in cutaneous vasodilation and inhibited thermogenesis (Nakamura 2011). The cooling-induced tachycardia is believed to facilitate the distribution of heat produced in thermogenic organs, as well as increase the availability of energy substrates to activated thermogenic organs. Furthermore, increased heart rate increases the amount of heat production in the heart (cardiac thermogenesis). But skin warming also increases heart rate. This warming-induced tachycardia helps the heat-dissipating effect of the simultaneous cutaneous vasodilation by maintaining cardiac output and arterial pressure at a sufficient level to assure optimal increase in cutaneous blood flow (Nakamura 2011).

Skin warming elicits cutaneous vasodilation, which is a heat defensive response: the increased skin blood flow facilitates the dissipation of body heat from the body surface. This response is mostly elicited through attenuation of cutaneous sympathetic nerve activity, whose tonicity regulates skin blood flow, even in a thermoneutral environment (Nakamura 2011).

### 3.2.3 Effect of ambient temperature on wake-sleep cycle

Environmental temperature exerts complex effects on mammalian sleep and wakefulness (Parmeggiani 1970). In general, shifting the ambient temperature toward the thermoneutral zone increases the time spent in NREMS and REMS.

Maintaining mice at a warm ambient temperature (30°C) increases time in both NREMS and REMS, whereas exposure to cold temperatures (10 or 18°C) reduces sleep (Roussel 1984).

In order to assess the effects of ambient temperature on sleep, Jhaveri et al. evaluated the impact of three environmental temperatures (22, 26, and 30°C) on C57BL/6J mice under normal conditions, after sleep deprivation (SD), and during influenza infection (Jhaveri 2007).

During the light phase, which corresponds to the rest period in nocturnal animals, the NREMS bout length was significantly longer in mice exposed to 30°C as compared to mice at 26°C, but the number of NREMS bouts did not differ significantly between the two Ta.

Moreover, the time spent in REMS was significantly higher at 26°C as compared with mice housed at 22°C. These data indicate that a higher ambient temperatures promote spontaneous NREMS. Thus, laboratories in which sleep in mice is studied should expose animals to ambient temperatures ranging from 22 to 29°C. Data collected from mice housed under different temperatures may vary depending on an interaction between the ambient temperature and the condition of the animal. In some rodent species, even small fluctuations in ambient temperature can change the physiology or behavior (Jhaveri 2007).

### 3.2.4 Effect of ambient temperature on cardiovascular parameters and clinical implications

Cardiovascular parameters such as BP and HR strongly depend on wake-sleep states but also on ambient temperature. Exposure of humans, rats, and mice to a cold ambient temperature results in elevated BP and HR. It appears as if the tachycardia and hypertension are the indirect result of sympathetic nervous system (SNS) activation of thermoregulatory mechanisms due to the fact that elevated plasma norepinephrine (NE) levels correlate with elevated blood pressure in the cold environment. Furthermore, propranolol, an adrenergic blocking agent, can blunt or completely reverse the cardiovascular effects of cold exposure. Cold-induced activation of the SNS, in turn, appears to elevate blood pressure through activation of the renin-angiotensin system (RAS). Genetic blockade of the RAS genetically in an angiotensinogen-knockout mouse model blunts or prevents cold-induced hypertension (Sun 2003), suggesting that RAS signalling pathways are necessary for Ta-induced effects on BP.

The elevation of Ta beyond typical housing temperatures also impacts on the cardiovascular system. Warming rats and mice from 23°C to 28-31°C, which is closer to, if not within, their thermoneutral zone (TNZ) results in a drop in heart rate and blood pressure.

Swoap and colleagues demonstrated that the cardiovascular effects of changes in Ta are larger in mice than in rats; this is not surprising, considering that mice are 10 times smaller than rats and therefore have a much greater rate of heat exchange relative to heat production than rats (Swoap 2004).

Cardiovascular parameters and metabolic rate were measured in rats and mice over a series of Ta, ranging from 18° to 30°C. At any given Ta, BP, HR, oxygen consumption, and activity were elevated in the dark period compared to the light period, and varied linearly with ambient housing temperature (Swoap 2004).

Under constant temperature conditions of 23°C, cardiovascular parameters and metabolic rate did not change in rats or mice over the same period of time (Swoap 2004) and small incremental changes in Ta resulted in statistically different cardiovascular parameters. These findings demonstrated that, in the normotensive rat and mouse strains studied, there was a highly sensitive and linear effect of raising or lowering Ta within the range of 18-30°C on cardiovascular function.

This cold-induced hypertension phenomenon appears to be dependent on thermoregulatory mechanisms, which operate via the SNS, as discussed above. Thus, there is strong evidence to suggesting that mammals increase SNS activity in response to thermogenic challenges (Swoap 2004).

These data have an enormous impact from a clinical point of view. Many studies underline a linear association between ambient temperature and cardiovascular risk.

The first study that estimated the impact of atmospheric variables on coronary heart disease morbidity in a population over a long period of time was published in 1999. Ambient temperature and pressure independently influenced myocardial infarction morbidity and mortality in the general population. A 10°C decrease in ambient temperature was associated with a 13% increase in total coronary event rates, an 11% increase in incident and coronary death rates, and a 26% increase in recurrent event rates (Danet 1999).

A statistically significant increased risk of myocardial infarction at colder temperatures has recently been confirmed in other studies (Bhaskaran 2010).

In this study, a broadly linear association between mean daily ambient temperature and risk of myocardial infarction was found, with a 1°C reduction in temperature associated with a cumulative 2% (95% confidence interval 1.1% to 2.9%) increase in risk of myocardial infarction over the current and subsequent 28 days (Bhaskaran 2010). Several mechanisms have been proposed as explanations of the effect of temperature reduction on myocardial infarction risk. Exposure to cold under controlled conditions has been associated with an increase in arterial pressure, blood viscosity, and cardiac workload. A mobilization of granulocytes has been observed,

and red cell counts and plasma cholesterol and fibrinogen concentrations, all of which may be thrombogenic, seem to be raised after exposure to cold. Finally, one study has suggested that the density distribution of blood platelet subpopulations may be affected. These small experimental studies combine to suggest that a pathway for cold induced thrombogenesis might involve a combination of factors, including hemoconcentration, an inflammatory response, and a tendency for an increased state of hypercoagulability (Bhaskaran 2010).

## 3.3 Interaction effect of Ta and wake-sleep state on cardiovascular parameters

It is important to bear in mind that Ta and behavior may together influence cardiovascular parameters. Impairment of thermoregulatory peripheral vasomotion during REMS has been seen in experiments on rats, rabbits and cats exposed to various Ta (Parmeggiani 1977; Franzini 1982). In particular, during REMS, they observed that skin vessels were dilated at a low Ta while vasoconstriction increased paradoxically, at a higher Ta.

The sympathetic nervous system, which controls skin blood vessels, was initially considered as playing an important role in thermoregulation, while sympathetic outflow to muscle blood vessels was involved in BP regulation. However, Fagius and Kay showed that, in humans, muscle sympathetic activity contributes to thermoregulation, and not only regarding the regulation of BP. Thus the elevation of BP and HR was considered to be related to heat production and to thermoregulatory vasomotion (Fagius 1991). Therefore, the thermoregulatory response to different Ta during sleep may include the change of muscle sympathetic nerve activity, thus affecting BP. However, Sei and Morita recorded the changes in mean arterial pressure (MAP) and HR during sleep at three Ta (16, 22 and 28C°) in rats. MAP and HR during sleep increased with the lowering of Ta. Indeed, the increase in MAP on passing from NREMS to REMS was diminished by lowering the Ta. At 28°C, HR

increased on passing from NREMS to REMS but decreased at 16°C (Sei 1996). This study suggested that Ta has a greater effect than sleep on cardiovascular parameters. These results highlight the idea that Ta and behavior have to be taken into account jointly when studying cardiovascular regulation; this is consistent with the findings that cardiovascular adaptations to ambient temperature changes are a part of neural pathways required for thermoregulation. This is particularly important in cases of hypocretin deficiency, given the pivotal role of the neuropeptide in thermoregulation, cardiovascular and behavior control.

# *4. Aim of the research*

The aim of this research was to investigate whether the effect of sleep and Ta on cardiovascular control depends on HCRT neurons.

The hypothesis was that a lack of HCRT signaling is involved in the impairment of hypothalamic physiologic regulation, and in particular of cardiovascular thermoregulatory adaptations to different Ta. To test this hypothesis, experiments were performed in a mouse model of HCRT deficiency, the HCRT-ataxin-3 transgenic mouse. HCRT-containing fibers and HCRT receptors are present in several brainstem regions including the rostral raphe pallidus, a region of the ventromedial medulla which is a principal site of sympathetic and somatic premotor neurons regulating the activity of thermal effectors. These premotor neurons play an important role in the regulation of body temperature and in controlling BAT thermogenesis, energy expenditure and heart rate (Cao 2003). The increase in body temperature following ICV injection of HCRT is accompanied by an increase in sympathetic outflow (Yoshimichi 2001) as well as in heart rate, blood pressure, and renal sympathetic nerve activity (Shirasaka 1999). Thus, lack of HCRT signaling could result in reduced energy expenditure through decreased thermogenesis and physical activity, leading to obesity, despite the presence of hypophagia (Hara 2001).

According to these data, core body temperature (Tb) in HCRT knockout mice with congenital HCRT deficiency was expected to be lower compared to controls (Mochizuki 2006). Surprisingly, Tb was the same in HCRT knockout mice and wild-type littermates during sustained wakefulness, whereas it fell much less than normal during sleep in HCRT knockout mice. This blunted fall in Tb during sleep was probably due to an inadequate activation of heat loss mechanisms or to a sustained activity in heat-generating system in absence of HCRT signaling (Mochizuki 2006).

Alterations in cardiovascular and thermoregulatory functions related to HCRT-deficiency have also been found in human subjects. In fact, thermoregulatory cardiovascular control is impaired in narcoleptic patients (Fronczek 2006) and the related core body and skin temperature might causally affect vigilance and sleepiness in these patients (Fronczek 2008a).

Recently in this Lab it has been demonstrated that in HCRT-deficient mice, differences in BP between wakefulness and sleep states were blunted with respect to control mice; in particular, sleep-related physiological hypertension was significantly reduced, especially during REMS. These results provide proof of principle that chronic lack of HCRT signaling entails consequences regarding BP that are potentially adverse and that differ among wake-sleep states (Bastianini 2011a). Thus, the rationale of this research was to evaluate whether the blunted BP difference between wakefulness and sleep states in narcoleptic mice are modulated by Ta. This result, if positive, would be relevant for narcoleptic patients, given that a non-dipping BP profile and a cold ambient temperature are associated to an elevated cardiovascular risk.

# *5. Methods*

The study protocol was approved by the Bologna University ethics committee on animal experimentation and complied according with International Institutes of Health guide for the care and use of laboratory animals.

# 5.1 Mice included in the experiment

Hybrid transgenic mice with postnatal ablation of HCRT neurons (hTG) and wild-type littermates (hWT) were included in these experiments. This strain was developed in the facilities of the Department of Human and General Physiology at the University of Bologna, with the purpose to obtain mice which were hemizygous for the HCRT-ataxin3 transgene with a mixed genetic background as in the original paper by Hara et al. (Hara 2005).

We chose this mice model because it represents a useful model for human narcolepsy. It reproduces the patients' phenotype well, with a global loss of HCRT containing neurons and residual gliosis in the perifornical region (Thannickal 2000), and presents smaller compensatory effects compared to congenital HCRT deficiency models.

Transgenic mice with postnatal ablation of HCRT neurons (TG) were developed in 2001 by Hara et al. (Hara 2001) by inserting a transgenic construct containing a toxin into mouse DNA. This transgenic construct is composed of the same human prepro-HCRT gene fragment ligated to a cDNA fragment, which encodes from amino acid 286 to the C terminus of elongated ataxin-3, isolated from a Machado Joseph Disease (MJD) patient. MJD is a rare autosomal dominant spinocerebellar type-3 ataxia in which neurons die because of the uncontrolled accumulation of the mutant form of ataxin-3 (Perez 1999).

This abnormal form of the human ataxin-3 has an expanded polyglutamine stretch that induces cell apoptosis. From a patient with MJD, Hara et al. isolated the N-terminally truncated portion of ataxin-3, which is sufficient to induce neuron apoptosis (Yoshizawa 2000) and put it next to the 5'-upstream region of the human prepro-HCRT gene. In these TG mice, which carry one copy of the transgene

containing the toxin (hemizygous mice), the hypothalamic HCRT-containing neurons gradually decrease in number, and are almost completely lost by 15 weeks of age.
Previous experiments performed in the PRISM lab utilized TG mice backcrossed for 13 generations and congenic to the C57Bl/6J strain (Bastianini 2011a). In the present study, in order to obtain hTG, male mice which were hemizygous for the HCRT-ataxin3 transgene and congenic to C57Bl/6J (13 generations of backcrossing) were crossed with female (C57Bl/6J × DBA/2J) F1 hybrid mice (strain B6D2F1/J).
hTG mice utilized in this experiment, similarly to mice studied by Hara et al. (2001) had 75% of their DNA which originated from the C57Bl/6J strain and 25% from DBA/2J strain.
HCRT neuron-ablated mice with a mixed genetic background allow researchers to point out the metabolic abnormalities associated with human narcolepsy. The impairment of energy homeostasis in HCRT neuron-deficient transgenic mice (HCRT-ataxin-3 mice) is revealed by the development of late-onset obesity associated with a reduction in food intake. Beside the genetic background, environmental factors are also important in the development of obesity, stressing the role of genetic variability (Hara 2005).

Experiments were performed on the following 2 age-matched groups of male mice:

- 11 hTG mice, age at surgery 13.9 ± 0.5 weeks.
- 12 hWT mice, age at surgery 15 ± 0.5 weeks.

Mice were kept on a light-dark cycle of 12-hour periods with ambient temperature set at 25°C and free access to water and food (4RF21 diet, Mucedola, Settimo Milanese, Italy).

# 5.2 Experimental protocol

PRISM lab has developed a combined, innovative, surgical technique which allows us to determine cardiovascular and sleep-wake phenotype simultaneously.

In experimental animals, as in humans, techniques for measuring BP have improved considerably over the past decade. BP can be directly measured using radiotelemetry techniques, with the implantation of catheters connected to telemetric transducers. Implantable telemetry has the advantage of allowing for continuous, direct measurement of BP without the need for restraint in conscious freely-moving laboratory animals (Kurtz 2005).

In this experiment, BP signal was obtained through a telemetric transmission device that is considered at the state of the art for the measurement of BP.

One of the methodological limits of this technique is that it is impossible to record other biosignals along with BP. That is why EEG and EMG were obtained by the classical via-cable acquisition method, using the same technique used by group leaders in animal sleep research (Franken 1999).

This combined technique allowed us to demonstrate that obesity may entail hypertensive derangements of BP, which are substantially modulated by the cardiovascular effects of the wake-sleep states (Silvani 2009), and to show that the chronic lack of HCRT signaling entails consequences regarding BP that are potentially adverse and that vary widely among wake-sleep states (Bastianini 2011a).

## 5.2.1 Surgical procedure

Mice underwent surgery under general anesthesia (isoflurane, Abbott S.p.a., Pomezia Roma, Italy, 1-2%, $NO_2$ 70%, and balance $O_2$) and sterile conditions, with the body temperature maintained at 37°C through a heating pad.

The mice were subcutaneously injected with 200 µl of Rimadyl (Carprofen, Pfizer) diluted in saline solution 0.9%.

The mice were then implanted with a catheter inserted into the abdominal aorta and connected to a telemetric BP transducer (TA11PA-C10, DSI, Roermond, the Netherlands). The telemetric system also yielded a motor activity signal by quantifying the spatial shift of the transducer. All the surgical instruments were sterilized for 10 minutes at 134°C in an autoclave (Beta 35 Easy-lock, PBI, Milano, Italy).

The right inguinal area of the mice was shaved and sterilized with iodine solution. One cm of the skin was cut and a subcutaneous pocket was created in the right flank of the animal. Using a Zeiss microscope at 16X magnifications, the femoral artery was carefully dissected from the nerve and the femoral vein, by removing the connective tissue. A 5-0 soft silk suture thread was permanently tied in the distal portion of the artery while a second suture thread was temporarily tied proximally to clamp the femoral artery, in order to arrest the blood flow. The catheter was then inserted in the distal portion of the artery, between the threads, through a hole made by a 90° bent needle (25 gauge). The tip of the catheter was placed between the iliac bifurcation and the renal arteries. The two silk suture threads were then tied in order to secure the catheter to the vessel. The telemetric transducer was inserted into the previously created flank pocket and fixed with surgical glue. Finally the skin incision was sutured with a 3-0 suture thread and covered with an antiseptic cream (Betadine solution 10%, Viatris, Milan, Italy).

In the second half of the surgical procedure, the mouse head was fixed on a stereotaxic apparatus (51600 Lab Standard™ Stereotaxic Instrument, 2biol, Varese, Italy). The head was shaved and sterilized with iodine solution. The skin was cut on the midline from the frontal bone to the nuchal muscles. The periosteum was removed and the bone surface was cleaned. A pair of Teflon-coated stainless-steel electrodes (Cooner Wire, Chatsworth, CA, USA) were positioned in contact with the dura mater through burr holes to obtain a differential EEG signal. The first wire was inserted 1 mm anterior and 1 mm right to Bregma in the frontal bone while the second wire was inserted 1 mm anterior and 1 mm right to Lambda in the parietal

bone. Another 3 holes were drilled on the left side of the skull in order to insert 3 small stainless-steel anchor screws (2.4 mm length, Plastics One, Roanoke, VA, USA).

A second pair of electrodes was inserted bilaterally in the nuchal muscles to obtain a differential EMG signal. All electrodes were connected to a miniature custom-built socket, which was then cemented to the skull with dental cement (Rely X ARC, 3M ESPE, Segrate, Milano, Italy), and dental acrylic (Respal NF, SPD, Mulazzano, Italy). At the end of the surgical procedure, an antibiotic solution (Benzilpenicillina Benzatinica, Biopharma) was administered subcutaneously. Then the mouse was left to recover for the rest of the day in a warmed cage at 30°C.

## 5.2.2 Biosignal recordings

After a 17-day recovery period and habituation to the recording apparatus, BP and motor activity, and EEG and EMG signals were recorded simultaneously while keeping each mouse at a warm (30°C) and cold (20°C) ambient temperature, with exposure order balanced in each experimental group. In a sub-group of experimental animals (hTG, n=8 and hWT, n=9), biosignals were also recorded at 25°C, which was used as the baseline condition. The temperature of the recording box was modified the day before the beginning of biosignal recordings at each ambient temperature, in order to let mice adapt to the new conditions.

Calibration of the telemetric BP transducers was performed using a high precision manometer (PCE P05, PCE Italy, Gragnano, Lucca, Italy), before implantation and after the end of the recordings. The TA11PA-C10 transducer was calibrated by inserting the catheter in a plastic chamber connected to a Riva-Rocci sphygmomanometer (PCE-P05, PCE Italy, Gragnano, Lucca, Italy). In the internal chamber different pressure values were set, from which it was possible to extrapolate a parabolic function then used to correct the BP signal. After this calibration, the TA11PA-C10 transducer was sterilized in a diluted solution (1:10) of peracetic acid (Nu Cidex®, Johnson & Johnson Medical, Rome, Italy).

The TA11PA-C10 transducer transmitted the BP signal by means of radio waves to a receiver (RPC-1, DSI) placed below the animal's cage. The BP signal was then routed to a calibrated BP analog adapter (R11CPA, DSI) with compensation for barometric pressure (APR-1, DSI). The RPC-1 receiver also yielded an activity signal by quantifying the spatial shifts of the transducer due to mouse movements.

The EEG and EMG signals were transmitted via cable. A rotating swivel (SL2+2C/SB, Plastics One) and a balanced suspensor arm prevented the cable from twisting and counterbalanced its weight, thus allowing the mice to make unhindered movements. The EEG and EMG signals were amplified and filtered (EEG: 0.3-100 Hz; EMG: 100-1000 Hz; 7P511J amplifiers, Grass, West Warwick, RI, USA).

All signals were digitized at 16-bit and 1024 Hz (PCI-6224 board, National Instruments, Austin, TX, USA). The EEG, EMG, and locomotion signals were down-sampled at 128 Hz and stored together with the 1024 Hz BP signal. Data acquisition was performed by means of custom software written in Labview (National Instruments).

### 5.2.3 Autopsy

At the end of the experimental procedure, animals were euthanized under deep anesthesia (4% isoflurane). Brains were rapidly removed, checked for any pathological alterations produced by electrodes and screw implantation, and then frozen at -80°C in dry ice for HCRT quantification. The inguinal incision and the subcutaneous pocket were observed for any infection and the catheter was removed with care and calibrated again in order to verify its integrity. Two tail biopsies were taken to confirm the genotype.

# 5.3 Discrimination of wake-sleep states

Data analysis was performed with custom software developed in Matlab and its Signal Processing Toolbox (The MathWorks Inc., Natick, MA, USA).

A semi-automatic scoring of wake-sleep states was carried out on all consecutive 4-s epochs (Figure 1).

A preliminary procedure was performed by identifying 3 clusters of epochs based on the root mean square (rms) of the EMG signal and the ratio between the EEG spectral power in the theta (6-9 Hz) and delta (0.5-4 Hz) frequency bands.

Then all the epochs were displayed on a 3D graph in which the x-axis corresponded to the root mean square of EMG, the y-axis corresponded to θ/δ EEG power ratio, and the z-axis represented the density of epochs with those XY coordinates (Figure 2). An algorithm set a cut-off region on the x-axis in correspondence of the minimal density between the two highest peaks. This cut-off automatically discriminated episodes of W (high EMG values) from those of sleep (low EMG values). A second cut-off region was fixed on the Y-axis between 0.75 and 1.25 based on preliminary data. All the epochs with higher values were automatically scored as REMS, whereas those with lower values were scored as NREMS. Episodes falling into the cut-off regions were marked as undetermined.

After this automatic analysis, a wake-sleep state manual scoring was also performed. Visual scoring was based on the following criteria: W was scored when the EMG tone was high and the EEG was at low voltage with possible δ and θ frequency components. NREMS was scored when the EMG tone was lower than in W and the EEG was at high voltage with prominent δ frequency components. REMS was scored when the EMG indicated muscle atonia with occasional muscle twitches and the EEG was at low voltage with predominant θ frequency components. Automatic scoring thus helped to speed up the visual scoring. Manual scoring was performed by three trained researchers.

Analysis of sleep macrostructure was performed with a threshold of 12 s (i.e., three consecutive 4-s epochs) for each wake-sleep episode duration, and results were expressed relative to recording time which were free of EEG and EMG artifacts. Artifacts in EEG signal represented only 0.0417 ± 0.3% of the recording time spent at 20°C and 0.0934 ± 0.6% spent at 30°C.

Cataplexy-like episodes were scored following the consensus criteria established by Scammell et al. (Scammell 2009):

- An abrupt episode of nuchal atonia lasting at least 12 seconds
- Theta activity dominating the EEG during the episodes
- At least 40 seconds of W preceding the episodes

The last point is very important to distinguishing cataplexy-like episodes from REMS episodes. Cataplexy-like episodes were then excluded from all subsequent analyses.

# 5.4 Analysis of cardiovascular variables

Data analysis was performed using custom software developed in our laboratory in Matlab (The Mathworks, Inc). While performing the visual scoring of the wake-sleep states, investigators identified the 4-s epochs, in which artifacts do not allow for an accurate automatic determination of the beat-to-beat values of HR. These artifacts in the BP signal represented the following percentage of the total recording time: 5.9 ± 2.4 % at 20°C and 9.6 ± 2.3 at 30°C. Cardiovascular analysis was performed on 2635 hours of artifact-free recordings with Ta set at 20°C and 2533 hours of artifact-free recordings with Ta set at 30°C.

Cardiovascular analysis was performed on 2635 hours of artifact-free recordings with Ta set at 20°C and 2533 hours of artifact-free recordings with Ta set at 30°C.

Beat-to-beat values of mean BP and heart rate were computed from the raw BP signal (sampling frequency 1024 Hz) in each artifact-free 4-s epoch. Beat-to-beat values of mean BP (MBP) were computed by averaging all digitized raw BP values in each cardiac cycle. HR was computed in each 4-s epoch as the reciprocal of the average

time between the onset of successive systolic upstrokes. MBP and HR differences between exposure to 20°C and 30°C Ta ($\Delta MBP_{20°C-30°C}$ and $\Delta HR_{20°C-30°C}$) were then calculated within each behavioral state. Finally MBP differences between wakefulness and NREMS ($\Delta MBP_{W-NREMS}$) and between wakefulness and REMS ($\Delta MBP_{W-REMS}$) and between sleep states ($\Delta MBP_{REMS-NREMS}$) were calculated for each Ta.

To investigate the contributions of the baroreflex and central autonomic commands to cardiac control, we analyzed the cross-correlation function (CCF) between heart period (HP, i.e. the reciprocal of HR) and systolic blood pressure (SBP) values. The CCF yields the correlation coefficient between HP and SBP as a function of the time-shift between these variables.

A positive correlation between BP and HP fluctuations with negative time shifts reflects a prevalence of baroreflex control of the heart whereas a negative correlation between these variables with positive time shifts indicates the prevalence of central autonomic commands (Silvani 2010). The CCF was computed for each mouse at each Ta.

## 5.5 Statistical analysis

Statistical analyses were performed using SPSS software (SPSS Inc., Chicago, IL, USA) with $P<0.05$ considered to be statistically significant. Data were analyzed by analysis of variance (ANOVA) and t-tests. Analyses involving within-subject factors were performed using the GLM procedure and the mixed-model design.

Wake-sleep cycle data were analyzed using 2-way ANOVA with mouse strain (with 2 levels: hTG and hWT) and Ta (with 2 levels: 20°C and 30°C) as factors. BP and HR data were analyzed with a 3-way ANOVA with mouse strain, wake-sleep state and Ta as factors. The wake-sleep state showed three levels: wakefulness, NREMS and REMS. In the case of significant interaction effects, the simple effect of the

mouse strain was tested using t-tests with $P<0.05$ considered to be statistically significant.

## 5.6 Measurement of brain HCRT-1

In order to confirm complete genetic ablation of HCRT neurons in hTG mice, brain HCRT-1 was measured at the end of the experiments in hTG (n=4) and hWT (n=5) mice included in the cardiovascular analysis.

The whole brain was removed immediately after decapitation under deep isoflurane/$N_2O$ anesthesia, and frozen at -80°C for subsequent processing. The brains were grossly dissected in 8 blocks, boiled for 10 minutes in 2.5 ml acetic acid 0.5 mol/l, and flash-cooled on ice. One aliquot (50 µl) of the supernatant was taken for measurement of total protein concentration in duplicate with the Lowry technique (DC protein assay, Bio-Rad Italy, Segrate, Italy, with bovine serum albumin as standard). Two 50 µl aliquots were dried overnight in siliconized (Sigmacote, Sigma-Aldrich Italy, Milano, Italy) cryotubes under a fume cupboard hood for HCRT-1 measurement. HCRT-1 measurement was performed in triplicate with a fluorescent immunoassay kit (FEK 003-30, Phoenix Pharm. Inc., Burlingame, CA, USA; range 0-10,000 pg HCRT1/ml) after reconstituting the dried peptide solutions in 125 µl assay buffer, following the manufacturer's instructions. All samples were processed simultaneously on the same immunoassay plate with a manufacturer-determined detection threshold of 20.3 pg HCRT-1/ml.

## 5.7 Quantitative Real Time PCR

An ancillary experiment was performed on 3 young mice (2 hTG and 1 hWT, aged 8 weeks) to test the expression of prepro-HCRT, Ataxin-3 and GAPDH genes quantifying their brain mRNA levels. The golden standard technique for the evaluation of RNA expression is the conversion of RNA into complementary DNA

(cDNA) followed by a Quantitative Real Time PCR (qRT-PCR). Briefly this procedure is based on the extraction of RNA from tissues of interest, its conversion into cDNA by reverse transcription and then quantification of genes of interest evaluating their amplification in a qRT-PCR with a DNA-intercalating fluorescent dye. For this experiment, genes of interests (i.e. prepro-HCRT and Ataxin-3) were normalized to the expression of GAPDH (housekeeping gene). Results were analyzed with a free trial version of qBase Plus and presented as relative expression to GAPDH ± SEM.

# *6. Results*

# 6.1 Validation of mouse model of narcolepsy

In order to assess the lack of HCRT signaling in the mice used in the experiments, we used three complementary approaches. Firstly, we verified the presence of the transgene using a polymerase chain reaction (Figure 3), (ref. *Appendix* for more detailed informations). The second approach was based on proteins measurement: we dosed brain HCRT-1 with a fluorescent immunoassay kit. In all hTG mice HCRT-1 brain levels were below the detection threshold, whereas they were over this threshold in hWT. In hTG, brain HCRT-1 concentration was 4.0 ± 0.2 fmol/mg total protein, whereas it was 51.0 ± 0.6 fmol/mg total protein in hWT mice (Figure 4). The third approach was based on the quantification of brain mRNA levels of prepro-HCRT and Ataxin-3, using a qRT-PCR technique. Data shown in Figure 5 confirmed that the hTG mice had drastically reduced prepro-HCRT expression levels and, moreover, they produced Ataxin-3, whereas hWT mice showed a higher expression of prepro-HCRT and a complete absence of Ataxin-3 expression.

# 6.2 Wake-sleep behavior at different Ta

The wake-sleep cycle structure changed as a function of Ta with no statistical difference between strains (Table 1), except for REMS latency. On passing from 20°C to 30°C the amount of time spent in wakefulness significantly decreased while the percentage of time spent in REMS significantly increased. Time spent in NREMS also tended to increase at 30°C ($P = 0.089$) (Table 1).

The density of W (i.e. number of episodes occurring in 24h) was higher in hTG mice both at 20°C and 30°C. The density of NREMS episodes was higher in hWT at 30°C. No statistically significant difference was found regarding the density of REMS episodes at either Ta (Table 2).

The duration of W episodes (sec.) was lower in hTG mice at both ambient temperature, in accordance with the difficulty of consolidating W that is presented in narcoleptic patients. NREMS episodes lasted longer at 30°C than at 20°C . The

duration of REMS episodes was lower at 20°C with no difference between mouse strains (Table 2).

Locomotor activity was not affected by Ta but depended on the mouse strain and it was higher in hWT mice at both 20°C and 30°C (data not shown).

To summarize, independently of Ta, hTG mice conserved their narcoleptic phenotype, showing a fragmentation of waking behavior (i.e. lower bout duration and higher number of episodes) and a significant reduction in REMS latency compared to hWT mice. Nevertheless the reduced REMS latency observed in hTG mice was significantly lower at 20°C.

The narcoleptic phenotype was also confirmed in hTG mice by the occurrence of SOREM periods (SOREMPs). hTG mice showed 4.9 ± 1.9 SOREMPs during total recording time with Ta set at 20°C, and 5.8 ± 3.8 with Ta set at 30°C. This difference was not statistically significant. No SOREMPs were found in hWT mice.

## 6.3 Effect of Ta on MBP and HR

Figure 6 and 7 illustrate the effect of Ta on cardiovascular variables during each behavioral state for both hTG and hWT mice. Measurements were performed at 20°C, 25°C, and 30°C. To simplify, the results of the 3-way ANOVA are reported in Table 3.

The ANOVA highlighted significant effects of the wake-sleep state and Ta on MBP and HR values ($P < 0.001$) whereas it failed to show any statistical differences between mouse strains ($P > 0.1$). In both hTG and hWT mice MBP and HR were significantly higher at 20°C, decreasing when passing from 20°C to 25°C and decreasing even further when the temperature rose to 30°C in all wake-sleep states (Figures 6 and 7).

The ANOVA also showed evidence of a significant interaction effect between wake-sleep state and Ta on cardiovascular variables: both the difference in MBP ($\Delta MBP_{20°C-30°C}$) and in HR ($\Delta HR_{20°C-30°C}$) between 20°C and 30°C exposure,

significantly changed as a function of wake-sleep state (Figure 8) and were highest in NREMS. This effect of Ta on MBP was significantly lower in REMS than in either NREMS or in wakefulness regardless of the mouse strain.

Finally, the ANOVA showed an interaction effect between wake-sleep state and mouse strain on MBP and HR values. The data shown in Figure 9 confirms that the physiological sleep-related decrease of MBP was blunted in hTG mice independently of Ta variations: $\Delta MBP_{W\text{-}NREMS}$ and $\Delta MBP_{W\text{-}REMS}$ were significantly smaller in hTG than in hWT, whether at 20°C or 30°C (Fig 9). At 30°C MBP differences between sleep states were enhanced.

Table 4 shows that $\Delta MBP_{REMS\text{-}NREMS}$ was much higher when all mice were kept at 30°C than when they were kept at 20°C. Interestingly, the magnitude of the $\Delta MBP_{REMS\text{-}NREMS}$ increase was exactly the same for hTG and hWT mice. The same pattern was the same for HR values (data not shown).

To summarize, MBP was higher in wakefulness than in either NREMS or REMS. This effect of sleep on blood pressure was significantly reduced in mice lacking HCRT neurons at each Ta, both in the dark and light periods, and particularly during REMS, confirming recently published data (Bastianini 2011a).

## 6.4 Cross correlation function (CCF) analysis

The average CCF profiles for both hTG and hWT mice kept at 20°C and 30°C are shown in figure 10. The maximum and minimum values of the CCF during each behavioral state at 20°C (red and blue lines) and 30°C (green and black lines) were similar between hTG and hWT. On the other hand, Ta showed an important effect on the CCF profiles of both strains in each behavioral state. This was particularly evident during REMS (Figure 10, bottom panel). Heart rhythm was mainly modulated by baroreflex control (cf. a prominent positive peak at negative time shift)

at 20°C, whereas central autonomic commands prevailed (cf. a deep throat at positive time shift) at 30°C.

To be more specific, during wakefulness at 20°C baroreflex and central commands equally contributed to heart rate control in both hTG and hWT mice. At 30°C central command contribution increased significantly in both strains.

During NREMS, baroreflex prevailed on HR control regardless of Ta. At 30°C central command contribution increased in both strains.

During REMS, in both strains, baroreflex control of HR prevailed at 20°C whereas central command control prevailed at 30°C.

Considering that no statistically significant effect of mouse strain was found, the balance between baroreflex and cental autonomic control of the heart is not dependent on HCRT neuron activity but changes as a function of wake-sleep states and ambient temperature (Silvani 2008).

# *7. Discussion*

# 7.1 Genetic ablation of HCRT neurons blunts the effects of sleep but not those of Ta on blood pressure in mice

The main finding of this research support the hypothesis that HCRT neurons are part of central neural pathways that mediate the phenomenon of BP dipping on passing from wakefulness to sleep, whereas they do not play a critical role in mediating the cardiovascular responses to different Ta.

Ta exerts a prominent influence on sleep. In rats and humans, low ambient temperatures generally impair sleep, whereas higher temperatures tend to promote sleep (Jhaveri 2007). Thermoregulation changes according to behavioral states. The thermoregulatory responses to ambient thermal loads are preserved during NREMS and are absent or depressed during REMS (Parmeggiani 2005). Furthermore, Ta and wake-sleep behaviour may together affect cardiovascular parameters (Sei 1994). Cardiovascular parameters such as BP and HR, strictly depend on Ta, especially in small animals like rodents in which the great surface area-to-volume ratio makes them more sensitive to Ta changes (Swoap 2004). Moreover, research performed in the PRISM lab has recently demonstrated that wake-sleep behavior deeply affects BP and HR values in different genetically-modified mouse strains (Silvani 2009; Bastianini 2011a).

Consistent data indicate a strong anatomical and functional integration between hypothalamic regulatory mechanisms of energy homeostasis, autonomic cardiovascular control, thermoregulation and wake-sleep states. HCRT is a good candidate for playing a pivotal role in the coordination of these entangled mechanisms.

We performed experiments on a group of narcoleptic mice with selective loss of HCRT neurons (hTG) and their wild-type littermates (hWT). In order to investigate the role of HCRT neurons in cardiovascular responses to different Ta, we

characterized the cardiovascular phenotype of the mice during the wake-sleep states at warm and cold Ta.

As expected, hTG mice showed a narcoleptic phenotype, with a fragmentation of waking behavior (i.e. lower bout duration and higher number of episodes) and a significant reduction or REMS latency compared to hWT mice.

Moreover, our results confirmed the influence of Ta on the wake-sleep cycle macrostructure; we found an increase in sleep for both hTG and hWT when thet were exposed to warm Ta. Numerous studies have suggested the involvement of HCRT neurons in the regulation of body temperature. Cardiovascular changes participate in mechanisms that mediate thermoregulation (Morrison 2011). Thus, we tested the hypothesis that the chronic lack of HCRT signaling may lead to abnormal cardiovascular responses to Ta changes.

In both humans and animals, the main thermoregulatory effectors include cutaneous blood vessels for control of heat loss, brown adipose tissue, skeletal muscle and increased HR for thermogenesis (Morrison 2011). The presence of cardiovascular adaptations to thermal loads is well known due to studies on small rodents. Exposure of rats and mice to cold Ta produces cutaneous vasoconstriction (ears and tail) and non-shivering thermogenesis in brown adipose tissue, which is activated by the sympathetic nervous system through the release of catecholamines (Swoap 2004). The activation of the sympathetic nervous system in response to a cold environment also has direct effects on the heart, increasing cardiac thermogenesis (Morrison 2011). As a consequence of this general sympathetic activation, exposure to cold Ta determines increases in BP and HR values possibly leading to hemodynamic derangements and increased myocardial risk, as also demonstrated on human subjects (Danet 1999).

The link between HCRT and thermoregulation has been deeply investigated. ICV injection of HCRT-1 evoked hyperthermia (Monda 2003). Neurons in the lateral hypothalamic area, where HCRT neurons are localized may influence brown adipose tissue sympathetic nerve activity, thermogenesis and HR through the activation of

neurons in the dorsomedial hypothalamus and raphe pallidus (Cerri 2005). This last result has been recently confirmed by Tupone et al., who have suggested a potent modulation of brown adipose tissue thermogenesis by HCRT released from the terminals of HCRT neurons into the raphe pallidus (Tupone 2011). The involvement of HCRT neurons in thermoregulation is also confirmed by the response of muscimol (GABA-A receptor agonist) injection in the preoptic hypothalamic area. The muscimol perfusion in POA induces c-Fos expression in HCRT neurons suggesting that subpopulations of the preoptic neurons give an inhibitory tone to the activities of the HCRT neurons in the perifornical/lateral hypothalamic areas and that removing this tone contributes to an increase in body temperature, HR and BP (Satoh 2004).
Consistently with these results, a chronic lack of HCRT would be expected to disrupt those responses required for thermoregulation. Results obtained in animal models of narcolepsy are contrasting, showing an increase in core body temperature during sleep in HCRT knockout mice (Mochizuki 2006) or a diurnal rhythm of core body temperature in HCRT-ataxin-3 transgenic mice similar to that recorded in their WT littermates (Zhang 2007).
Studies in narcoleptic patients reported no defects in thermoregulation suggesting a marginal role of HCRT in this regulation (Grimaldi 2010a). On the other hand, some studies found instead that thermoregulatory cardiovascular control is impaired in narcoleptic patients (Fronczek 2006); it has been suggested that the related core body and skin temperature alterations might causally affect vigilance and sleepiness in these patients (Fronczek 2008a).

The rationale of the present study was that HCRT deficiency in HCRT-ataxin-3 transgenic mice would suppress the general sympathetic activation induced by HCRT, leading to impairments in thermoregulatory responses to thermal loads and in particular in cardiovascular responses to Ta changes. Nevertheless, no significant difference in the cardiovascular responses to the different Ta was found, suggesting that HCRT neurons are not necessary for cardiovascular adaptations to different Ta.

Additional experiments, aimed at measuring core body temperature, could reveal other disturbances in thermoregulatory function as a consequence of HCRT deficiency.

hTG mice and hWT mice showed the same cardiovascular responses to Ta changes. Both MBP and HR increased in a cold environment and decreased gradually on passing from 20°C to a warm environment (30°C).

Thermoregulatory and cardiovascular responses are strictly dependent on wake-sleep states. Changes in blood flow during NREMS reflect thermoregulatory vasomotor adjustment and depend on Ta. In contrast, REMS appears to be a state in which changes in regional blood flow indicate a disruption in thermal homeostasis. This assumption is confirmed by the obtained results: cardiovascular adaptation to Ta changes ($\Delta MBP_{20°C\text{-}30°C}$ and $\Delta HR_{20°C\text{-}30°C}$) is highest during NREMS in both mouse strains. Accordingly, NREMS is characterized by relative autonomic stability, with vagal nerve dominance and reduced sympathetic nerve activity (Somers 1993), and shows an enhanced homeostatic control compared to wakefulness. On the other hand, both $\Delta MBP_{20°C\text{-}30°C}$ and $\Delta HR_{20°C\text{-}30°C}$ significantly decreased on passing from NREMS to REMS, in agreement with the depressed thermoregulation associated with the latter behavioral state (Parmeggiani 2005). Sei and Morita (Sei 1996) demonstrated that in rats BP difference on passing from NREMS to REMS decreases at low Ta. Our data confirmed these observations in mice. This response was the same in hTG mice and in hWT mice, thus sustaining the hypothesis that HCRT neurons are not implicated in this phenomenon.

HCRT neurons participate in the maintenance of energy homeostasis (Funato 2009) on different time scales. In the long term, in the absence of HCRT, mismatches between metabolic rate and caloric intake may lead to the increased body weight that often characterizes narcoleptic patients. On the other hand, the short-term balance between production and energy dissipation is a function of temperature regulation, and, as a consequence any imbalance should imply a change in body temperature.

Narcolepsy is often associated with an increase in body weight/body mass index (BMI); weight gain usually seems to be independent of caloric intake, which is paradoxically lower in narcolepsy patients (Lammers 1996). On the basis of an increased heart rate variability, a reduced sympathetic tone has been proposed in narcoleptic patients (Fronczek 2008b ). The autonomic system innervates white and brown (BAT) adipose tissue, inducing catabolic processes through sympathetic inputs, and anabolic processes, through parasympathetic inputs, Thus, a sympathetic tone reduction could explain the increased fat accumulation observed in narcolepsy. The mouse strain that we used in these experiments is particularly suitable for evaluating the presence of an imbalance in the short-term energy homeostasis maintenance. In fact, HCRT-ataxin-3 transgenic mice with a mixed C57BL/6J plus DBA/2J (3:1) background display a marked late-onset obesity compared to their controls (Hara 2005), despite eating less; they are also more sensitive to the development of metabolic alterations than narcoleptic mice with a pure genetic background. This predisposition to the development of obesity is not yet fully expressed in young adult subjects, and accordingly, the weight of the studied animals did not differ significantly between mouse strains, hTG mice weighing 29.2 ± 0.7 g and in hWT mice weighing 28.6 ± 0.8 g.

Finally, using the CCF analysis between spontaneous fluctuation of systolic BP and heart period, the relative contribution of baroreflex and central autonomic commands on heart control was assessed. The interplay between these two control mechanisms was deeply affected by the wake-sleep cycle in both mouse strains, confirming previous observations (Silvani 2010). In mice, as in rats and humans, the contribution of central autonomic command is prevalent during REMS, particularly at 30°C. No evident difference was found between hTG and hWT mice either in the maximum or minimum values of CCFs. In REMS, during the exposition to 20°C, the related contribution of central autonomic commands to cardiac control is less evident in both mouse strains and this is probably due to the elevated basal values of HR, induced by lower

# 7.2 Conclusion

In conclusion, this study demonstrated that in mice cardiovascular adaptations to Ta changes strongly depend on the wake-sleep states whereas HCRT neurons do not seem to be involved in this process.

These results confirm the recent finding that HCRT-deficient narcoleptic mice have blunted BP differences between wakefulness and sleep states (Bastianini 2011a). Moreover, BP derangements induced by HCRT deficiency seem to be highly reproducible regardless of Ta changes.

A marked hypertension in HCRT-deficient mice has never been found before. Rather, a marked hypotension was reported both in HCRT knockout mice and HCRT-ataxin-3 transgenic mice compared to control mice (Kayaba 2003; Zhang 2006). These discrepancies could be attributed to methodological differences but also to the fact that in previous studies sleep-dependent cardiovascular modifications were disregarded. Moreover, a marked hypotension has never been reported in narcoleptic patients, who suffer from a selective loss of HCRT neurons. Rather, narcoleptic patients were hypertensive compared to patients with idiopathic hypersomnia (Baker 1986) or there was no difference in BP measurements throughout the night compared to normal control subjects (Guilleminault 1986).

To conclude, the genetic ablation of HCRT neurons blunts only the effect of sleep but not those of Ta on BP in mice, leading to a neurogenic hypertension during sleep and in particular during REMS. The possible explanation of this result is that, although HCRT neurons almost stop firing in NREMS and REMS, brain interstitial HCRT remains substantial during sleep in the interstitial fluid (i.e. volume transmission). Thus when synaptic activity of HCRT neurons is almost nil, as in NREMS and REMS, the residual volume transmission exerted by interstitial HCRT may entail a vasodepressive tone, in the absence of which BP increases. The second hypothesis is that the lack of HCRT signaling entails compensatory mechanisms during W that also

continue to act during sleep, thus compensating for the loss of HCRT effects during wakefulness.

HCRT neurons seem to be involved in a neural pathway that mediates the phenomenon of blood pressure dipping on passing from wakefulness to sleep.

Several studies have revealed an association between non-dipping BP profile and increased cardiovascular risk (Ohkubo 2002). This concept may have clinical implications in narcolepsy patients with cataplexy, who lack HCRT neurons.

# *8. Perspectives*

There is evidence that hypocretinergic neurotransmission interacts with many other hypothalamic regulatory systems to mediate different physiological functions.

In particular, the relationship between hypocretinergic function and histaminergic function seems to be involved in the regulation of the wake-sleep cycle and cardiovascular regulation.

HCRT neurons project downstream to the tuberomamillary nucleus (TMN) in the posterior hypothalamus, where histaminergic neurons are localized. The histaminergic system is exclusively localized within the posterior hypothalamus with projection to almost all the major regions of the central nervous system. Strong and consistent evidence exists to suggest that histamine (HA), acting via H(1) and/or H(3) receptors has a pivotal role in the regulation of sleep-wake cycle. Administration of histamine or H(1) receptor agonists induces wakefulness, whereas administration of H(1) receptor antagonists promotes sleep. The activation of H(3) receptor reduces histamine release and promotes sleep. Conversely, the blockade of H(3) receptor promotes wakefulness. The histaminergic neurons display maximal activity during the state of high vigilance, and cease their activity during NREMS and REMS. The cerebrospinal levels of histamine are reduced in diseased states where hypersomnolence is a major symptom. HA-deficient l-histidine decarboxylase knockout (HDC KO) mice display sleep fragmentation and increased REM sleep during the light period along with profound wakefulness deficit at dark onset, and in a novel environment. These studies strongly implicate the critical role of histaminergic neurons of the TMN in the maintenance of high vigilance state during wakefulness (Thakkar 2011).

Anaclet et al. studied the behavioral and sleep-wake phenotypes of histidine-decarboxylase (HDC, HA-synthesizing enzyme) (-/-) mice. HDC-KO mice showed sleep fragmentation and increased REMS, similarly to HCRT-KO narcoleptic mice. On the contrary, only HCRT-KO mice, but not HDC-KO mice, displayed a complete narcolepsy phenotype and deficient W when faced with motor challenges. In particular, when placed on a wheel, wild-type (WT), but not littermate HCRT-KO

mice, voluntarily spent their time turning it and as a result, remained highly awake; this was accompanied by dense c-fos (marker of neuronal activity) expression in many areas of their brain, including HCRT neurons in the dorsolateral hypothalamus. This was evident from the fact that intraventricular infusion of HCRT-1 restored their W amount and motor performance (Anaclet 2009). These data indicate that HCRT, but not HA, promotes W through enhanced locomotion and suggest that HA and HCRT neurons exert a distinct, but complementary and synergistic control of W; the neuropeptide is more involved in its behavioral aspects, whereas the amine is mainly responsible for its qualitative cognitive aspects and cortical EEG activation. Moreover, the activation of central nervous histamine receptors is involved in cardiovascular regulation (Suzuki 2005), with different effects on blood pressure and heart rate, mediated by different receptor mechanisms.

It would be interesting to compare the sleep-dependent cardiovascular phenotype of HCRT knockout mice, HDC knockout mice and double HCRT/HDC knockout mice in order to establish the effect of the interaction between hypocretin and histamine on cardiovascular regulation during sleep.

# *9. Appendix*

# 9.1 Mouse colony management

Mouse colonies were maintained in the facilities of the Department of Human and General Physiology at the University of Bologna, Italy.

Mice were kept on a light-dark cycle of 12-hour periods with ambient temperature set at 25°C and free access to water and food (4RF21 diet, Mucedola, Settimo Milanese, Italy).

Founder mice were generously provided by Prof. E. Mignot (Stanford University, USA) and consisted of the following individuals: 1 male and 2 female mice which were hemizygous for the HCRT-ataxin3 transgene and fully congenic (12 generations of backcrossing) to C57Bl/6J. The colony was expanded in order to perform the experiments recently published (Bastianini 2011a; Bastianini 2011b). Founder TG mice were first backcrossed once to C57Bl/6J female mice, purchased from Charles River, (Calco, Italy) and then maintained by hemizygote X wild-type mating. Animals were studied in an inbred background. Inbreeding is the process of breeding closely-related individuals for sequential generations and results in alleles throughout the genome fixed in homozygosity. A *fully inbred* strain is one that has undergone enough generations of inbreeding that it is mathematically predicted to have 100% homozygous genome. Thus, all members of an inbred strain are considered essentially genetically identical (Berry 2007).

Two possible mating schemes were used to expand the colony: the sibling system, in which 1 male was mated with a female, and harem mating or trio, in which multiple female were mated with a single male. This system increases male reproductive performances.

TG mice were always crossed with wild-type mice in order to avoid the birth of homozygous TG mice. All the mice were fully congenic to C57Bl/6J, with a genomic homology of 99,9%, because they were within the $10^{th}$ generation of the sibling system and no substrain was created. Colonies separated by more than 20 generations

from the progenitor strain are considered to be genetically distinct (Berry 2007). The average litter size for the TG colony was 5.0 ± 0.4.

In order to obtain hTG, male mice, hemizygous for the HCRT-ataxin3 transgene and congenic to C57Bl/6J (13 generations of backcrossing) were crossed with female (C57Bl/6J × DBA/2J)F1 hybrid mice (strain B6D2F1/J). C57Bl/6J mice and B6D2F1/J mice were purchased from Charles River, (Calco, Italy). Hybrid female mice had a mixed genetic background, carrying 50% of the DNA of C57Bl/6J and 50% of DBA/2J strain. Thus, F2 mice had 75% of DNA coming from C57Bl/6J strain and 25% DNA of DBA/2J strain. Hybrid mice resulted as more resistant to diseases and this characteristic is known as hybrid vigor. This is why the average litter size for the hTG colony was 7.4 ± 0.5.

After a female got pregnant, male mice were removed from the breeding cage. Pregnant females were left alone or with other pregnant females in order to promote the cross-fostering of offspring and thus improve litter size.

Male mice were bred until they reached 50 weeks of age, females until 40 weeks of age. Usually, reproductive performance begins to decrease in mice older than 6 or 7 months (Berry 2007).

When newborn mice were 25 days old, they were separated from their mothers and males were separated from females to avoid further breeding. Then, they were anaesthetized, so that their left ears could be marked with a puncher and a small tail biopsy, to be used for genotyping, could be obtained. After the genotyping procedure, mice were again anaesthetized to mark their right ear, following the legend in Figure 11, to visually distinguish their genotype.

# 9.2 Genotyping procedure

Mouse genotype was assessed in the facilities of the Centre for Applied Biomedical Research - CRBA, S. Orsola University Hospital, Bologna, Italy. DNA was extracted from bioptic tissue, amplified by polymerase chain reaction and resolved by gel electrophoresis.

- ✓ DNA extraction from tail biopsies

Tail biopsies, with a length of about 0.5 cm, were put in eppendorf tubes and stored at -80°C. To extract DNA, a protease (proteinase K 10 mg/ml, Sigma Aldrich, Italia), was added to the tail biopsies, to digest all the native proteins. The material was incubated overnight in a thermomixer at 56°C in orbital shaking. In the morning, at the end of the digestion, DNA was precipitated using the salting-out technique and dissolved in TE buffer (Tris Hcl 1 M, EDTA 0.5M in water).

- ✓ Polymerase chain reaction

Subsequently, a range of DNA between 10-100ng was amplified with a polymerase chain reaction. The reaction solution was composed of: buffer 1X, deoxinucleotides (0.2 mM), MgCl (1.5 mM), Taq Polymerase (0.02 U/µl, Promega, Italy), and specific primers (0.5 µM, Sigma Aldrich, Italy).
Primer sequences used for hWT and hTG were:

5'-TCACCCCCTTGGGATAGCCCTTCC-3'(WT, allele 5')

5'-GACGACGGCCTCAGACTTCTTGGG-3'(WT, allele 3')

5'-GCAGCGGCCATTCCTTGG-3'(transgene, 5')

5'-AAGTCGACGGTGTCTGGCGCTCAGGGTG-3'(transgene, 3')

- ✓ Gel electophoresis

At the end of the PCR reaction, PCR products were loaded in an agarose gel (2%) and resolved by gel electrophoresis. DNA bands were visible because of the Etidium Bromide (EtBr) added to the gel during its preparation. EtBr is fluorescent under UV light when intercalated into the major grooves of DNA. By running DNA through an EtBr-treated gel and visualizing it with UV light, any band containing more than ~20ng DNA becomes distinctly visible. The products of amplification reaction of hTG mice samples were visualized as two bands: a small one, 250 base pairs (bp) long (transgene allele) and another, 400 bp long (wild-type allele), whereas hWT showed

only one 400 bp long band (wild-type allele), as results from Figure 3. The size of unknown DNA molecules was estimated through the comparison with a DNA ladder, (Sigma Aldrich), a solution of DNA molecules of different lengths that separate during gel electrophoresis.

## 9.3 qRT-PCR

A preliminary experiment was performed on 2 hTG mice and 1 hWT to verify the expression of three genes of interest, GAPDH, prepro-HCRT and Ataxin-3 by quantifying the mRNA in their brains. The brain of each animal was removed and immediately dissected to separate the diencephalon from the rest, then frozen at -80°C. Total m-RNA was extracted using a phenol-chloroform solution (Tri-Reagent, Applied Biosystem), following the manufacturer's protocol. The RNA obtained was dissolved in sterile water, in a previously determined volume, in order to obtain a concentration in the range between 500 and 1000ng/µl. The concentration was verified by testing UV absorption with nanodrop 2000. The extracted RNA was checked for its quality, taking into consideration both purity (absence of protein and DNA contamination, absence of inhibitors) and integrity (Nolan 2006). Nanodrop allowed for the identification of the contamination on the part of any solvent or protein, through the analysis of the A260/A280 ratio. Furthermore, to assess the quality of extracted RNA, 1µl of RNA was loaded in a 1% gel and after a gel electrophoresis run, three bands were visualized corresponding to the major quantity of RNA, the 3 subunits of the ribosomal RNA. The presence of other bands indicated that RNA was degraded. After the extraction, an aliquot of RNA was purified to eliminate genomic DNA by enzymatic digestion with DNAsi I (Sigma Aldrich, Italy). For each sample, 2 aliquots were purified, in order to use one for the retro-transcription and the other as a control. After verification of sample purity and quality, RNA was converted in c-DNA using a retro-transcriptase (High capacity RNA-to-c-DNA kit, Applied Biosystem). 1µg of RNA was retro-transcripted in 1 µg of DNA. One tube was retro-transcripted; in the other, all the reagents were added, except for the retro-transcriptase. In order to assess the retro-transcription, c-DNA

and control samples were amplified with a PCR reaction for a control gene (GAPDH). The retro-transcripted tube showed an amplification, which was absent in the control. After the retrotranscription, qRT-PCR was performed with the Sybr green method (Go Taq q-PCR, Promega). The Sybr green, a green dye, binds to double-stranded (ds) DNA, thus providing a fluorescent signal that reflects the amount of double strand DNA products generated during PCR. The direct fluorescence was revealed by a special thermocycler Iq5, Biorad.

Primers must be chosen carefully in order to avoid non-specific amplification. When designing gene-specific real-time PCR primers, it is important to keep in mind the fact that amplicon length should be approximately 80-250 bp. In general, primers should be 18-24 nucleotides in length and should have comparable melting temperatures (within 5°C). Moreover, primers should be specific for the target sequence and be free of internal secondary structure. If possible, it is preferable to design primers that anneal on both sides of an intron to avoid the amplification of genomic DNA (Bustin 2007). In this experiment, the ENSEMBL database (www.ensembl.org) was used to custom design primers.

To confirm the specificity of chosen primers (primers should recognize only the target of interest), the sequences were tested in silico for any unspecific alignment either in the human or mouse genome (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Primers were purchased from Sigma-Aldrich, Italy, as a powder, and then reconstituted in sterile water.

Primers chosen for the experiments were:

5’-CGTGCCGCCTGGAGAAACC-3’ (GAPDH, 5’)

5’-TGGAAGAGTGGGAGTTGCTGTTG-3’ (GAPDH, 3’)

5’-ACGCTGCTGCTGCTGCTAC-3’ (prepro-HCRT, 5’)

5’-CCGCCGCTTTCCCAGAGTC-3’ (prepro-HCRT, 3’)

5’-GGGACCTATCAGGACAGAGTTCAC-3’ (Ataxin-3, 5’)

5’-AGCATCACCTAGATCACTCCCAAG-3’ (Ataxin-3, 3’)

Amplification efficiencies of these primers were tested with calibration curves. The following efficiencies were obtained:

GAPDH efficiency = **92%**

Prepro-HCRT efficiency = **83%**

ATAXIN3 efficiency = **82%**

For every diencephalon the 3 genes were tested in triplicate. At the end of the qRT-PCR a further test was carried out on PCR products. For every tube, a melting curve was created in order to evaluate the presence of nonspecific products of amplification. A melting curve charts the change in fluorescence observed when double-stranded DNA (dsDNA) with incorporated dye molecules dissociates, or "melts" into single strand DNA (ssDNA) as the temperature of the reaction is raised. These curves are based on gradual increments (0.5°C) of temperature. If nonspecific products are present more than one melting value will be found in each well. Only one melting value for every tube was found in my samples and these values were reproducible among samples for every tested gene.

# *10. Figures and Tables*

# Figure 1

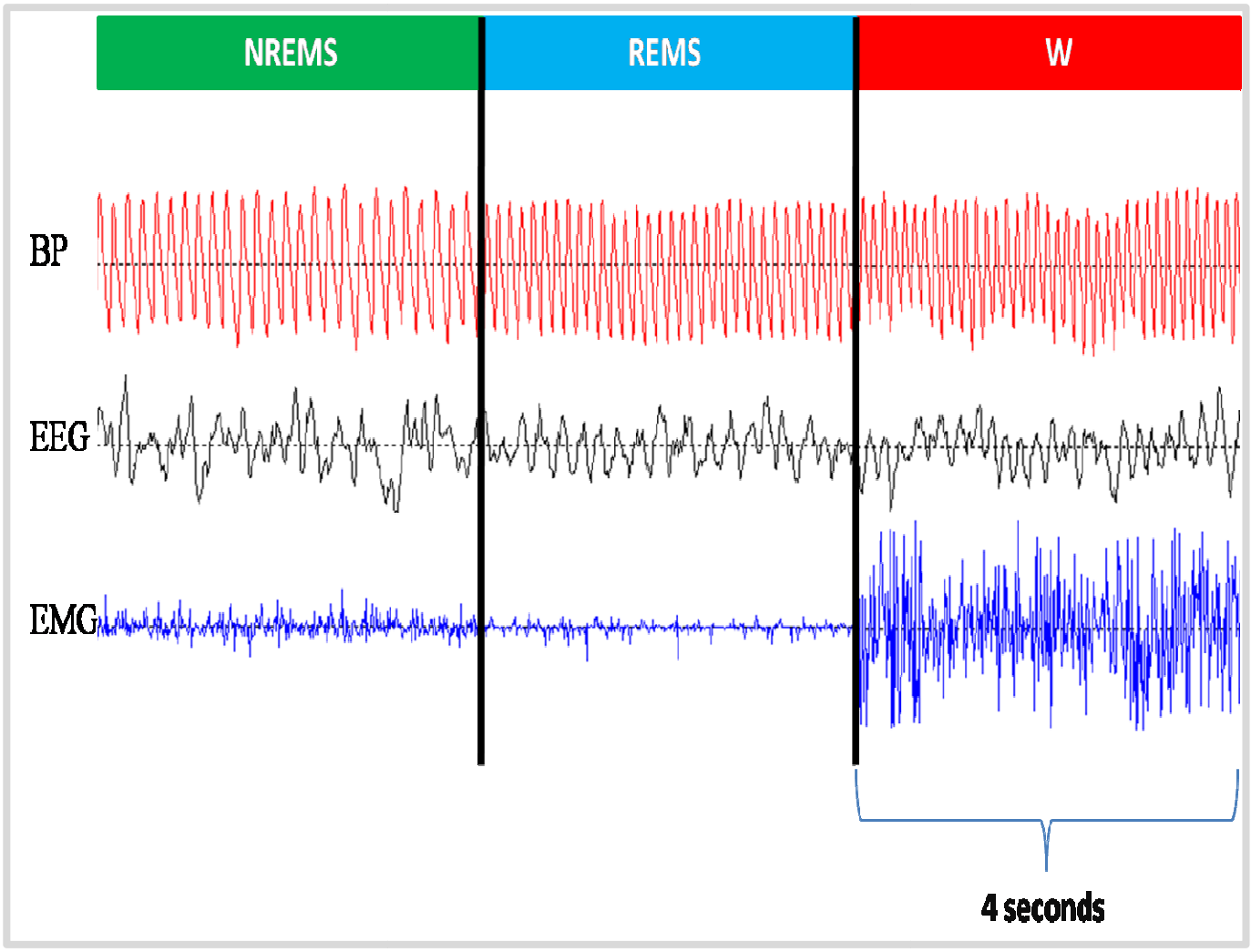

**Figure 1**. Example of scoring window. The automatic scoring procedure automatically classified the behavioral state (top of this window: W, wakefulness; NREMS, NREM sleep; REMS, REM sleep) for each 4-s epoch; this classification is then confirmed or denied by the investigator. BP, blood pressure.

# Figure 2

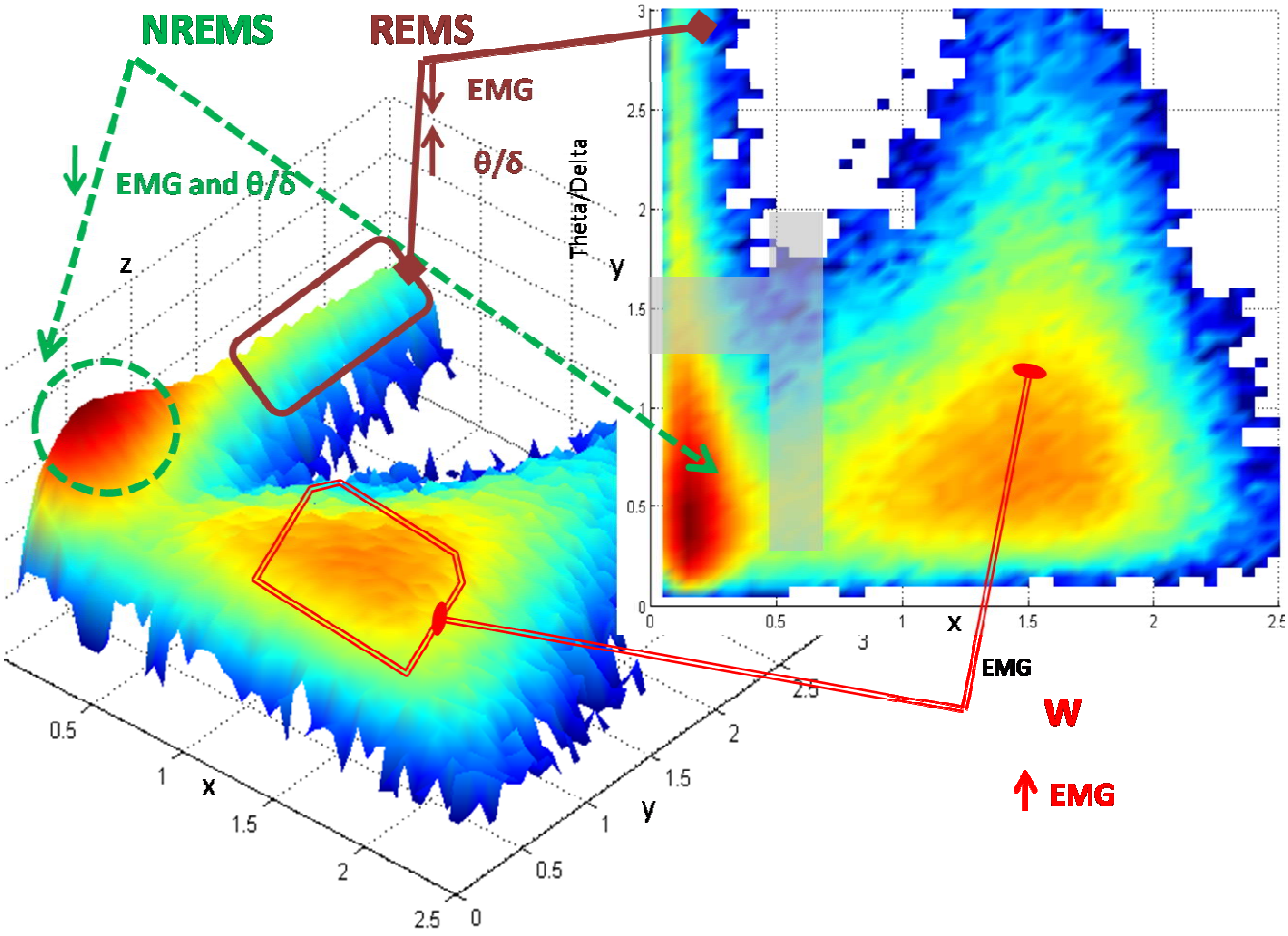

**Figure 2**. Three dimentional (on the left) and bidimendional (on the right) graphs showing all the 4s epochs recorded in a single mice. X-axis, reports the root mean square of EMG; Y-axis reports θ/δ EEG ratio; Z-axis reports the density of epochs. The color scale used on z-axis reflects the number of epochs for each couple of x-y coordinates, with blue color indicating a low number, and red color indicating a high number.

Wakefulness (W) is characterized by high EMG tone; NREM sleep (NREMS) is characterized by low EMG tone and low theta/delta (θ/δ) EEG ratio; REM sleep (REMS) is characterized by low EMG tone and high θ/δ EEG ratio.

# Figure 3

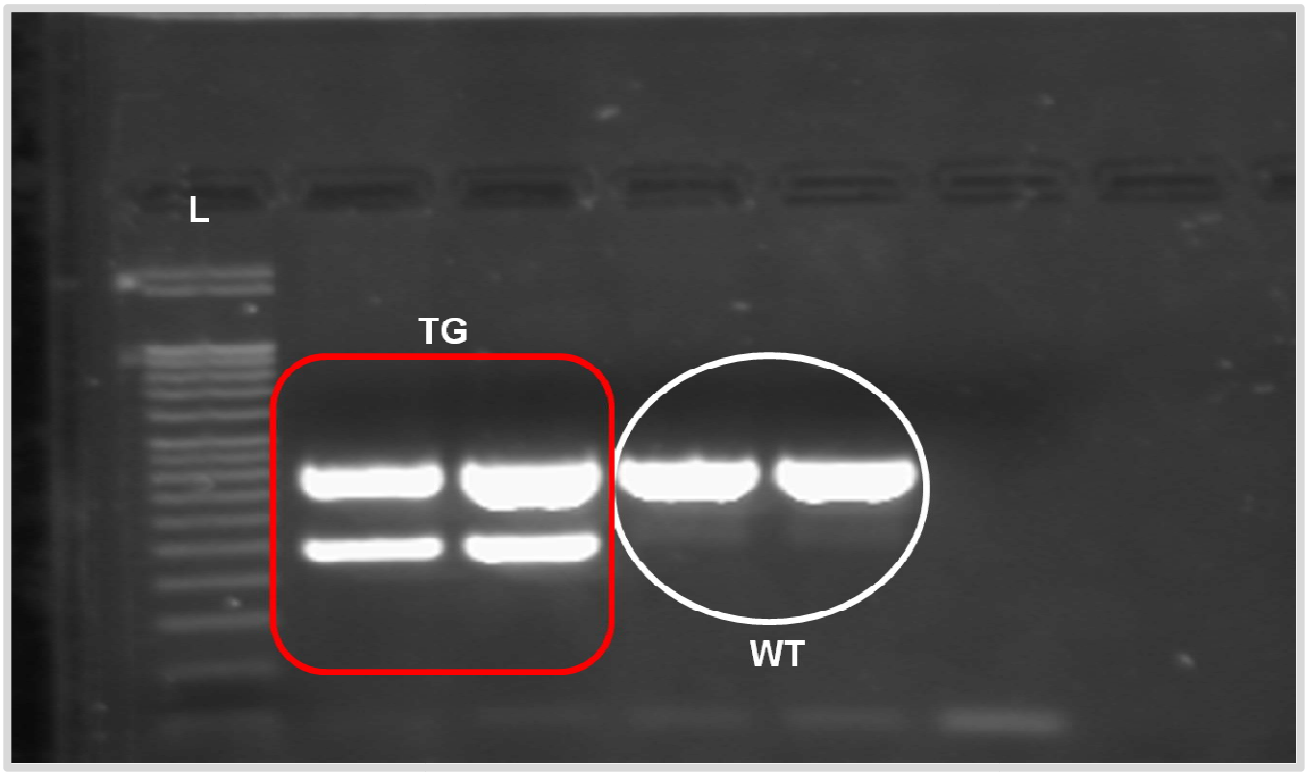

**Figure 3**. Example of visualization of the results of PCR (polymerase chain reaction) with gel electrophoresis. DNA ladder is loaded in the first well and after DNA migration, it is possible to visualize different bands (50 base pairs). Samples of two transgenic mice (hTG) are loaded in the second and third wells whereas samples of two wild-type control mice (hWT ) are loaded in the last two wells. The upper band is 250 base pairs long (transgene allele), whereas the bottom one is 400 base pairs long (wild-type allele). hWT mice does not display the band corresponding to the transgenic allele.

# Figure 4

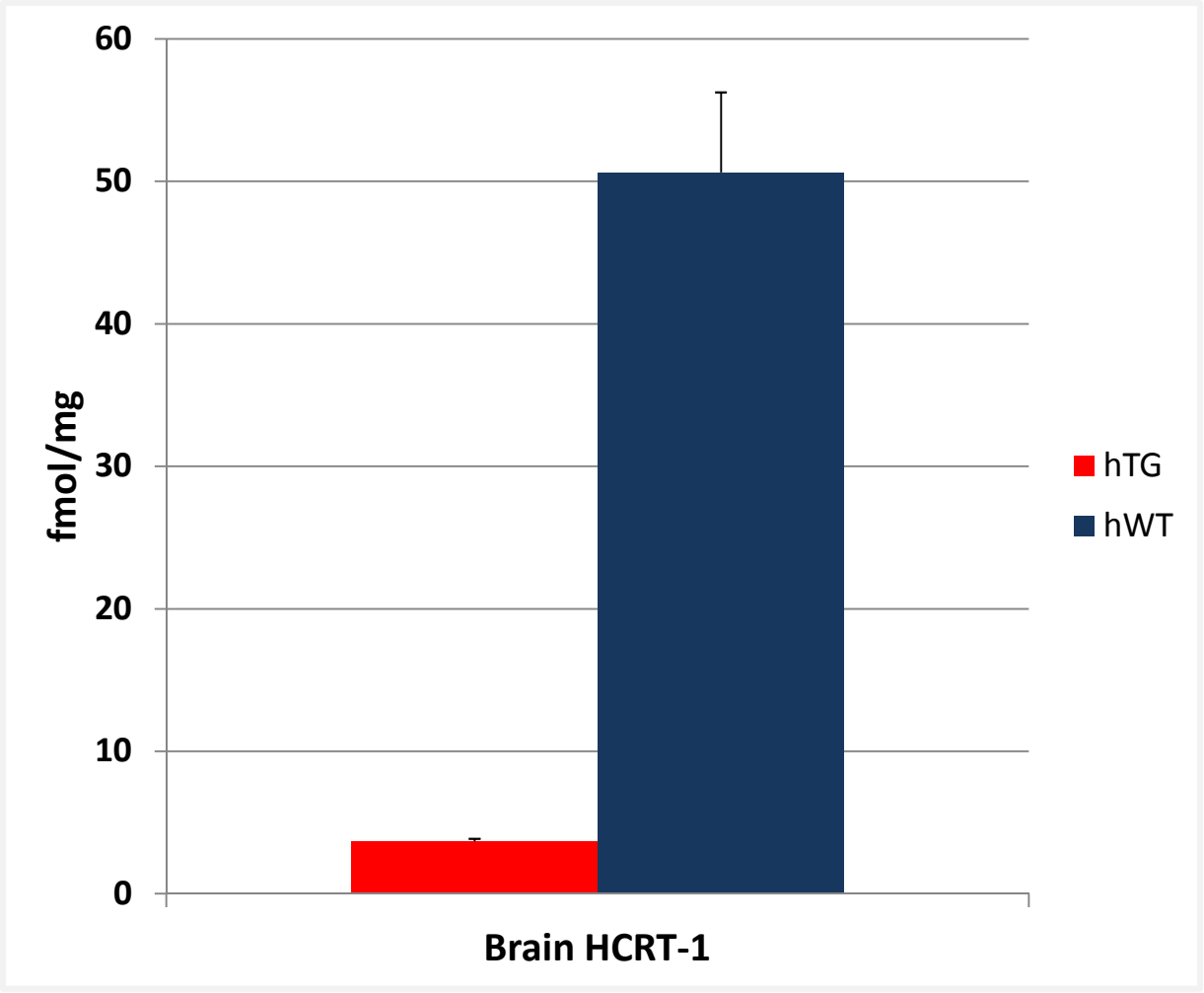

**Figure 4**. Cerebral HCRT-1 concentration obtained in 4 transgenic mice ((hTG, red bars) and 5 wild-type control mice (hWT, blue bars) determined with a fluorescent immunoassay kit (FEK 003-30, Phoenix Pharm. Inc., Burlingame, CA, USA). Data are reported in *fmol* over total brain proteins (mg). Error bars represent SEM.

# Figure 5

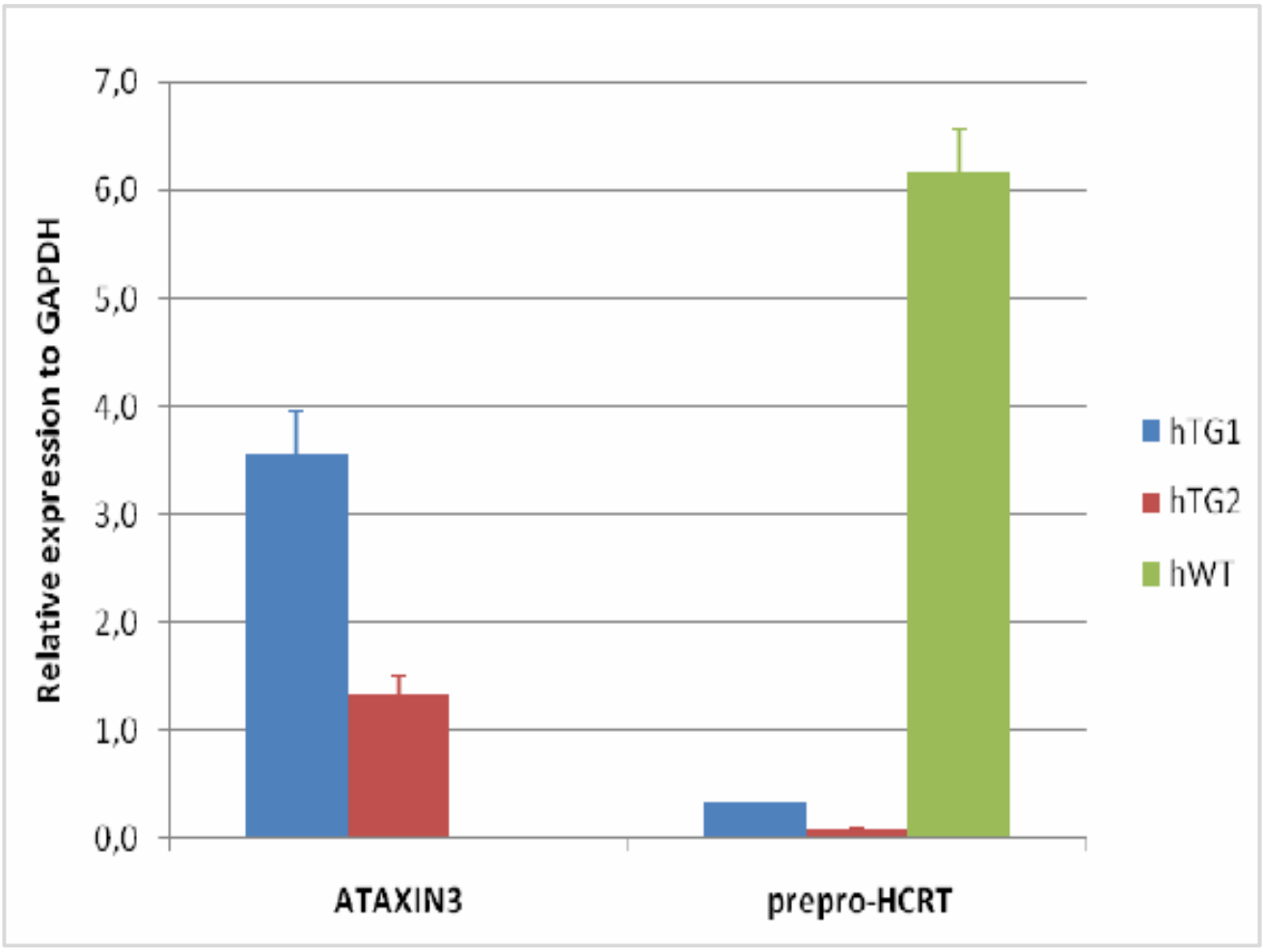
7,0
6,0
5,0
4,0
3,0
2,0
1,0
0,0
Relative expression to GAPDH
ATAXIN3
prepro-HCRT
hTG1
hTG2
hWT

**Figure 5**. Relative expression of genes of interest (Ataxin3 and prepro-HCRT) compared to an housekeeping gene (GAPDH) in 3 young hybrid mice (1 wild-type control mouse, hWT, and 2 transgenic mice, hTG). hWT mouse showed high expression of prepro-HCRT and complete absence of Ataxin3. Both hTG1 and hTG2 showed drastically reduction in prepro-HCRT expression and besides they produced Ataxin3.

# Figure 6

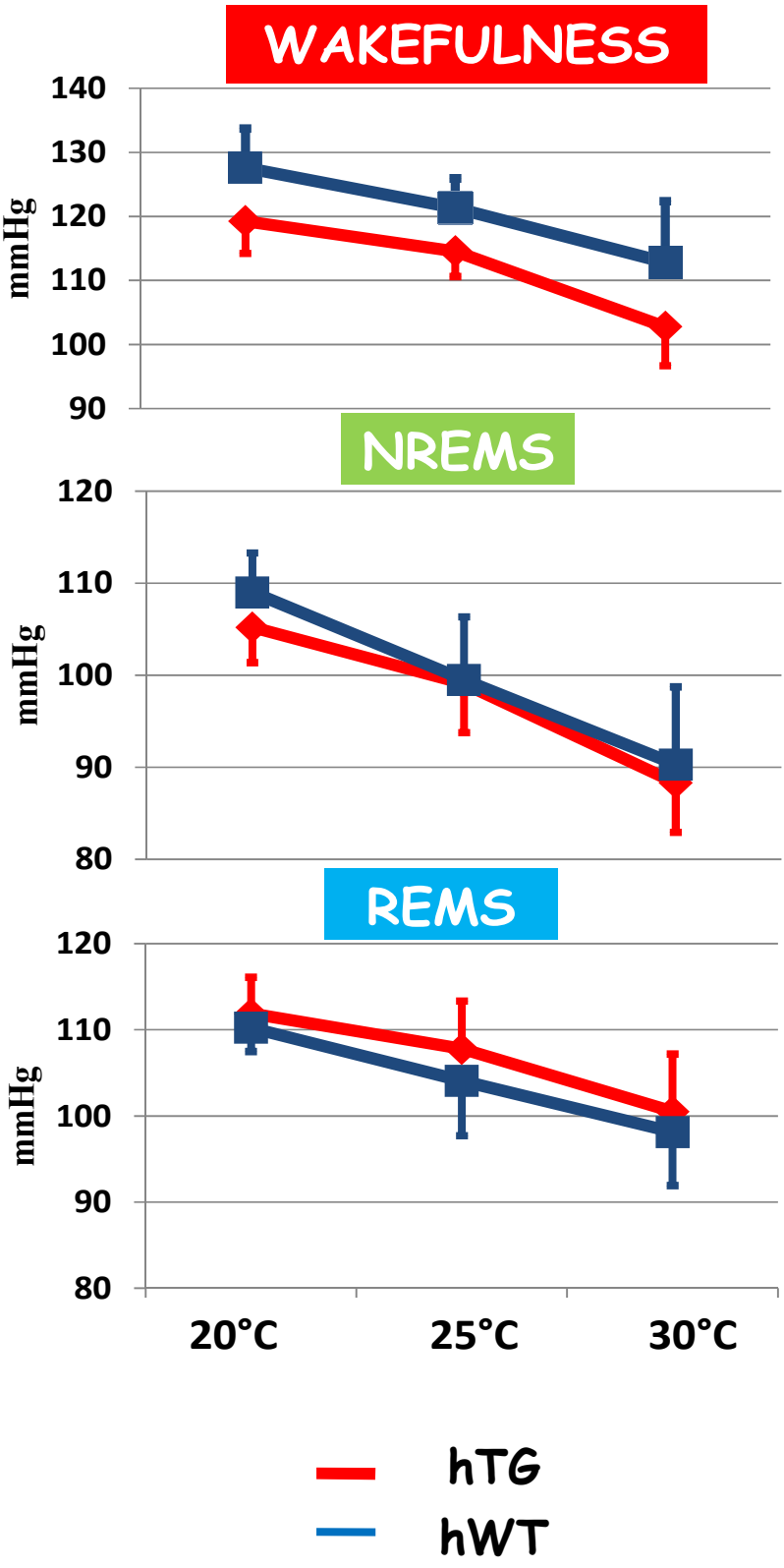
WAKEFULNESS
140
130
120
110
100
90
mmHg
NREMS
120
110
100
90
80
mmHg
REMS
120
110
100
90
80
mmHg
20°C
25°C
30°C
hTG
hWT

**Figure 6**. Mean blood pressure values in transgenic mice (hTG, n=11) and wild-type control mice (hWT, n=12) in wakefulness, non rapid-eye movement sleep (NREMS) and in rapid-eye-movement sleep (REMS) at different ambient temperature (20°C, 25°C, 30°C). Error bars represent SEM. ANOVA showed a significant interaction effect between wake-sleep state and Ta on MBP ($p<0,001$), and significant main effects of wake-sleep states and Ta on MBP ($p<0,001$).
Data recorded during at 25°C were obtained from 8 hTG and 9 hWT.

**Figure 7**

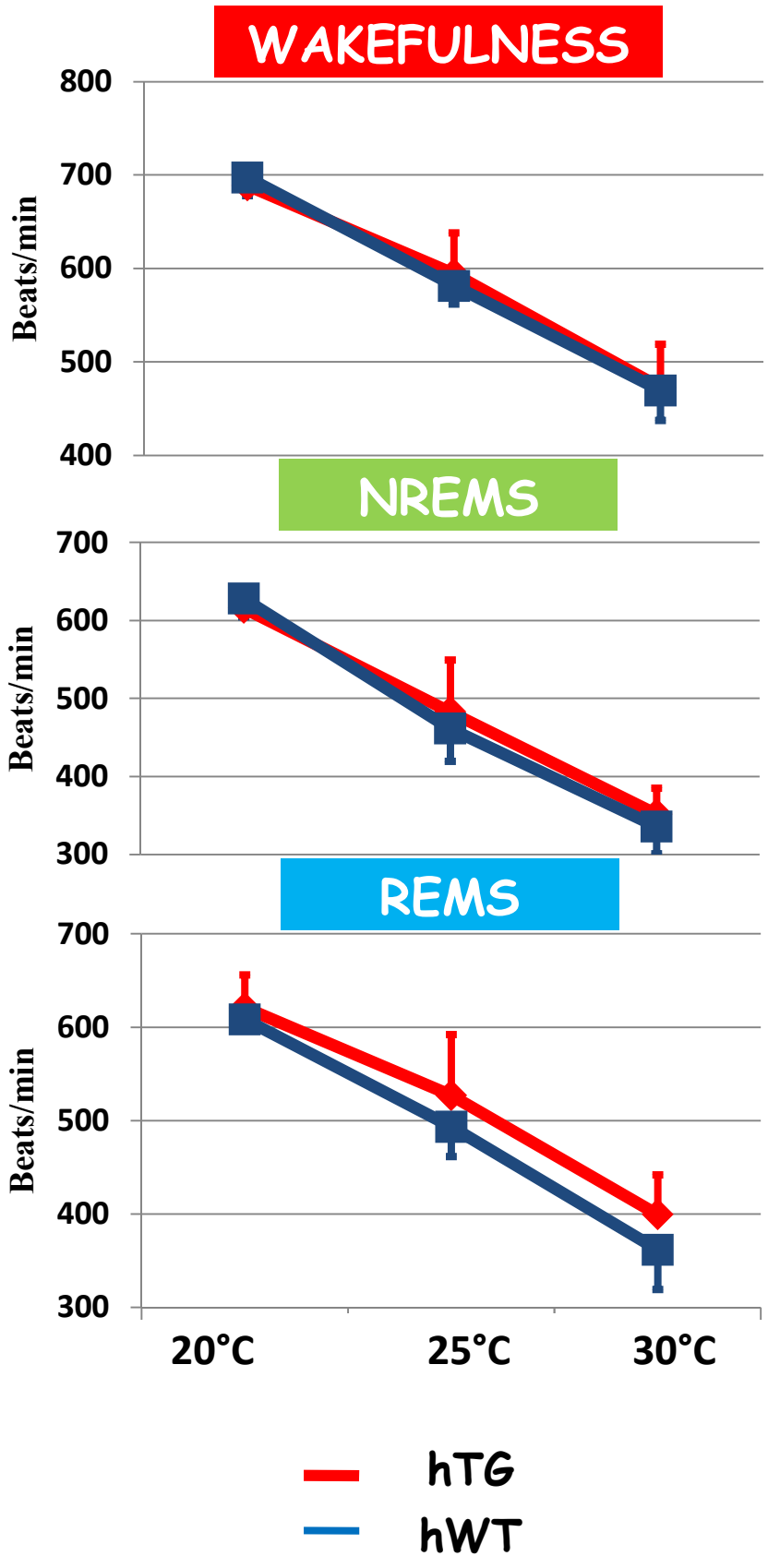
WAKEFULNESS
800
700
600
500
400
Beats/min
NREMS
700
600
500
400
300
Beats/min
REMS
700
600
500
400
300
Beats/min
20°C
25°C
30°C
hTG
hWT

**Figure 7**. Heart rate (HR) values in transgenic mice (hTG, n=11) and wild-type control mice (hWT, n=12) in wakefulness, non rapid-eye movement sleep (NREMS) and in rapid-eye-movement sleep (REMS) at different ambient temperature (20°C, 25°C, 30°C). Error bars represent SEM. ANOVA showed a significant interaction effect between wake-sleep state and Ta on HR ($p<0,001$), and significant main effects of W-S states and Ta on HR ($p<0,001$).

Data recorded at 25°C were obtained from 8 hTG and 9 hWT.

# Figure 8

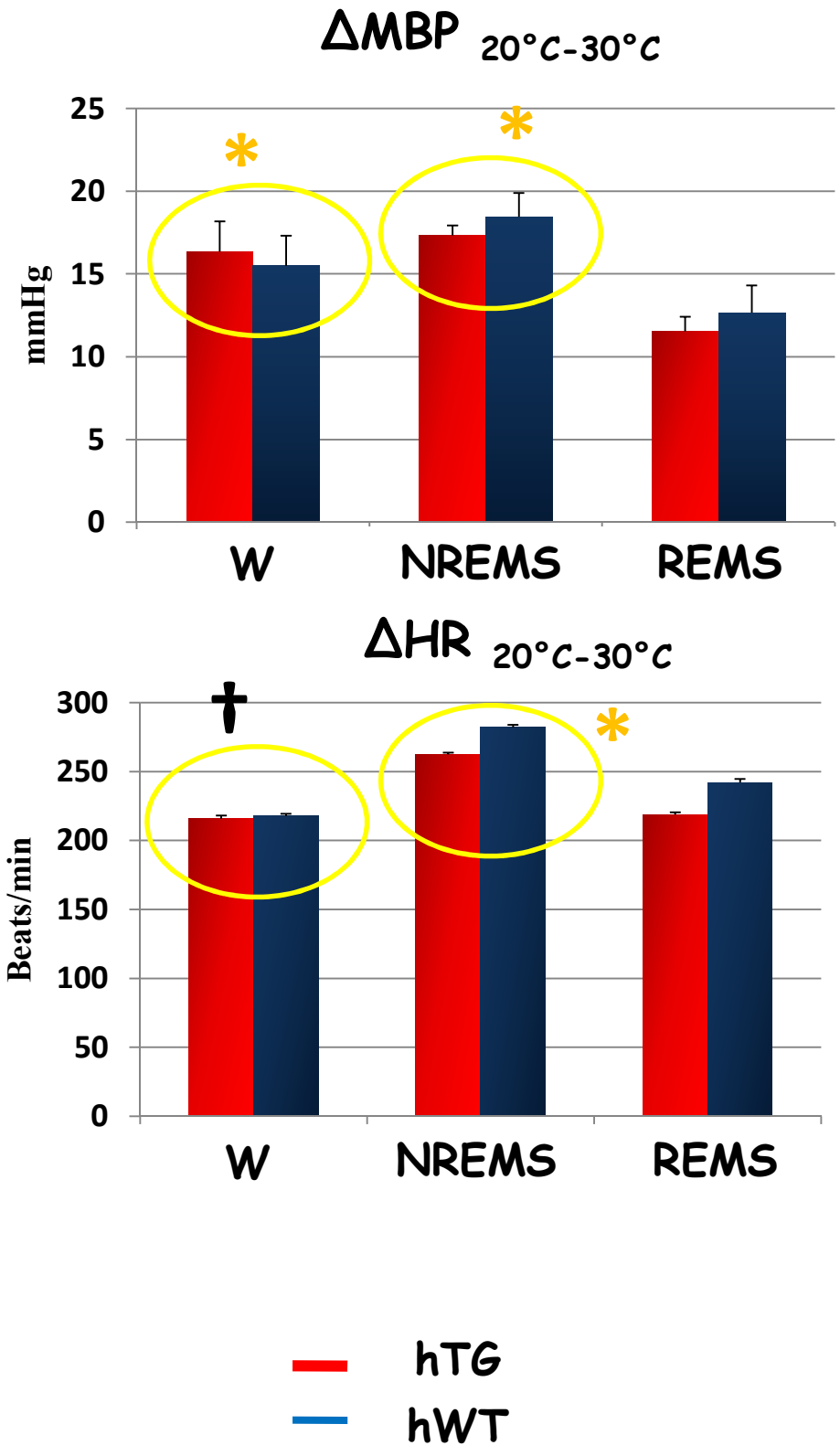
ΔMBP 20°C-30°C
mmHg
25
20
15
10
5
0
W
NREMS
REMS
ΔHR 20°C-30°C
Beats/min
300
250
200
150
100
50
0
W
NREMS
REMS
hTG
hWT

**Figure 8**. Differences in mean blood pressure (MBP) (upper graph) and heart rate (HR) (bottom graph) between the exposure at 20°C and 30°C in transgenic mice (hTG, n=11) and wild-type control mice (hWT, n=12) during wakefulness, non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS). Error bars represent SEM. * $p<0,05$ vs REMS † $p<0,05$ vs NREMS

# Figure 9

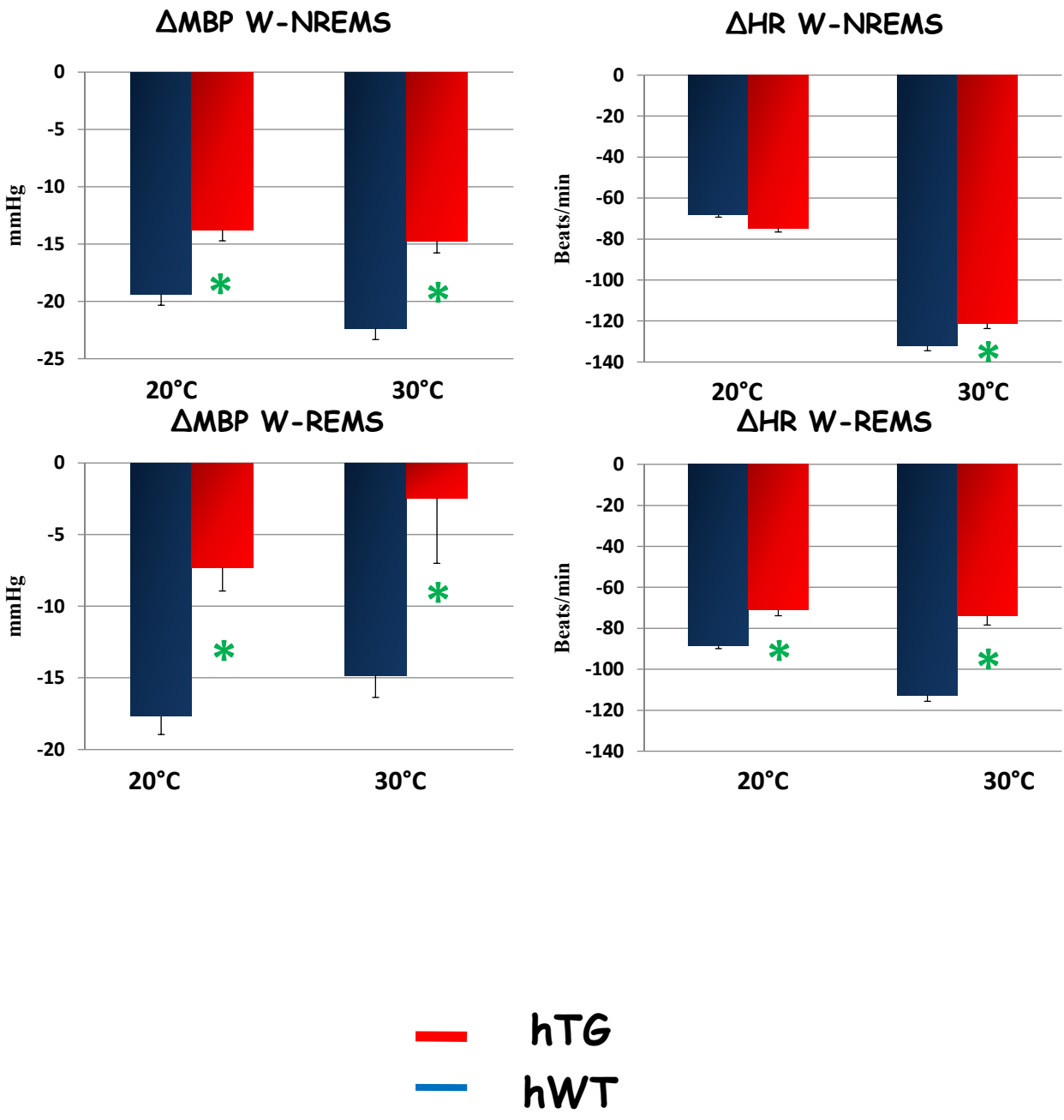
ΔMBP W-NREMS
mmHg
0
-5
-10
-15
-20
-25
20°C
30°C
ΔHR W-NREMS
Beats/min
0
-20
-40
-60
-80
-100
-120
-140
20°C
30°C
ΔMBP W-REMS
mmHg
0
-5
-10
-15
-20
20°C
30°C
ΔHR W-REMS
Beats/min
0
-20
-40
-60
-80
-100
-120
-140
20°C
30°C
hTG
hWT

**Figure 9**. Differences in mean blood pressure (MBP) (on the left) and heart rate (HR) (on the right) between wakefulness and non-rapid-eye-movement sleep (NREMS) and between wakefulness and rapid-eye-movement sleep (REMS) in transgenic mice (hTG, n=11) and wild-type control mice (hWT, n=12), at 20°C and 30°C. Error bars represent SEM. *p<0,05 vs hWT

# Figure 10

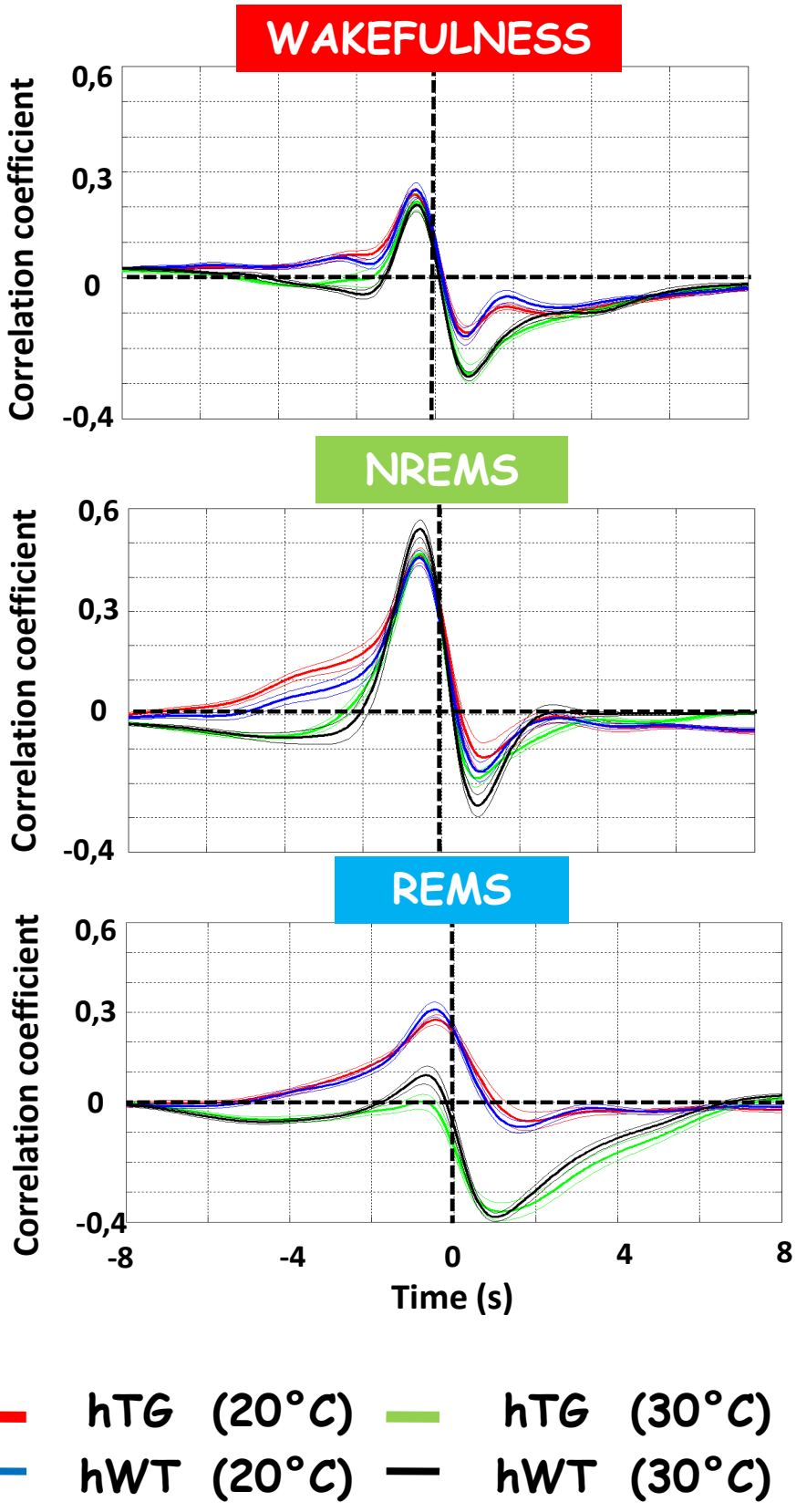
WAKEFULNESS
NREMS
REMS
Correlation coefficient
0,6
0,3
0
-0,4
-8
-4
0
4
8
Time (s)
hTG (20°C)
hWT (20°C)
hTG (30°C)
hWT (30°C)

**Figure 10**. Cross-correlation function between systolic blood pressure (SBP) and heart rate (HR) in wakefulness, non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) in transgenic mice (hTG, n=11) at 20°C and 30°C (red and green lines, respectively) and wild-type control mice (hWT, n=12) at 20°C and 30°C (blue and black lines, respectively). ANOVA showed a significant main effect of wake-sleep (W-S) state and ambient temperature (Ta), and an interaction effect between W-S state and Ta.

# Figure 11

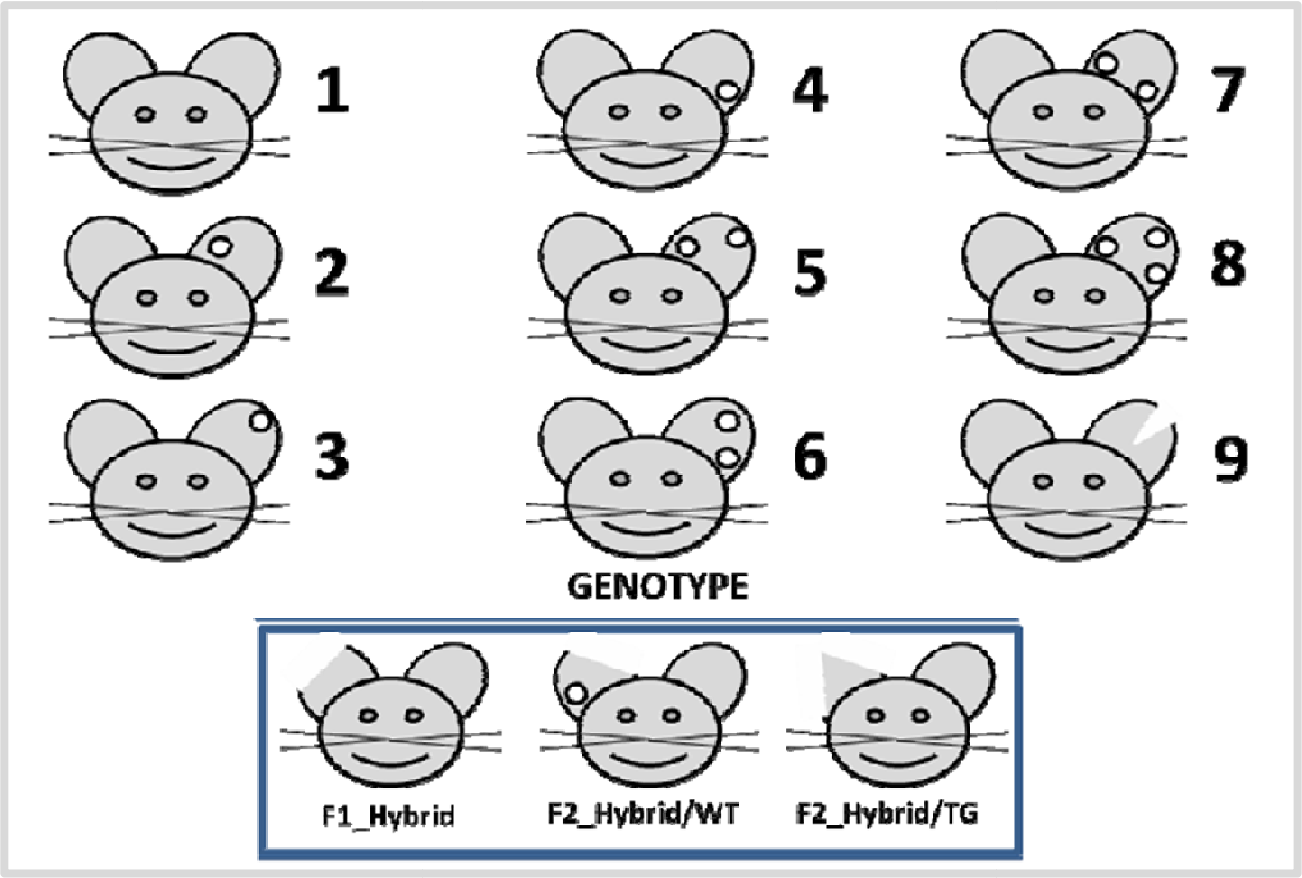

**Figure 11**. Legend of ear punching. Animal in the same cage were identified making a different number of holes in different position on the left ear.
After genotyping, mice were marked for their genotype on the right ear.

# Table 1

| Behavioral State | Ta | hTG | hWT |
|---|---|---|---|
| W (%) | **20°C *** | 48.9 ± 1.0 | 47.6 ± 1.3 |
| | **30°C** | 46.5 ± 4.1 | 40.4 ± 1.5 |
| NREMS (%) | **20°C** | 43.2 ± 1.0 | 45.0 ± 1.5 |
| | **30°C** | 44.8 ± 3.7 | 50.8 ± 1.4 |
| REMS (%) | **20°C *** | 5.0 ± 0.3 | 4.8 ± 0.4 |
| | **30°C** | 6.4 ± 0.7 | 5.8 ± 0.3 |

**Table 1**. Percentage of wake and sleep states during the recording period: W, Wakefulness; NREMS, non rapid-eye-movement sleep; REMS, rapid-eye-movement sleep. Values are reported as mean ± SEM in transgenic mice (hTG, n=11) and wild-type control mice (hWT, n=12). *, $P < 0.05$ Vs 30°C at same behavioral state.

# Table 2

| | Ta | hTG | hWT |
|---|---|---|---|
| **n°of W (episodes over 24h)** | **20°C** | 214.6 ± 15.7 * | 138.3 ± 7.9 |
| | **30°C** | 202.6 ± 13.6 * | 143.3 ± 4.2 |
| **n°of NREMS (episodes over 24h)** | **20°C** | 478.0 ± 19.7 | 449.5 ± 25.4 |
| | **30°C** | 414.0 ± 32.1 | 486.4 ± 23.3 |
| **n°of REMS (episodes over 24h)** | **20°C** | 74.3 ± 7.1 | 66.8 ± 5.3 |
| | **30°C** | 76.6 ± 8.3 | 71.3 ± 5.8 |
| **W bout duration (sec)** | **20°C** | 200.9 ± 14.0 * | 300.6 ± 24.2 |
| | **30°C** | 205.2 ± 29.1 * | 236.4 ± 10.4 |
| **NREMS bout duration (sec)** | **20°C** | 78.5 ± 3.2 | 89.7 ± 6.9 |
| | **30°C** | 92.5 ± 4.8 | 91.9 ± 4.8 |
| **REMS bout duration (sec)** | **20°C** | 60.6 ± 3.6 | 61.5 ± 2.3 |
| | **30°C** | 72.2 ± 4.5 | 71.7 ± 2.9 |
| **REMS latency (sec)** | **20°C** | 280.1 ± 25.0 * | 497.7 ± 42.9 |
| | **30°C** | 376.0 ± 717.2 * | 717.2 ± 72.6 |

**Table 2**. Density (number in 24 h) and duration of wake and sleep states episodes: W, Wakefulness; NREMS, non rapid-eye-movement sleep; REMS, rapid-eye-movement sleep. Values are reported as mean ± SEM in transgenic mice (hTG, n=11) and wild-type control mice (hWT, n=12). *, $P < 0.05$ Vs hWT at same ambient temperature.

# Table 3

| Factors | MBP | HR |
|---|---|---|
| W-S states | P<0,001 | P<0,001 |
| Ta | P<0,001 | P<0,001 |
| Strain x Ta | P=0, 882 | P=0,176 |
| Strain x W-S states | P<0,001 | P=0,020 |
| W-S states x Ta | P<0,001 | P<0,001 |
| Strain x Ta x W-S states | P=0,488 | P=0,144 |

**Table 3**. 3-way Analysis of variance (ANOVA) results for mean blood pressure (MBP) and heart rate (HR): in red are reported the significant results, whereas non significant ones are reported in blue. Ta, ambient temperature; W-S states: wake-sleep states.

# Table 4

| Ta | Mouse Strain | MBP difference between REMS and NREMS (mmHg) |
|---|---|---|
| **20°C** | **hTG** | 6.4 ± 1.1 |
| | **hWT** | 1.7 ± 0.9 |
| **30°C*** | **hTG** | 12.3 ± 1.4 |
| | **hWT** | 7.5 ± 0.9 |

**Table 4**. Mean blood pressure (MBP) difference between non-rapid-eye movement sleep (NREMS) and rapid-eye-movement sleep (REMS) in transgenic mice (hTG, n=11) and wild-type control mice (hWT, n=12) at 20°C and 30°C.

MBP difference between NREMS and REMS is significantly higher at 30°C in both experimental groups (*, $p<0.05$ vs 20°C).

# *11. References*

**Anaclet**, C., Parmentier, R., Ouk, K., Guidon, G., Buda, C., Sastre, JP., Akaoka, H., Sergeeva, OA., Yanagisawa, M., Ohtsu, H., Franco, P., Haas, HL., Lin, JS. (2009). "Orexin/hypocretin and histamine: distinct roles in the control of wakefulness demonstrated using knock-out mouse models." J Neurosci. **29**(46): 14423-38.

**Baker**, T., Guilleminault, C., Nino-Murcia, G., Dement, WC. (1986). "Comparative polysomnographic study of narcolepsy and idiopathic central nervous system hypersomnia." Sleep **9**(1-2): 232-42.

**Balaskò**, M., Szelènyi, Z., Szèkely, M. (1999). "Central thermoregulatory effects of neuropeptide Y and orexin A in rats." Acta Physiol Hung **86**: 219-222.

**Bassetti**, C. (2005). "Selective hypocretin (orexin) loss and multiple signaling deficiences." Neurology **65**: 1152-1153.

**Bastianini**, S., Silvani, A., Berteotti, C., Elghozi, JL., Franzini, C., Lenzi, P., Lo Martire, V., Zoccoli, G. (2011a). "Sleep related changes in blood pressure in hypocretin-deficient narcoleptic mice." Sleep **34**(2): 213-18.

**Bastianini**, S., Silvani, A., Berteotti, C., Lo Martire, V., Zoccoli, G. (2011b). "High-amplitude theta wave bursts during REM sleep and cataplexy in hypocretin-deficient narcoleptic mice." J Sleep Res. **doi: 10.1111/j.1365-2869.2011.00945.x.** .

**Baust**, W., Bohnert, B. (1969). "The regulation of heart rate during sleep." Exp. Brain Res. **7**: 169-180.

**Berry**, M., Linder, CC. (2007). Breeding Systems: Considerations, Genetic Fundamentals, Genetic Background, and Strain Types. The Mouse in Biomedical Research. J. Fox, Barthold, SW., Davisson, MT., Newcomr, CE., Quimby, FW., Smith, AL., Academic Press. **I**.

**Berthoud**, H., Patterson, LM., Sutton, GM., Morrison C., Zheng, H. (2005). "Orexin inputs to caudal raphe neurons involved in thermal, cardiovascular, and gastrointestinal regulation." Histochem. Cell. Biol. **123**: 147-156.

**Bhaskaran**, K., Hajat, S., Haines A.,Herrett, E., Wilkinson, P., Smeeth, L. (2010). "Short term effects of temperature on risk of myocardial infarction in England and Wales: time series regression analysis of the Myocardial Ischaemia National Audit Project (MINAP) registry." BMJ **341**: c3823.

**Bourgin**, P., Huitron-Resendiz, S., Spier, AD., Fabre, V., Morte, B., Criado, JR., Sutcliffe, JG., Henriksen, SJ., de Lecea, L. (2000). "Hypocretin-1 modulates rapid eye movement sleep through activation of locus ceruleus neurons." Journal of Neuroscience **20**(7760-65).

**Bristow**, J., Honour, AJ, Pickering, TG., Sleight, P. (1969). "Cardiovascular and respiratory changes during sleep in normal and hypertensive subjects." Cardiovasc. Res. **3**: 476-85.

**Burgess**, C., Tse, G., Gillis, L., Peever, JH. (2010). "Dopaminergic regulation of sleep and cataplexy in a murine model of narcolepsy." Sleep **33**(10): 1295-304.

**Bustin**, S. (2007). A-Z of Quantitative PCR.

**Campen**, M., Tagaito, Y., Jenkins, TP., Smith, PL., Schwartz, AR., O'Donnell, CP. (2002). "Phenotypic differences in the hemodynamic response during REM sleep in six strains of inbred mice." Physilogical Genomics **11**(3): 227-34.

**Cannon**, W. (1929). "Organization for physiological homeostasis." Physiol Rev **9**: 399-431.

**Cao**, W., Morrison, SF. (2003). "Disinhibition of rostral raphe pallidus neurons increases cardiac sympathetic nerve activity and heart rate." Brain Research **980**: 1-10.

**Carrington**, M., Barbieri, R., Colrain, IM., Crowley, KE., Kim, Y., Trinder, J. (2005). "Changes in cardiovascular function during the sleep onset period in young adults." Journal of applied physiology **98**(2): 468-76.

**Carskadon**, M., Mitler, MM., Roth, T. (1986). "Guidelines for the Multiple Sleep Latency Test (MSLT): a standard measure of sleepiness." Sleep **9**: 519-24.

**Cerri**, M., Morrison S.F. (2005). "Activation of lateralhypothalamic neurons stimulates brown adipose tissue thermogenesis." Neuroscience **135**: 627-638.

**Chemelli**, R., Willie, JT., Sinton, CM., Elmquist, JK., Scammell, T., Lee, C., Richardson, JA., Williams, SC., Xiong, Y., Kisanuki, Y., Fitch, TE., Nakazato, M., Hammer, RE., Saper, CB., Yanagisawa, M. (1999). "Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation." Cell **98**(4): 437-51.

**Chokroverty**, S. (1986). "Sleep apnea in narcolepsy." Sleep **9**(1 pt 2): 250-53.

**Cianci**, T., Zoccoli, G., Lenzi, P., Franzini, C. (1991). "Loss of integrative control of peripheral circulation during desynchronized sleep." Am. J. Physiol. Regul. Integr. Comp. Physiol. **261**: R373-7.

**Ciriello**, J., McMurray, JC., Babic, T., de Oliveira, CV. (2003). "Collateral axonal projections from hypothalamic hypocretin neurons to cardiovascular sites in nucleus ambiguus and nucleus tractus solitarius." Brain Research **991**(1-2): 133-41.

**Crocker**, A., Espana, R.A., Papadopoulou, M., Saper, C.B., Faraco, J., Sakurai, T., Honda, M., Mignot, E., Scammell, T.E. (2005). "Concomitant loss of dynorphin, NARP, and orexin in narcolepsy." Neurology **65**: 1184-88.

**Danet**, S., Richard, F., Montaye, M., Beauchant, S., Lemaire, B., Graux, C., Cottel, D., Marécaux, N., Amouyel, P. (1999). "Unhealthy effects of atmospheric temperature and pressure on the occurrence of myocardial infarction and coronary deaths: a 10-year survey: the lille-world health organization

MONICA project (monitoring trends and determinants in cardiovascular disease)." Circulation **100**: e1-e7.

**Dauvilliers**, Y., Arnulf, I., Mignot, E. (2007). "Narcolepsy with cataplexy." Lancet **369**: 499-511.

**Davies**, C., Crosby, JH., Mullins, RL., Barbour, C., Davies, RJ., Stradling, JR. (2000). "Case-control study of 24 hour ambulatory blood pressure in patients with obstructive sleep apnoea and normal matched control subjects." Thorax **55**(9): 726-28.

**De Lecea**, L., Kilduff, T.S., Peyron, C., Gao, X.-B., Foye, P.E., Danielson, P.E., Fukuhara, C., Battenberg, E.L.F., Gautvik, V.T., Barlett, F.S., Frankel, W.N., Van Den Pol, A.N., Bloom, F.E., Gautvik, K.M. & Sutcliffe, J.G. (1998). "The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity." Proceedings of the National Academy of Sciences **95**: 322-327.

**Dement**, W. (2005). History of Sleep Physiology and Medicine. Principles and Practice of Sleep Medicine. M. Kryger, Roth, T., Dement, WC., Elsevier**:** 1-12.

**Fagius**, J., Kay, R. (1991). "Low ambient temperature increases baroreflex-governed sympathetic outflow to muscle vessels in humans." Acta Physiol Scand. **142**: 201-209.

**Ferini-Strambi**, L., Spera, A., Oldani, A., Zucconi, M., Bianchi, A., Cerutti, S., Smirne, S. (1997). "Autonomic function in narcolepsy: power spectrum analysis of heart rate variability." Journal of Neurology **244**(4): 252-55.

**Franken**, P., Malafosse, A., Tafti, M. (1999). "Genetic determinants of sleep regulation in inbred mice." Sleep **22**(2): 155-69.

**Franzini**, C., Cianci, T., Lenzi, PL., Guidalotti, PL. (1982). "Neural control of vasomotion in rabbit ear is impaired during desynchronized sleep." Am J Physiol **243**: 142-146.

**Fronczek**, R., Overeem, S., Lammers, GJ., van Dijk, JG., Van Someren, EJW. (2006). "Altered Skin-Temperature Regulation in Narcolepsy Relates to Sleep Propensity." Sleep **29**(11): 1444-1449.

**Fronczek**, R., Overeem, S., Reijntjes, R., Lammers, GJ., van Dijk, JG., Pijl, H. (2008b ). "Increased heart rate variability but normal resting metabolic rate in hypocretin/orexin-deficient human narcolepsy." Journal of Clinical Sleep Medicine **4**(3): 248-254.

**Fronczek**, R., Raymann, RJ., Romeijn, N., Overeem, S., Fischer, M., van Dijk, JG., Lammers, GJ., Van Someren, EJ. (2008a). "Manipulation of core body and skin temperature improves vigilance and maintenance of wakefulness in narcolepsy." Sleep **31**(2): 233-40.

**Funato**, H., Tsai, AL., Willie, JT., Kisanuki, Y., Williams, SC., Sakurai, T., Yanagisawa, M. (2009). "Enhanced orexin receptor-2 signaling prevents diet-induced obesity and improves leptin sensitivity." Cell Metabolism **9**(1): 64-76.

**Futuro-Neto**, H., Coote, JH. (1982). "Changes in sympathetic activity to heart and blood vessels during desynchronized sleep." Brain Res. **252**: 259-68.

**Gerashchencko**, D., Murillo-Rodriguez, E., Lin, L. et al. (2003). "Relationship between CSF hypocretin levels and hypocretin neuronal loss." Exp Neurol. **184**: 1010-6.

**Grimaldi**, D., Agati, P., Pierangeli, G., Franceschini, C., Guaraldi, P., Barletta, G., Vandi, S., Cevoli, S., Plazzi, G., Montagna, P., Cortelli, P. (2010a). "Hypocretin deficiency in narcolepsy with cataplexy is associated with a normal body core temperature modulation." Chronobiology International **27**(8): 1596-608.

**Grimaldi**, D., Pierangeli, G., Barletta, G., Terlizzi, R., Plazzi, G., Cevoli, S., Franceschini, C., Montagna, P., Cortelli, P. (2010b). "Spectral analysis of heart rate variability reveals an enhanced sympathetic activity in narcolepsy with cataplexy." Clinical Neurophysiology **121**(7): 1142-47.

**Guilleminault**, C., Salva, MA., Mancuso, J., Hayes, B. (1986). "Narcolepsy, cataplexy, heart rate, and blood pressure." Sleep **9**(1-2): 222-26.

**Guyton**, A., Hall, J. (2006). Body temperature, temperature regulation and fever. Textbook of medical physiology. Philadelphia, Elseviers Saunders.

**Hallanger**, A., Levey, AI., Lee, HJ., Rye, DB., Wainer, BH. (1987). "The origins of cholinergic and other subcortical afferents to the thalamus in the rat." The Journal of Comparative Neurology **262**: 104-24.

**Hara**, J., Beuckmann, CT., Nambu, T., Willie, JT., Chemelli, RM., Sinton, CM., Sugiyama, F., Yagami, K., Goto, K., Yanagisawa, M., Sakurai, T. (2001). "Genetic ablation of orexin neurons in mice results in narcolepsy, hypophagia, and obesity." Neuron **30**(2): 345-54.

**Hara**, J., Yanagisawa, M., Sakurai, T. (2005). "Difference in obesity phenotype between orexin-knockout mice and orexin neuron-deficient mice with same genetic background and environmental conditions." Neuroscience Letters **380**(3): 239-42.

**Hublin**, C., Matikainen, E., Partinen, M. (1994). "Autonomic nervous system function in narcolepsy." The Journal of Sleep Research **3**(3): 131-37.

**Ida**, T., Nakahara, K., Katayama, T., Murakami, N., Nakazato, M. (1999). "Effect of lateral cerebroventricular injection of the appetite-stimulating neuropeptide, orexin and neuropeptide Y, on the various behavioral activities of rats. ." Brain Research **821**: 526-529.

**Jhaveri**, K., Trammell, RA., Toth, LA. (2007). "Effect of environmental temperature on sleep, locomotor activity, core body temperature and immune responses of C57BL/6J mice." Brain, Behavior, and Immunity **21**: 975-987.

**Kayaba**, Y., Nakamura, A., Kasuya, Y., Ohuchi, T., Yanagisawa, M., Komuro. I., Fukuda, Y., Kuwaki, T. (2003). "Attenuated defense response and low basal blood pressure in orexin knockout mice." American Journal of Physiology - Regulatory Integrative and Comparative Physiology. **285**(3): R519-21.

**Khatri**, I., Freis, ED. (1967). "Hemodynamic changes during sleep." J. Appl. Physiol. **22**: 867-73.

**Kohara**, K., Nishida, W., Maguchi, M., Hiwada, K. (1996). "Autonomic nervous function in non-dipper essential hypertensive subjects. Evaluation by power spectral analysis of heart rate variability." Hypertension **26**(5): 808-14.

**Kok**, S., Overeem, S., Lammers, JG., Seidell, JC., Pijl, H., Meinders, AE. (2003). "Hypocretin deficiency in narcoleptic humans is associated with abdominal obesity." Obes. Res. **11**(9): 1147-54.

**Kurtz**, T., Griffin, KA., Bidani, AK., Davisson, RL., Hall, JE. (2005). "Recommendations for blood pressure measurement in humans and experimental animals part 2: blood pressure measurement in experimental animals. A statement for professionals from the Subcommittee of Professional and Public Education of the American Heart Association Council on High Blood Pressure Research." Hypertension **45**: 299-310.

**Kuwaki**, T. (2008). "Orexinergic modulation of breathing across vigilance states." Respiratory physiology and neurobiology **164**(1-2): 204-12.

**Lammers**, G., Pijl, H., Iestra, J., Langius, JA., Buunk, G., Meinders, AE. (1996). "Spontaneous food choice in narcolepsy." Sleep **19**(1): 75e6.

**Lee**, M., Hassani, OK., Jones, BE. (2005). "Discharge of Identified Orexin/Hypocretin Neurons across the Sleep–Waking Cycle." Journal of Neuroscience **25**(28): 6716-20.

**Lin**, L., Faraco, J., Li, R., Kadotani, H., Rogers, W., Lin, X., Qiu, X., de Jong PJ., Nishino, S., Mignot, E. (1999). "The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene." Cell **98**(4): 409-12.

**Lin**, Y., Matsumura, K., Tsuchihashi, T., Abe, I., Iida, M. (2002). "Chronic central infusion of orexin-A increases arterial pressure in rats." Brain Research Bulletin **57**(5): 619-22.

**Liu**, R., van den Pol, AN. & Aghajanian, GK. (2002). "Hypocretins (orexins) regulate serotonin neurons in the dorsal raphe nucleus by excitatory direct and inhibitory indirect actions." J. Neurosci. **22**: 9453-9464.

**Loredo**, J., Nelesen, R., Ancoli-Israel, S., Dimsdale, JE. (2004). "Sleep quality and blood pressure dipping in normal adults." Sleep **27**(6): 1097-103.

**Lu**, J., Greco, MA., Shiromani, P., Saper, CB. (2000). "Effect of lesions of the ventrolateral preoptic nucleus on NREM and REM sleep." Journal of Neuroscience **20**: 3830-42.

**Lubkin**, M., Stricker-Krongrad, A. (1998). "Independent feeding and metabolic actions of orexins in mice." Biochem Biophys Res Commun **253**: 241-245.

**Mancia**, G., Baccelli, G. et al. (1971). "Vasomotor regulation during sleep." Am. J. Physiol. **220**: 1086-1093.

**Marcus**, J., Aschkenasi, CJ., Lee, CE., Chemelli, RM., Saper, CB., Yanagisawa, M., Elmquist, JK. (2001). Differential expression of orexin receptors 1 and 2 in the rat brain. J Comp Neurol. . **435:** 6-25.

**Matsumura**, K., Tsuchihashi, T., Abe, I. (2001). "Central orexin-A augments sympathoadrenal outflow in conscious rabbits." Hypertension **37**(6): 1382-87.

**Miki**, K., Kato, M., Kajii, S. (2003). "Relationship between renal sympathetic nerve activity and arterial pressure during REM sleep in rats." Am. J. Physiol.Regul. Integr. Comp. Physiol. **284**: R467-73.

**Miki**, K., Oda, M., Kamijyo, N., Kawahara, K., Yoshimoto, M. (2004). "Lumbar sympathetic nerve activity and hindquarter blood flow during REM sleep in rats." J. Physiol. **557**: 261-71.

**Mochizuki**, T., Klerman, EB., Sakurai, T., Scammell, TE. (2006). "Elevated body temperature during sleep in orexin knockout mice." American Journal of Physiology **291**: R533-40.

**Monda**, M., Viggiano, A., Viggiano, A., Fuccio, F., De Luca, V. (2003). "Paradoxical effect of orexin A: hypophagia induced by hyperthermia." Brain Research **961**(2): 220-28.

**Morrison**, F., Nakamura, K. and Madden, CJ. (2008). "Central control of thermogenesis in mammals." Exp Physiol. **93**(7): 773-797.

**Morrison**, S., Nakamura, K. (2011). "Central neural pathways for thermoregulation." Frontiers in Bioscience **16**: 74-104.

**Nakamura**, A., Zhang, W., Yanagisawa, M., Fukuda, Y., Kuwaki, T. (2007). "Vigilance state-dependent attenuation of hypercapnic chemoreflex and exaggerated sleep apnea in orexin knockout mice." Journal of applied physiology **102**(1): 241-48.

**Nakamura**, K. (2011). "Central circuitries for body temperature regulation and fever." Am J Physiol Regul Integr Comp Physiol. **301**: R1207-R1228.

**Nakamura**, K., Morrison, SF. (2008). "A thermosensory pathway that controls body temperature." Nat. Neurosci. **11**: 62-71.

**Nambu**, T., Sakurai, T., Mizukami, K., Hosoya, Y., Yanagisawa, M., Goto, K. (1999). "Distribution of orexin neurons in the adult rat brain." Brain Research **827**(1-2): 243-260.

**Nishino**, S. (2007). "Clinical and Neurobiological Aspects of Narcolepsy." Sleep Med **8** (4): 373-399.

**Nishino**, S., Ripley, B., Overeem, S., Lammers, GJ., Mignot, E. (2000). "Hypocretin (orexin) deficiency in human narcolepsy." Lancet **355**(9197): 39-40.

**Nolan**, R., Hands, RE., Bustin, SA. (2006). "Quantification of mRNA using RT-PCR." Nature Protocols **1**: 1559-1582.

**Ohkubo**, T., Hozawa, A., Yamaguchi, J., Kikuya, M., Ohmori, K., Michimata, M., Matsubara. M., Hashimoto, J., Hoshi, H., Araki, T., Tsuji, I., Satoh, H., Hisamichi, S., Imai, Y. (2002). "Prognostic significance of the nocturnal decline in blood pressure in individuals with and without high 24-h blood pressure: the Ohasama study." Journal of Hypertension **20**(11): 2183-89.

**Ohno**, K., Sakurai, T. (2008). "Orexin neuronal circuitry: Role in the regulation of sleep and wakefulness." Frontiers in Neuroendocrinology **29**: 70-87.

**Parmeggiani**, P. (1980). "Behavioral phenomenology of sleep (somatic and vegetative)." Experientia **36**: 6-11.

**Parmeggiani**, P. (2005). Physiologic regulation in sleep. Principles and practice of Sleep Medicine. M. Kryger, Roth, T., Dement, WC. Philadelphia, W.B Saunders Company**:** 185-191.

**Parmeggiani**, P., Rabini, C. (1970). "Sleep and environmental temperature." Arch. Ital. Biol. **108**: 369-387.

**Parmeggiani**, P. L., Zamboni, G., Cianci, T., Calasso, M. (1977). "Absence of thermoregulatory vasomotor responses during fast wave sleep in cats." Elec. Clin. Neurophysiol. **42**: 372-380.

**Perez**, M., Paulson, HL., Pittman, RN. (1999). "Ataxin-3 with an altered conformation that exposes the polyglutamine domain is associated with the nuclear matrix." Human Molecular Genetics **8**(13): 2377-85.

**Peyron**, C., Faraco, J., Rogers, W., Ripley, B., Overeem, S., Charnay, Y., Nevsimalova, S., Aldrich, M., Reynolds, D., Albin, R., Li, R., Hungs, M., Pedrazzoli, M., Padigaru, M., Kucherlapati, M., Fan, J., Maki, R., Lammers, GJ., Bouras, C-, Kucherlapati, R., Nishino, S., Mignot, E. (2000). "A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains." Nature Medicine **6**(9): 991-7.

**Peyron**, C., Tighe, DK., van den Pol, AN., de Lecea, L., Heller, HC., Sutcliffe, JG., Kilduff, TS. (1998). "Neurons containing hypocretin (orexin) project to multiple neuronal systems." Journal of Neuroscience **18**(23): 9996-10015.

**Plazzi**, G., Moghadam, KK., Maggi, LS., Donadio, V., Vetrugno, R., Liguori, R., Zoccoli, G., Poli, F., Pizza, F., Pagotto, U., Ferri, R. (2011). "Autonomic disturbances in narcolepsy." Sleep Medicine Review **15**: 187-196.

**Poli**, F., Plazzi, G., Di Dalmazi, G., Ribichini, D., Vicennati, V., Pizza, F., Mignot, E., Montagna, P., Pasquali, R., Pagotto, U. (2009). "Body mass index-independent metabolic alterations in narcolepsy with cataplexy." Sleep **32**(11): 1491-97.

**Rechtschaffen**, A. a. K., A. (1968). A manual of standardized terminology: Techniques and Scoring system for sleep stages of human subjects. Los Angeles (California), Brain Information Service/ Brain Information Institute.

**Redgate**, E. S., Gellhorn, E. (1958). "Respiratory activity and the hypothalamus." Am J Physiol. **193**: 189-194

**Roussel**, B., Turrillot, P., Kitahama, K., (1984). "Effect of ambient temperature on the sleep-waking cycle in two strains of mice." Brain Res. **294** (1): 67-73.

**Rusyniak**, D., Zaretsky, DV., Zaretskaia, MV., DiMicco, JA. (2011). "The role of orexin-1 receptors in physiologic responses evoked by microinjection of PgE2 or muscimol into the medial preoptic area." Neurosci Lett. **498**(2): 162-6.

**Sachs**, C., Kaijser, L. (1982). "Autonomic regulation of cardiopulmonary functions in sleep apnea syndrome and narcolepsy." Sleep **5**(3): 227-38.

**Sakurai**, T. (2007). "The neural circuit of orexin (hypocretin): maintaining sleep and wakefulness." Nature Reviews Neuroscience **8**: 171-81.

**Sakurai**, T., Amemiya, A., Ishii, M., Matsuzaki, I., Chemelli, R.M., Tanaka, H., Williams, S.C., Richardson, J.A., Kozlowski, G.P., Wilson, S. et al. (1998). "Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior." Cell **92**: 573-85.

**Sakurai**, T., Moriguchi, T., Furuya, K., Kajiwara, N., Nakamura, T., Yanagisawa, M., Goto, K. (1999). "Structure and function of human prepro-orexin gene." The Journal of Biological Chemestry **274**(25): 17771-76.

**Saper**, C., Chou, TC., Scammell, TE. (2001). "The sleep switch: hypothalamic control of sleep and wakefulness." Trends in Neurosciences **24**: 726-731.

**Saper**, C., Scammell, TE., Lu, J. (2005). "Hypothalamic regulation of sleep and circadian rhythms." Nature **437**(27): 1257-63.

**Satoh**, S., Matsumura, H. , Fujioka, A., Nakajima, T., Kanbayashi, T., Nishino, S., Shigeyoshi, Y. , Yoneda, H. (2004). "FOS expression in orexin neurons following muscimol perfusion of preoptic area." Neuroreport **15**: 1127-1131.

**Scammell**, T., Willie, JT., Guilleminault, C., Siegel, JM. (2009). "A consensus definition of cataplexy in mouse models of narcolepsy." Sleep **32**(1): 111-16.

**Scammell**, T. E. (2003). "The neurobiology, diagnosis, and treatment of narcolepsy." Ann. Neurol. **53**: 154-66.

**Schaub**, C., Tankersley, C., Schwartz, AR., Smith, PL., Robotham, JL., O'Donnell, CP. (1998). "Effect of sleep/wake state on arterial blood pressure in genetically identical mice." Journal of applied physiology **85**: 366-71.

**Schiffman**, P., Trontell, MC., Mazar, MF., Edelman, NH. (1983). "Sleep deprivation decreases ventilatory response to CO2 but not load compensation." Chest **84**(6): 695-98.

**Schneider**, H., Schaub, CD., Andreoni, KA. (1997). "Systemic and pulmonary hemodynamic responses to normal and obstructed breathing during sleep. ." J. Appl. Physiol. **83**: 1671-80.

**Schuld**, A., Hebebrand, J., Geller, F., Pollmächer, T. (2000). "Increased body-mass index in patients with narcolepsy." The Lancet **355**(9211): 1274-1275.

**Sei**, H., Morita, Y. (1996). "Effect of ambient temperature on arterial pressure variability during sleep in the rat." J. Sleep Res. **5**: 37-41.

**Sei**, H., Sakai, K., Kanamori, N., Salvert, D., Vanni-Mercier, G., Jouvet, M. (1994). "Long-term variations of arterial blood pressure during sleep in freely moving cats." Physiology and Behavior **55**: 673-79.

**Shih**, C., Chuang, YC. (2007). "Nitric oxide and GABA mediate bi-directional cardiovascular effects of orexin in the nucleus tractus solitarii of rats." Neuroscience **149**(3): 625-35.

**Shirasaka**, T., Kunitake, T., Takasaki, M., Kannan, H. (1999). "Sympathetic and cardiovascular actions of orexins in conscious rats." Am J Physiol Regulatory Integrative Comp Physiol. **277**: 1780-1785.

**Shirasaka**, T., Kunitake, T., Takasaki, M., Kannan, H. (2002). "Neuronal effects of orexins: relevant to sympathetic and cardiovascular functions." Regulatory Peptides **104**(1-3): 91-95.

**Shirasaka**, T., Kunitake, T., Takasaki, M., Kannan, H. (2003). "Cardiovascular effects of leptin and orexins." Am J Physiol Regul Integr Comp Physiol **284**: 639-651.

**Silvani**, A. (2008). "Physiological sleep-dependent changes in arterial blood pressure: central autonomic commands and baroreflex control." Clinical and Experimental Pharmacology and Physiology **35**(9): 987-94.

**Silvani**, A., Bastianini, S., Berteotti, C., Franzini, C., Lenzi, P., Lo Martire, V., Zoccoli, G. (2009). "Sleep modulates hypertension in leptin-deficient obese mice." Hypertension **53**(2): 251-55.

**Silvani**, A., Bastianini, S., Berteotti, C., Franzini, C., Lenzi, P., Lo Martire, V., Zoccoli, G. (2010). "Dysregulation of heart rhythm during sleep in leptin-deficient obese mice." Sleep **33**(3): 355-61.

**Somers**, V., Dyken, ME., Mark, AL., Abboud, FM. (1993). "Sympathetic-nerve activity during sleep in normal subjects." The New England Journal of Medicine **328**(5): 303-7.

**Sun**, Z., Cade, R., Zhang, Z., Alouidor, J., Van, H. (2003). "Angiotensinogen gene knockout delays and attenuates cold-induced hypertension." Hypertension **41**: 322-327.

**Suzuki**, H., Mobarakeh, JI., Nunoki, K., Sukegawa, J., Watanabe, H., Kuramasu, A., Watanabe, T., Yanai, K., Yanagisawa, T. (2005). "Effects of activation of central nervous histamine receptors in cardiovascular regulation; studies in H(1) and H(2) receptor gene knockout mice." Arch Pharmacol. **371**(2): 99-106.

**Swoap**, S., Overton, JM., Garber, G. (2004). "Effect of ambient temperature on cardiovascular parameters in rats and mice: a comparative approach." American Journal of Physiology - Regulatory Integrative and Comparative Physiology. **287**: 391-96.

**Székely**, M., Pétervári E., Balaskó M., Hernádi I., Uzsoki B. (2002). "Effects of orexins on energy balance and thermoregulation." Regulatory Peptides **104**(1-3): 47-53.

**Tanaka**, M., Tonouchi, M., Hosono, T., Nagashima, K., Yanase-Fujiwara, M., Kanosue, K. (2001). "Hypothalamic region facilitating shivering in rats." Jpn J Physiol **51**: 625-629.

**Tanida**, M., Niijima, A., Shen, J., Yamada, S., Sawai, H., Fukuda, Y., Nagai, K. (2006). "Dose-different effects of orexin-A on the renal sympathetic nerve and blood pressure in urethane-anesthetized rats." Experimental Biology and Medicine **231**(10): 1616-25.

**Thakkar**, M. (2011). " Histamine in the regulation of wakefulness." Sleep Med Rev. **15**(1): 65-74.

**Thannickal**, T., Lai, YY., Siegel, JM. (2007). "Hypocretin (orexin) cell loss in Parkinson's disease." Brain **130**: 1586-95.

**Thannickal**, T., Moore, RY., Nienhuis, R., Ramanathan, L., Gulyani, S., Aldrich, M., Cornford, M., Siegel, JM. (2000). "Reduced number of hypocretin neurons in human narcolepsy." Neuron **27**(3): 469-74.

**Tupone**, D., Madden, CJ., Cano, G., Morrison, SF. (2011). "An orexinergic projection from perifornical hypothalamus to raphe pallidus increases rat brown adipose tissue thermogenesis." J Neurosci. **31**(44): 15944-55.

**Verrier**, R., Harper, RL., Hobson, JA. (2005). Cariovascular Physiology: Central and Autonomic Regulation. Philadelphia, W.B. SAUNDERS COMPANY.

**Wang**, S., Paton, JFR., Kasparov, S. (2007). "Differential sensitivity of excitatory and inhibitory synaptic transmission to modulation by nitric oxide in rat nucleus tractus solitarii." Experimental Physiology **92**: 371-382.

**Williams**, R., Burdakov, D. (2008). "Hypothalamic orexins/hypocretins as regulators of breathing." Expert reviews in molecular medicine **10**: e28.

**Willie**, J. T., Chemelli, RM., Sinton, CM. (2003). "Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes." Neuron **38**: 715-30.

**Willie**, J. T., Chemelli, R. M., Sinton, C. M. Yanagisawa, M. (2001). "To eat or to sleep? Orexin in the regulation of feeding and wakefulness." Annula Review of Neuroscience **24**: 429-58.

**Yamanaka**, A. (2002). "Orexins activate histaminergic neurons via the orexin 2 receptor." Biochem. Biophys. Res. Commun. **290**: 1237-1245.

**Yamanaka**, A., Beuckmann, CT., Willie, JT., Hara, J., Tsujino, N., Mieda, M., Tominaga, M., Yagami, K., Sugiyama, F., Goto, K., Yanagisawa, M., Sakurai, T. (2003). "Hypothalamic orexin neurons regulate arousal according to energy balance in mice." Neuron **38**(5): 701-13.

**Yamanaka**, A., Kunii, K., Nambu, T., Tsujino, N., Sakai, A., Matsuzaki, I., Miwa, Y., Goto, K., Sakurai, T. (2000). "Orexin-induced food intake involves neuropeptide Y pathway." Brain Research **859**: 404-409.

**Yoshida**, K., McCormack, S., Espana, RA., Crocker, A. & Scammell, TE. (2006). "Afferents to the orexin neurons of the rat brain." J. Comp. Neurol. **494**: 845-861

**Yoshimichi**, G., Yoshimatsu, H., Masaki, T., Sakata, T. (2001). "Orexin-A Regulates Body Temperature in Coordination with Arousal Status." Exp Biol Med Vol. **226**(5): 468-476.

**Yoshimoto**, M., Sakagami, T., Nagura, S., Miki, K. (2004). "Relationship between renal sympathetic nerve activity and renal blood flow during natural behavior in rats. ." Am. J. Physiol. Regul. Integr. Comp. Physiol. **286**: R881-7.

**Yoshizawa**, T., Yamagishi, Y., Koseki, N., Goto, J., Yoshida, H., Shibasaki, F., Shoji, S., Kanazawa, I. (2000). "Cell cycle arrest enhances the in vitro cellular toxicity of the truncated Machado-Joseph disease gene product with an expanded polyglutamine stretch." Human Molecular Genetics **9**(1): 69-78.

**Zaretsky**, D., Zaretskaia, MV., DiMicco, JA. (2003). "Stimulation and blockade of GABAA receptors in the raphe pallidus: effects on body temperature, heart rate, and blood pressure in conscious rats." Am J Physiol Regul Integr Comp Physiol **285**: R110-R116.

**Zhang**, S., Zeitzer, JM., Sakurai, T., Nishino, S., Mignot, E. (2007). "Sleep/wake fragmentation disrupts metabolism in a mouse model of narcolepsy." The Journal of Physiology **581(pt 2)**: 649-63.

**Zhang**, W., Fukuda, Y., Kuwaki, T. (2005). "Respiratory and cardiovascular actions of orexin-A in mice." Neuroscience Letters **385**: 131-36.

**Zhang**, W., Sakurai, T., Fukuda, Y., Kuwaki T. (2006). "Orexin neuron-mediated skeletal muscle vasodilation and shift of baroreflex during defense response in mice." American Journal of Physiology **290**: R1654-63.

**Zhang**, Y., Yanase-Fujiwara, M., Hosono, T., Kanosue, K. (1995). "Warm and cold signals from the preoptic area: which contribute more to the control of shivering in rats?" J Physiol. **485**: 195-202.

**Zhu**, Y., Miwa, Y., Yamanaka, A., Yada, T., Shibahara, M., Abe, Y., Sakurai, T., Goto K. (2003). "Orexin receptor type-1 couples exclusively to pertussis toxin-insensitive G-proteins, while orexin receptor type-2 couples to both pertussis toxin-sensitive and -insensitive G-proteins." J Pharmacol Sci. **92**(3): 259-66.

**Zoccoli**, G., Amici, R., Silvani, S. (2011). The hypothalamus and its function. Narcolepsy. C. Baumann, Bassetti, CL., Scammel, TE. NY, Springer.

**Zoccoli**, G., Andreoli, E., Bojic, T. (2001). "Central and baroreflex control of heart rate during the wake-sleep cycle in rat." Sleep **24**: 753-8.