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# AUTONOMIC MECHANISMS OF BLOOD PRESSURE ALTERATIONS DURING SLEEP IN OREXIN/HYPOCRETIN-DEFICIENT NARCOLEPTIC MICE

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## ABSTRACT

**Study Objectives:** increases in arterial pressure (AP) during sleep and smaller differences in AP between sleep and wakefulness have been reported in orexin (hypocretin)-deficient mouse models of narcolepsy type 1 (NT1) and confirmed in NT1 patients. We tested whether these alterations are mediated by parasympathetic or sympathetic control of the heart and/or resistance vessels in an orexin-deficient mouse model of NT1.

**Methods:** 13 orexin knock-out (ORX-KO) mice were compared with 12 congenic wild-type (WT) mice. The electroencephalogram, electromyogram, and AP of the mice were recorded in the light (rest) period during intraperitoneal infusion of atropine methyl nitrate, atenolol, or prazosin to block muscarinic cholinergic,  $\beta_1$ -adrenergic, or  $\alpha_1$ -adrenergic receptors, respectively, while saline was infused as control.

**Results:** AP significantly depended on a 3-way interaction among the mouse group (ORX-KO vs WT), the wake-sleep state, and the drug or vehicle infused. During the control vehicle infusion, ORX-KO had significantly higher AP values during REM sleep, smaller decreases in AP from wakefulness to either non-rapid-eye-movement (non-REM) sleep or REM sleep, and greater increases in AP from non-REM sleep to REM sleep compared to WT. These differences remained significant with atropine methyl nitrate, whereas they were abolished by prazosin and, except for the smaller AP decrease from wakefulness to REM sleep in ORX-KO, also by atenolol.

**Conclusions:** sleep-related alterations of AP due to orexin deficiency significantly depend on alterations in cardiovascular sympathetic control in a mouse model of NT1.

**Keywords:** orexin/hypocretin, narcolepsy, arterial pressure, sympathetic, vagal, dipping, mouse

## STATEMENT OF SIGNIFICANCE

In patients with narcolepsy type 1 (NT1), loss of orexin-producing neurons causes neurological signs including excessive daytime sleepiness and cataplexy. Inappropriately high values of arterial pressure during rapid-eye-movement sleep, blunted arterial pressure differences between sleep states and wakefulness, and a non-dipping arterial pressure pattern have also been found in patients with NT1. Here, we report that arterial pressure alterations during sleep in the orexin-deficient mouse model of NT1, which closely mirror those in NT1 patients, resulted from alterations in sympathetic control of the heart and resistance vessels. These data raise specific hypotheses on the autonomic mechanisms of sleep-related cardiovascular alterations in patients with NT1.

## INTRODUCTION

Narcolepsy type 1 (NT1) is a chronic neurological disease mainly characterized by excessive daytime sleepiness, cataplexy, disrupted nocturnal sleep, sleep-related hallucinations and paralysis, and reduced latency of rapid-eye-movement (REM) sleep with sleep-onset REM sleep periods.<sup>1</sup> Although less often appreciated, sleep-related cardiovascular alterations have long been described in patients with NT1.<sup>2,3</sup> These alterations include inappropriately high values of arterial pressure (AP) during nocturnal REM sleep<sup>4</sup> and a non-dipping AP pattern, whereby AP falls less than expected during nocturnal sleep compared to daytime wakefulness.<sup>5</sup> Recent data suggest that children and adolescents with NT1 close to disease onset already show a blunted fall in AP from wakefulness to either non-rapid-eye-movement (NREM) sleep or REM sleep.<sup>6</sup> Obstructive sleep apnea, which is known to increase AP, may be particularly deleterious for cardiovascular health in people with prevalent cardiovascular disease when it occurs primarily during REM sleep.<sup>7</sup> Cardiovascular control during REM sleep may thus be relevant for cardiovascular risk. Moreover, inappropriately high values of AP during nocturnal sleep and a non-dipping AP pattern are clinical indices of increased cardiovascular risk in the general population,<sup>8</sup> and may contribute to the increase in cardiovascular risk, which has been recently reported in patients with narcolepsy.<sup>9</sup> However, the mechanisms of AP alterations associated with narcolepsy in general, and with NT1 in particular, still represent an open question.<sup>10</sup>

The key event in NT1 pathophysiology is the functional loss, possibly triggered by an autoimmune reaction,<sup>11</sup> of the hypothalamic neurons that release the orexin (also called hypocretin) peptides.<sup>12</sup> Orexin knock-out (ORX-KO) mice have congenital orexin deficiency<sup>13</sup> but their orexin neurons remain viable, albeit with altered neurophysiological properties.<sup>14</sup> ORX-KO mice show key features of human NT1, including excessive daytime

sleepiness with sleep attacks fragmenting wakefulness, reduced REM sleep latency, and cataplexy,<sup>15</sup> demonstrating that the loss of orexin peptides is sufficient to cause the key clinical features of NT1.

Inappropriately high values of AP during REM sleep and a blunted fall in AP from wakefulness to either NREM sleep or REM sleep also occur in ORX-KO mice, and were, actually, discovered in this animal model by our group before their occurrence was reported in NT1 patients.<sup>16</sup>

Recently, we have developed a novel technique for continuous intraperitoneal infusion of different autonomic blockers during spontaneous sleep in mice instrumented for electrophysiologic and cardiovascular monitoring. With this technique, we have obtained evidence that the effects of sleep on AP in wild-type (WT) mice with the C57Bl/6J genetic background are critically mediated by changes in sympathetic vasoconstriction, with a minor role also played by changes in activity to the heart.<sup>17</sup> The C57Bl/6J genetic background is one of the most studied in mouse functional genomics and the first to have its genome sequenced. Here, we applied the same technique to ORX-KO mice with the C57Bl/6J background and compared the results with those previously obtained on WT mice. We aimed to test whether the sleep-related AP alterations in ORX-KO mice are due to alterations in parasympathetic control of the heart, sympathetic control of the heart, or sympathetic control of resistance vessels.

## METHODS

### **Ethical approval**

The study protocol was complied with the EU Directive 2010/63/EU for animal experiments and was approved by the Committee on the Ethics of Animal Experiments of the University of Bologna (prot. n. 30209) and of the Italian Ministry of Health (prot. n. 141/2018-PR). Surgery was performed under deep anesthesia, and all efforts were made to minimize suffering.

### **Mice**

Experiments were performed on 13 male ORX-KO mice<sup>13</sup> fully congenic (>10 generations of backcrossing) to the C57Bl/6J strain. Results were compared with those previously obtained with the same experimental protocol on 12 age-matched male WT mice of the C57Bl/6J strain ( $32.3 \pm 1.5$  vs  $33.6 \pm 0.4$  weeks of age, mean  $\pm$  SEM,  $P = 0.413$ , t-test), recently published.<sup>17</sup> All mice were housed under a 12:12-h light–dark cycle with ambient temperature set at 21–23°C and free access to water and food (4RF21diet; Mucedola, Settimo Milanese, Italy) in the facilities of the Department of Biomedical and Neuromotor Sciences, University of Bologna, Italy. The ORX-KO mice were obtained with heterozygote x homozygote or homozygote x homozygote mating. The genotype of ORX-KO mice was assessed in the facilities of the Centre for Applied Biomedical Research – CRBA, S. Orsola University Hospital, Bologna, Italy from ear tissue biopsies with polymerase chain reaction as previously described.<sup>16</sup>

## Experimental protocol

The details of the experimental protocol have been recently published<sup>17</sup> and a graphical summary is provided in Figure 1. Briefly, mice underwent surgery under isoflurane anesthesia (1.8–2.4% in O<sub>2</sub>, inhalation route) with intra-operative analgesia (Carprofen 0.1 mg subcutaneously, Pfizer Italy, Latina). The mice were implanted with two electroencephalographic (EEG) screw electrodes (frontal-parietal differential lead) and two electromyographic (EMG) wire electrodes in the neck muscles. A calibrated telemetric AP transducer (TA11-PAC10, DSI, New Brighton, MN, USA) was also implanted subcutaneously with the catheter inserted in the abdominal aorta below the renal arteries via the femoral artery. Finally, a catheter crafted in the laboratory from medical-grade silicone (Silclear Degania, Defries Industries, Australia) with length of 82 mm and volume of approximately 20  $\mu$ L was inserted in the peritoneal cavity and tunneled to the head.

After 3 weeks' recovery from surgery and habituation to the recording environment, each mouse was scheduled to undergo 6 recording sessions, each one taking place during the first 8 h of the light cycle. Three recording sessions (test sessions) were performed with intraperitoneal infusion of atropine methyl nitrate, atenolol, or prazosin, in random order at 1-week intervals. The other 3 recording sessions (control sessions) were performed with intraperitoneal infusion of saline, which was the vehicle of each drug, 2 days before each test session. After the sixth recording session, mice were euthanized by cervical dislocation under deep anesthesia (isoflurane 4% in O<sub>2</sub>) and autopsied.

During each recording session, the AP signal was acquired via telemetry, whereas the EEG and EMG signals were acquired via an electrical cable connected to a rotating swivel (Plastics One, Roanoke, VA, USA), which was mounted on a balanced suspensor arm to allow unhindered movement to the mice. Ambient temperature was set at 25°C during the



recordings. Data acquisition was performed with LabVIEW software (National Instruments, Austin, TX, USA). At the start of each recording session, a remote infusion pump (model 22 multiple syringe pump, Harvard Apparatus, Holliston, MA, USA) was connected to the intraperitoneal catheter by means of an extracorporeal tube that could easily accommodate twisting because of its length, making a fluid swivel unnecessary. This tube was pre-filled with either sterile saline or drug solutions before each recording session. Before the start of each recording session, a rapid infusion of saline or drug solution was performed for 5 min at a rate of  $30 \mu\text{L min}^{-1}$  to fill the intraperitoneal catheter. This infusion also transferred approx.  $50 \mu\text{L}$  of saline or drug solution to the peritoneal cavity in approx. 1.7 min. After this initial bolus loading, recordings were started, and infusion of saline or drug solution was performed at  $100 \mu\text{L h}^{-1}$  before recordings were eventually stopped.

## Drugs

Atropine methyl nitrate (SML0732, Sigma-Aldrich, St Louis, MO, USA), a muscarinic acetylcholine receptor antagonist, was infused at  $0.5 \text{ mg mL}^{-1}$  to block parasympathetic activity on the heart. Atenolol (A7655, Sigma-Aldrich), a hydrophilic selective  $\beta_1$ -adrenergic receptor antagonist with limited blood–brain barrier transport, was infused at  $0.25 \text{ mg mL}^{-1}$  to block sympathetic activity on the heart. Prazosin hydrochloride (P7791, Sigma-Aldrich), an  $\alpha_1$ -adrenoceptor antagonist, was infused at  $0.25 \text{ mg mL}^{-1}$  to block sympathetic vasoconstrictor activity. Each drug was dissolved in sterile saline before each recording session. The drugs and their concentrations were selected based on published evidence as previously discussed in detail.<sup>17</sup> The mouse weight was not measured before each recording session because this would have involved stressing the mouse due to handling and tubing/cable disconnection and connection, with consequences for sleep and cardiovascular

control during the subsequent recordings. Drug concentrations or infusion rates were, therefore, not scaled to the mouse weight.

## Data analysis

Data analysis was performed with software written in Matlab (Mathworks, Natick, MA, USA). The states of wakefulness, NREM sleep and REM sleep were discriminated with 4-s time resolution by trained investigators based on inspection of raw EEG and EMG signals following published criteria.<sup>16,17</sup> Sleep structure was assessed by computing the percentage of recording time spent in wakefulness, NREM sleep and REM sleep, the average duration of spontaneous episodes of these wake-sleep states, and the average latency from the end of a wakefulness episode to the onset of the subsequent REM sleep episode. Cataplexy-like episodes were scored according to consensus criteria.<sup>18</sup> Episode duration, REM sleep latency and cataplexy-like episodes were analyzed taking into account only episodes with duration  $\geq 12$  s as previously described.<sup>16</sup> This was meant to limit the confounding effect of the very short wake-sleep bouts, which are common in mice.<sup>19</sup> The values of mean AP (MAP, the average AP in each cardiac cycle) and heart period (HP, the time between the onset of successive systolic upstrokes, akin to the electrocardiogram-derived R-R interval) were computed from the raw AP signal for each heartbeat, and averaged for each wake-sleep state and each mouse.<sup>20</sup> To narrow down the search for mechanistic links between orexin deficiency and cardiovascular autonomic changes during sleep, we also estimated cardiac baroreflex sensitivity (cBRS) based on beat-to-beat values of systolic AP and HP with the sequence technique.<sup>21</sup> The analysis of cBRS was performed on all artifact-free episodes of wakefulness, NREM sleep, and REM sleep of duration  $\geq 60$  s, consistently with our previous work on cardiovascular variability in mice.<sup>21,22</sup> Data obtained during the first hour of

recordings were excluded from analysis as a precaution to avoid the effects of the initial transient in the circulating drug concentration.

### Statistical analysis

The statistical analysis was performed with SPSS Statistics (IBM Corp., Armonk, NY, USA) with the level of significance (alpha level) set at  $P < 0.05$ . To simplify interpretation of the results, we preliminarily tested for systematic differences in MAP and HP among vehicle infusions of different control recording sessions. A 3-way mixed-model analysis of variance (ANOVA) with the mouse groups (2 levels), recording sessions (3 levels), and wake-sleep states (3 levels) as factors did not detect significant main effects or interactions of the recording sessions (data not shown). The results obtained during the different control recording sessions were, therefore, averaged for each mouse. Three-way mixed model ANOVAs were then performed on MAP, HP, and cBRS with mouse groups (2 levels), drugs/vehicle (4 levels: average control recording session with vehicle infusion, atropine methyl nitrate, atenolol, and prazosin), and wake-sleep states (3 levels) as factors. In case of significance of the 3-way interaction, simple effects of the mouse groups were assessed with independent-sample t-tests. Independent-sample t-tests were also employed to test for differences in age and weight between ORX-KO and WT mice. The data on sleep structure were analyzed with 2-way ANOVA with the mouse groups (2 levels) and drugs/vehicle (4 levels, cf. above) as factors. In case of significant effects of the drugs/vehicle factor without significant interactions, the effect of each drug was compared with that of the average control recording session with ANOVA simple contrasts. The Huynh-Feldt correction was applied if the ANOVA sphericity assumption was not met. All data are reported as means  $\pm$  SEM.

Because of battery failure of AP telemetric transducers, we lost data from at least one drug infusion in 4 WT mice and in 2 ORX-KO mice. As a result, ANOVAs were performed with a

sample size of 8 WT vs 11 ORX-KO mice with the complete drug infusion set. Simple effects of the mouse group were analyzed with t-tests with sample sizes of 12 WT vs 13 ORX-KO mice for the average control recording session, of 10 WT vs 12 ORX-KO mice for atropine methyl nitrate and atenolol infusions, and of 10 WT vs 13 ORX-KO mice for prazosin infusions. A statistical power analysis (G\*power web application)<sup>23</sup> was performed based on the effect size of the difference in MAP during REM sleep previously reported between ORX-KO and WT mice.<sup>16</sup> This analysis indicated that sample sizes of 10 vs 12 mice with alpha level of  $P < 0.05$  and two-tailed independent-sample t-test afforded a 99% statistical power.

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## RESULTS

At surgery, the body weight of ORX-KO mice was significantly higher than that of WT mice ( $34.5 \pm 0.7$  g vs.  $31.7 \pm 0.7$  g,  $P = 0.012$ , t-test).

### Wake-sleep architecture

The effects of drugs on the wake-sleep architecture in ORX-KO and WT mice are shown in Table 1. ANOVA did not reveal any significant interaction between drugs and mouse groups for any variable under study ( $P \geq 0.148$ ). ORX-KO significantly differed from WT mice in terms of lower mean duration of wakefulness and REM sleep episodes and of shorter REM sleep latency, consistent with their NT1 phenotype ( $P = 0.001$ ,  $P = 0.025$ , and  $P = 0.005$ , respectively; ANOVA main effects). Drugs significantly affected the percentage of recording time spent in REM sleep ( $P = 0.002$ , ANOVA main effect), which was significantly lower during atropine methyl nitrate than during saline administration ( $P = 0.0002$ , ANOVA simple contrast). Drugs also significantly affected the average duration of wakefulness, NREM sleep and REM sleep episodes ( $P = 0.000001$ ,  $P = 0.0005$ , and  $P = 0.004$ , respectively; ANOVA main effects) and REM sleep latency ( $P = 0.005$ , ANOVA main effect). Each of these variables was significantly lower during administration of any drug than during saline administration ( $P \leq 0.040$ , ANOVA simple contrasts; cf. Supplementary Table 1 for detailed  $P$  values). Cataplexy-like episodes did not occur during the recordings, except for 1 episode in 1 ORX-KO mouse during prazosin administration.

## Mean arterial pressure

The effects of autonomic receptor blockers on MAP as a function of the wake-sleep state in ORX-KO and WT mice are shown in Figure 2. ANOVA on MAP values revealed a significant 3-way interaction of mouse groups, drugs, and wake-sleep states ( $P = 0.001$ ), significant 2-way interactions between wake-sleep states and either mouse groups or drugs, and significant main effects of drugs and wake-sleep states (all  $P < 0.001$ ). The main effect of mouse groups ( $P = 0.204$ ) and the 2-way interaction between mouse groups and drugs ( $P = 0.639$ ) were not statistically significant. Analysis of simple effects with t-tests indicated that MAP was significantly higher in ORX-KO than in WT mice during REM sleep during saline ( $P = 0.018$ , Figure 2A) and atropine methyl nitrate ( $P = 0.036$ , Figure 2B) administration, but not during atenolol ( $P = 0.131$ , Figure 2C) or prazosin ( $P = 0.074$ , Figure 2D) administration. To better characterize the significant interactions involving mouse groups and wake-sleep states, a further analysis was performed on the differences in MAP ( $\Delta$ MAP) between NREM sleep and wakefulness, between REM sleep and wakefulness, and between NREM sleep and REM sleep (Figure 3). During saline administration (Figure 3A),  $\Delta$ MAP between either NREM sleep or REM sleep and wakefulness was significantly smaller in ORX-KO compared with WT mice, whereas  $\Delta$ MAP between NREM sleep and REM sleep was significantly greater in ORX-KO compared with WT mice ( $P = 0.008$ ,  $P < 0.001$ , and  $P = 0.047$ , respectively; t-test). The significance and direction of these differences between ORX-KO and WT mice were preserved during atropine methyl nitrate administration ( $P < 0.001$ ,  $P < 0.001$ , and  $P = 0.004$ , respectively; t-test; Figure 3B). During atenolol administration (Figure 3C),  $\Delta$ MAP between REM sleep and wakefulness was significantly smaller in ORX-KO compared with WT mice ( $P = 0.039$ , t-test), whereas  $\Delta$ MAP between NREM sleep and wakefulness and  $\Delta$ MAP between NREM sleep and REM sleep did not differ significantly

between ORX-KO and WT mice ( $P = 0.784$  and  $P = 0.130$ , respectively; t-test). During prazosin administration (Figure 3D),  $\Delta$ MAP between NREM sleep and wakefulness,  $\Delta$ MAP between REM sleep and wakefulness, and  $\Delta$ MAP between NREM sleep and REM sleep did not differ significantly between ORX-KO and WT mice ( $P = 0.189$ ,  $P = 0.258$ , and  $P = 0.909$ , respectively; t-test).

### **Heart period and cardiac baroreflex sensitivity**

The effects of autonomic receptor blockers on HP are shown in Figure 4. ANOVA on HP values revealed a significant 2-way interaction between drugs and wake-sleep states as well as significant main effects of the drugs and of the wake-sleep states (all  $P < 0.001$ ). The 3-way interaction of the mouse groups, drugs, and wake-sleep states ( $P = 0.311$ ), the 2-way interactions between mouse groups and either drugs ( $P = 0.664$ ) or wake-sleep states ( $P = 0.591$ ), and the main effects of mouse groups ( $P = 0.925$ ) were not statistically significant.

The effects of autonomic receptor blockers on cBRS are shown in Figure 5. ANOVA on cBRS values revealed significant main effects of the drugs ( $P = 0.011$ ) and of the wake-sleep states ( $P < 0.001$ ). The 3-way interaction of the mouse groups, drugs, and wake-sleep states ( $P = 0.797$ ), the 2-way interactions between drugs and wake-sleep states ( $P = 0.075$ ) and between mouse groups and either drugs ( $P = 0.709$ ) or wake-sleep states ( $P = 0.075$ ), and the main effects of mouse groups ( $P = 0.343$ ) were not statistically significant.

In the absence of significant interactions involving the mouse groups and the wake-sleep states, no further analysis was performed on simple effects of the mouse group on HP and cBRS values in each wake-sleep state and on the differences in HP and cBRS between wake-sleep states.

## DISCUSSION

The main findings of our study were that the sleep-related differences in MAP between ORX-KO and WT mice were entirely abolished by prazosin and mostly abolished by atenolol.

During saline administration, the differences we found in MAP and sleep structure between ORX-KO and WT mice confirmed previous findings of our group.<sup>16</sup> In our previous study, however, ORX-KO mice also showed increased MAP during non-REM sleep, increased heart rate during each wake-sleep state, and increased rate of cataplexy-like episodes compared to WT mice.<sup>16</sup> Differences in the mouse age (14-15 vs. 32-33 weeks of age), surgical preparation, and mouse volume loading (in our previous work,<sup>16</sup> mice were not instrumented for intraperitoneal infusions) may possibly account for discrepancies in these variables. A 2017 review of the available evidence concluded that the association between alterations in cardiac (as opposed to AP) control and NT1 in human patients and animal models was a matter of controversy and inconsistency.<sup>3</sup> The same review concluded that NT1 may be associated with AP values that are lower than normal in wakefulness and normal during sleep, or normal in wakefulness and higher than normal during sleep, particularly during REM sleep.<sup>3</sup> On the other hand, the lack of cataplexy-like episodes in ORX-KO mice in this study may have been, at least in part, because recordings were restricted to the light period.<sup>24</sup> Finally, the lack of significant differences in cBRS between ORX-KO and WT mice in this study was in line with the results of a previous study by our group<sup>21</sup> and, more generally, with the available data on patients with NT1 and animal models thereof.<sup>3</sup>

The lack of significant differences in MAP and in  $\Delta$ MAP between ORX-KO and WT mice during prazosin administration indicates that alterations in the sympathetic control of resistance vessels may be sufficient to mediate sleep-related AP alterations due to orexin deficiency in NT1. Although based on lack of statistical significance, this suggestion is



strengthened by the significance of the ANOVA 3-way interaction of drugs, wake-sleep states, and mouse groups, and supported by the high estimated statistical power of the tests (cf. Methods). This conclusion is also in line with our previous analysis of data on WT mice, which showed that modulation of sympathetic vasoconstriction is critical for the effects of sleep on AP.<sup>17</sup> However, blunted, non-significant differences in AP between wakefulness and NREM sleep have been reported in patients with NT1 in the face of significant sleep-related decreases in sympathetic nerve activity to skeletal muscle and skin blood vessels measured with microneurography.<sup>25</sup> This discrepancy may be only apparent, as data on rats indicate that sympathetic nerve activity to the kidneys also decreases from wakefulness to NREM sleep, reaches its minimum values during REM sleep,<sup>26</sup> and causes an effective renal vasodilatation compared with wakefulness.<sup>27</sup> These data raise the hypothesis that sleep-related alterations in AP due to orexin deficiency in NT1 critically depend on an insufficient sleep-related reduction of renal sympathetic nerve activity. Intracerebroventricular administration of orexins increases AP, heart rate, and renal sympathetic nerve activity in conscious rats.<sup>28</sup> Our findings might thus result from over-compensation by non-orexinergic control of renal sympathetic nerve activity during sleep, and particularly during REM sleep. It should also be remarked that orexin deficiency did not altogether abolish the sleep-related  $\Delta$ MAP in ORX-KO mice. This result was in line with our previous results on ORX-KO mice,<sup>16</sup> which lack orexin peptides, and on orexin-ataxin 3 transgenic mice,<sup>16,29</sup> which lack the whole orexin neurons.<sup>15</sup> This result was also in line with the finding that a non-dipping pattern of diastolic AP, i.e., a nocturnal diastolic AP less than 10% lower than daytime AP, occurred only in 31% of adult patients with NT1, who are orexin-deficient by definition.<sup>5</sup> Together, these results highlight that the orexin system is not the sole determinant of the sleep-related changes in AP.<sup>30</sup>

A recent study found no evidence of cardiac sympathetic denervation at myocardial scintigraphy in patients with NT1,<sup>31</sup> but left open the question of functional differences in cardiac sympathetic nerve activity. In our study, the lack of significant differences in MAP and in  $\Delta$ MAP (with the exception of that in  $\Delta$ MAP between wakefulness and REM sleep) between ORX-KO and WT mice during atenolol administration indicates that alterations in the sympathetic control of the heart may be sufficient for sleep-related AP alterations due to orexin deficiency in NT1. However, we did not find alterations in HP control in ORX-KO compared to WT mice. Atenolol blocks the  $\beta_1$ -adrenergic receptors that not only decrease HP, but also increase contractility in response to sympathetic nerve activity to the heart.<sup>32</sup> Although opposite changes in HP and cardiac contractility often occur, the nervous system can affect each variable independently.<sup>33,34</sup> Our data thus raise the hypothesis that the sleep-related AP alterations in ORX-KO mice mainly depend on sympathetic increases in cardiac contractility. The significantly blunted  $\Delta$ MAP between wakefulness and REM sleep in ORX-KO mice during atenolol administration, taken together with its lack during prazosin administration, may indicate that the effects of cardiac sympathetic response alterations were somewhat less robust than that of alterations in sympathetic activity to resistance vessels. Surprisingly, little is known on physiological changes in cardiac sympathetic activity during sleep.<sup>35</sup> Indirect information obtained with the analysis of heart rate variability must be interpreted with caution because of the lack of consistent estimators of cardiac sympathetic modulation.<sup>36,37</sup> In our previous analysis of results on WT mice, we concluded that changes in sympathetic activity to the heart contributed to sleep-related changes in HP and to the increase in AP from NREM sleep to REM sleep.<sup>17</sup> However, our prior analysis focused on transitions from wakefulness to relatively long episodes of NREM sleep and vice versa.<sup>17</sup> Conversely, here we focused on  $\Delta$ MAP between episodes of wakefulness and NREM sleep

of any duration, allowing direct comparison with our original work on cardiovascular differences between ORX-KO and WT mice.<sup>16</sup>

We found that the sleep-related AP differences between ORX-KO and WT mice were fully preserved during administration of atropine methyl nitrate. These results demonstrate that alterations in cardiac parasympathetic control are not necessary to explain the sleep-related AP alterations associated with orexin deficiency and NT1. This conclusion is in line with the results of our previous analysis of data in WT mice, which indicated that changes in cardiac parasympathetic activity play a role in the sleep-related changes in HP, but not in those of AP.<sup>17</sup>

The administration of atropine methyl nitrate, atenolol, and prazosin partially disrupted sleep architecture. Sleep and arousal are significantly modulated by arterial baroreceptor afferents.<sup>38</sup> Therefore, sleep architecture changes may have resulted, at least in part, from the substantial AP and HP changes caused by the drugs. Nevertheless, it appears unlikely that sleep structure alterations could explain our results on AP. On the one hand, the reduction in REM sleep episode duration that was associated selectively with prazosin administration was relatively small, in the order of 10-20%. On the other hand, the sleep-related differences in AP between ORX-KO and WT mice were fully preserved during administration of atropine methyl nitrate, which fragmented wakefulness and NREM sleep episodes, and which decreased REM sleep latency and REM sleep time.

Our study has limitations. We did not study ORX-KO mice at the same time as WT littermates, but, rather, compared ORX-KO mice with WT control mice that had been previously studied.<sup>17</sup> However, the ORX-KO and WT mice had the same genetic background and were studied in the same facilities with an identical research protocol. This approach helped to reduce the number of animals employed for research, and was sensitive enough to confirm, during saline administration, the previously reported differences between ORX-KO

and WT mice<sup>16</sup> that are most robustly characteristic of NT1 in human patients and animal models.<sup>3</sup> However, as we studied only male mice, our work did not provide information on potential sex-related differences in the autonomic correlates of orexin deficiency and NT1.

We did not measure alterations in sympathetic and parasympathetic activities of ORX-KO mice directly, but, rather, estimated their effects by subtraction, evaluating differences between ORX-KO and WT mice during administration of autonomic receptor blockers. Direct measurements of autonomic nerve activity in freely behaving mice during spontaneous sleep have never been performed to our knowledge and would be challenging in these small animals. Nevertheless, our approach did not allow us to exclude that central nervous system effects particularly of prazosin<sup>39</sup> and, to a lesser extent, of atenolol,<sup>40</sup> contributed to our results. Moreover, our approach did not identify the central neural pathways that link the loss of orexin peptides to sleep-related changes in AP. Nevertheless, our results did narrow the focus on the central pathways that control the sympathetic nervous system and receive orexinergic input, including the medullary raphe obscurus, which may be key in the differential changes in regional sympathetic nerve activity during sleep.<sup>41</sup>

We did not adjust drug doses to mouse body weight. ORX-KO mice, which were significantly heavier than WT mice at surgery, may therefore have received lower doses per unit body weight than WT mice. Nevertheless, the differences in body weight between ORX-KO and WT mice were small, in the order of 10%, and if anything, they would be expected to enhance the cardiovascular differences between ORX-KO and WT mice.

We did not obtain dose-response curves for the effects of each drug on AP and HP. However, prazosin caused a clear-cut decrease in AP in each wake-sleep state. Moreover, we have previously obtained evidence that our doses of atropine and atenolol at least approximated complete receptor blockade.<sup>17</sup>

Our analysis of simple effects relied on a relatively large number of independent-sample t-tests. Recognizing that this may inflate the type I error rate, we analyzed simple effects only for MAP, for which ANOVA revealed a highly significant 3-way interaction including the mouse group, and not for HP or cBRS, for which such interaction was not significant. Our sample size, although substantial considering the technical complexity and workload of the experiments, was small in absolute terms. With the statistical approach that we followed, our statistical power analysis suggested that the type II error rate associated with lack of statistical significances in MAP and  $\Delta$ MAP during prazosin or atenolol administration was low. Had we also applied a correction for multiple comparisons, we would have risked being overly. It is worth remarking that all significant differences that we found concerned variables that also differed significantly between ORX-KO and WT mice in our previous work.<sup>16</sup> Moreover, we took care to report the numerical P value of each significant difference, thus providing readers with all the pieces of information useful to evaluate our results.

We performed this study on ORX-KO mice, which have congenital deficiency of orexin peptides.<sup>13</sup> We obtained ORX-KO mice with heterozygote x homozygote or homozygote x homozygote matings. Keeping track of the pedigree of each individual mouse is not standard practice in our internal mouse facility. As a result, we cannot determine which of the ORX-KO mice we studied was delivered by an ORX-KO dam. This may be a limitation. A research paper claimed in utero effect of maternally derived orexin on pups' phenotype, and particularly on brown adipose tissue thermogenesis, based on the finding of differentiation defect in ORX-KO pups, which was circumvented by orexin injections to ORX-KO dams.<sup>42</sup> However, these results were not replicated in recent work.<sup>43</sup> On the other hand, patients with NT1 lose the whole orexin neurons, including their released co-transmitters,<sup>44</sup> after birth. While the study of mouse models of NT1 that lose the whole orexin neurons after birth,<sup>15,45</sup> or of dog models of NT1,<sup>46</sup> may have yielded different results, ORX-KO mice still represent

the best mouse model to single out the contribution of orexin peptide deficiency to the key behavioral<sup>13</sup> and cardiovascular<sup>16</sup> features of NT1. Nevertheless, confirmation of the present results on human patients with NT1 is necessary before one can speculate on their clinical implications. Interestingly, these implications may also pertain to the choice of NT1 treatment options and management, as recent findings indicate higher diastolic AP and heart rate in patients with NT1 treated with psychostimulants than in untreated patients.<sup>47</sup>

In conclusion, our study indicates that alterations in sympathetic activity are sufficient to explain the sleep-related alterations of AP associated with orexin peptide deficiency in ORX-KO mice, with no evidence of alterations in cardiac baroreflex control. These results narrow down the search for a complete set of mechanistic links between orexin peptide deficiency and sleep-related AP alterations, and await replication in other animal models of NT1 and in patients with NT1. These studies may ultimately shed light on the sleep-related AP alterations associated with NT1, a potential contributor to cardiovascular risk in patients with NT1, with the goal of identifying druggable targets.

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## FIGURE CAPTIONS

### **Figure 1. Summary of the research protocol**

EEG: electroencephalogram. EMG: electromyogram. AP: arterial pressure. IP: intraperitoneal. Wks, weeks. RS: recording sessions.

### **Figure 2. Effects of autonomic receptor blockers on mean arterial pressure during wakefulness and sleep in orexin knock-out mice and wild-type control mice**

MAP: mean arterial pressure. W: wakefulness. NREM: non-rapid-eye-movement sleep. REM, rapid-eye-movement sleep. WT: wild-type mice. ORX-KO: orexin knock-out mice. Atropine methyl nitrate is indicated as atropine for brevity. Data are shown as mean  $\pm$  SEM. For WT mice, N = 12 (saline) or N = 10 (atropine, atenolol, and prazosin). For ORX-KO mice, N = 13 (saline and prazosin) or N = 12 (atropine and atenolol). \*: P < 0.05 vs. WT (t-test).

### **Figure 3. Effects of autonomic receptor blockers on differences in mean arterial pressure between wake-sleep states in orexin knock-out mice and wild-type control mice**

$\Delta$ MAP: difference in mean arterial pressure between wake-sleep states. W: wakefulness. NREM: non-rapid-eye-movement sleep. REM, rapid-eye-movement sleep. WT: wild-type mice. ORX-KO: orexin knock-out mice. Atropine methyl nitrate is indicated as atropine for brevity. Data are shown as mean  $\pm$  SEM. For WT mice, N = 12 (saline) or N = 10 (atropine, atenolol, and prazosin). For ORX-KO mice, N = 13 (saline and prazosin) or N = 12 (atropine and atenolol). \*: P < 0.05 vs. WT (t-test).

**Figure 4. Effects of autonomic receptor blockers on heart period during wakefulness and sleep in orexin knock-out mice and wild-type control mice**

HP: heart period. W: wakefulness. NREM: non-rapid-eye-movement sleep. REM, rapid-eye-movement sleep. WT: wild-type mice. ORX-KO: orexin knock-out mice. Atropine methyl nitrate is indicated as atropine for brevity. Data are shown as mean  $\pm$  SEM. For WT mice, N = 12 (saline) or N = 10 (atropine, atenolol, and prazosin). For ORX-KO mice, N = 13 (saline and prazosin) or N = 12 (atropine and atenolol).

**Figure 5. Effects of autonomic receptor blockers on cardiac baroreflex sensitivity during wakefulness and sleep in orexin knock-out mice and wild-type control mice**

cBRS: cardiac baroreflex sensitivity. W: wakefulness. NREM: non-rapid-eye-movement sleep. REM, rapid-eye-movement sleep. WT: wild-type mice. ORX-KO: orexin knock-out mice. Atropine methyl nitrate is indicated as atropine for brevity. Data are shown as mean  $\pm$  SEM. For WT mice, N = 12 (saline), N = 9 (atropine in wakefulness), or N = 10 (otherwise). For ORX-KO mice, N = 13 (saline), N = 12 (prazosin in NREM, atropine, atenolol), or N = 11 (prazosin in W or REM).

# TABLES

**Table 1. Effects of autonomic receptor blockers on sleep and wakefulness in orexin knock-out mice and wild-type controls**

		Saline		Atropine		Atenolol		Prazosin	
		WT	ORX-KO	WT	ORX-KO	WT	ORX-KO	WT	ORX-KO
W	%	32±2	31±2	31±3	34±4	33±1	31±1	33±2	28±2
	D (s)	116±11	83±7*	71±8†	54±4*†	83±5†	70±6*†	79±7†	44±6*†
NREM	%	56±2	57±2	60±3	59±3	56±1	59±1	56±2	62±2
	D (s)	76±4	71±2	68±4†	57±5†	59±4†	67±4†	56±5†	54±5†
REM	%	9±1	9±0	7±1†	6±1†	8±0	8±0	7±1	8±1
	D (s)	54±2	45±1*	55±5	42±3*	51±4	48±2*	44±5†	39±2*†
	L (s)	456±50	242±19*	315±37	212±30	280±43	207±14	278±44	154±21
	(s)			†	*†	†	*†	†	*†

W: wakefulness. NREM: non-rapid-eye-movement sleep. REM, rapid-eye-movement sleep. WT: wild-type mice. ORX-KO: orexin knock-out mice. %: percentage of recording time spent in each wake-sleep state. D: episode duration. L: latency. Atropine methyl nitrate is indicated as atropine for brevity. Data are shown as mean ± SEM. For WT mice, N = 12 (saline) or N = 10 (atropine, atenolol, and prazosin). For ORX-KO mice, N = 13 (saline and prazosin) or N = 12 (atropine and atenolol). \*: P < 0.05 vs. WT (ANOVA main effect). †: P < 0.05 vs. saline (ANOVA main effect and simple contrast).



Figure 1






SURGERY	RECOVERY and HABITUATION	RECORDING SESSIONS (RS)					
EEG electrodes EMG electrodes AP transducer IP catheter		eight hours' recordings of EEG, EMG, AP 100 $\mu\text{L h}^{-1}$ IP infusions <b>DRUG 1-2-3:</b> atropine methylnitrate, atenolol, or prazosin, in random order					
		SALINE	DRUG 1	SALINE	DRUG 2	SALINE	DRUG 3
							
Time 0 (32-34 wks of age)	(3 wks)	+21 days	+23 days	+28 days	+30 days	+35 days	+37 days

Figure 2

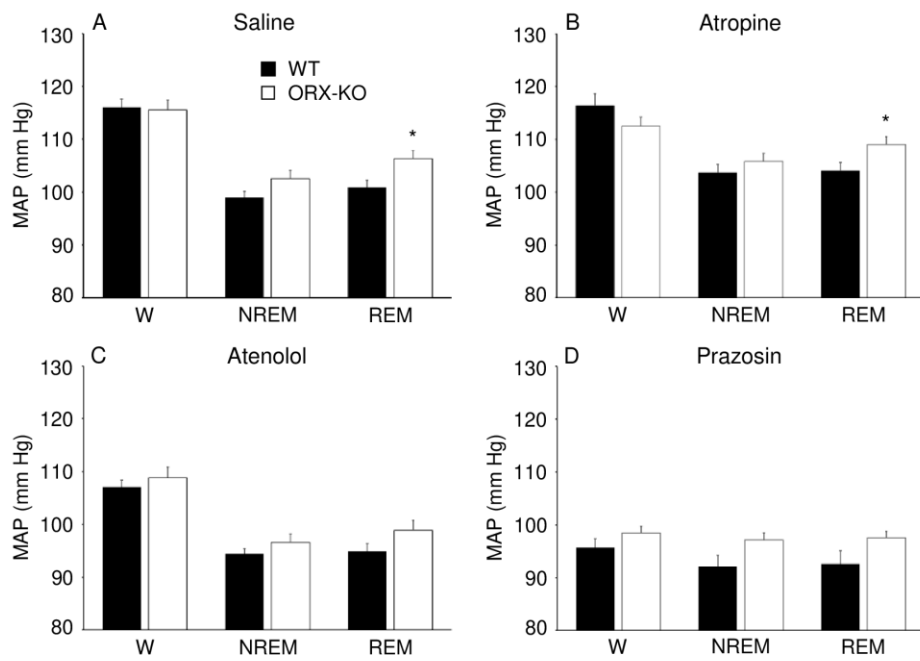


Figure 3

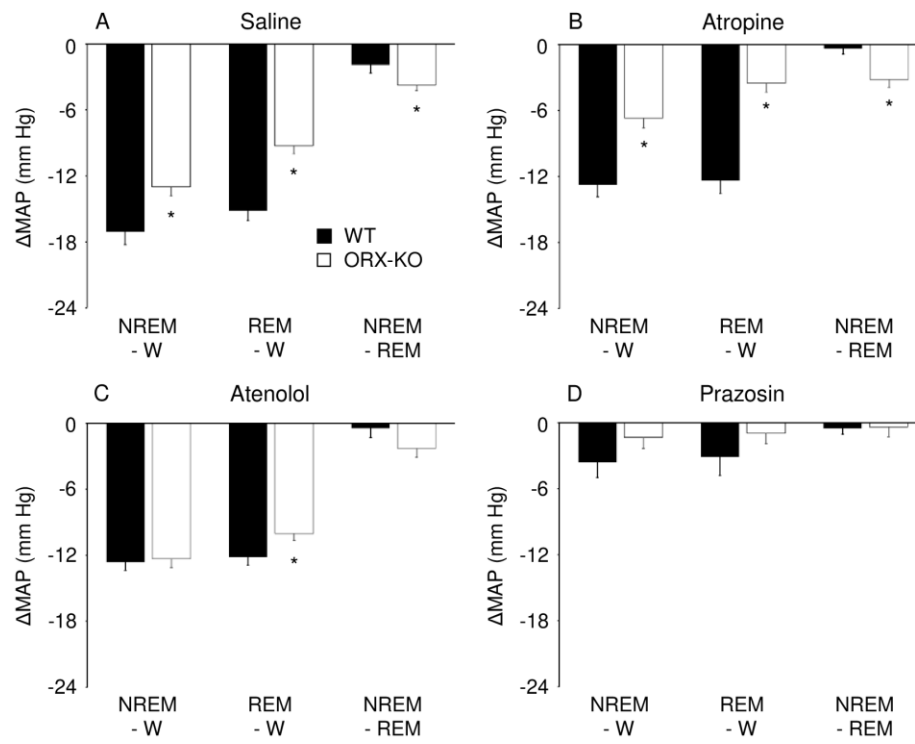


Figure 4

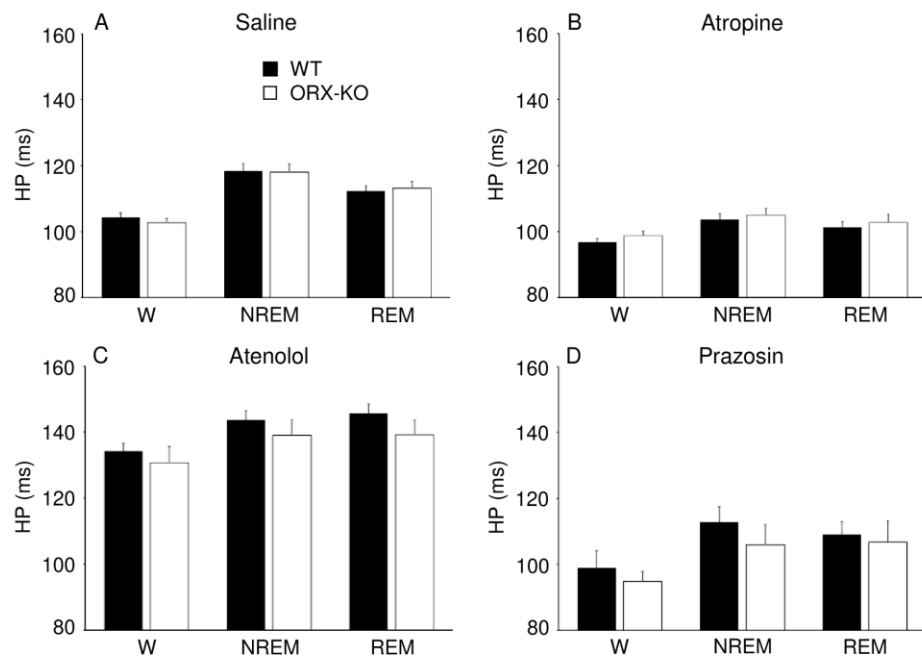


Figure 5

