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Hypothalamic orexin prevents hepatic insulin resistance induced by social defeat stress in mice

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ABSTRACT

Depression is associated with insulin resistance and type 2 diabetes, although the molecular mechanism behind the pathological link remains unclear. Orexin, a hypothalamic neuropeptide regulating energy and glucose homeostasis, has been implicated in the endogenous antidepressant mechanism. To clarify whether orexin is involved in the coordination between mental and metabolic functions, we investigated the influence of orexin deficiency on social interaction behavior and glucose metabolism in mice subjected to chronic social defeat stress. Chronic stress-induced glucose intolerance and systemic insulin resistance as well as social avoidance were ameliorated by calorie restriction in an orexin-dependent manner. Moreover, orexin-deficient mice maintained under ad libitum-fed conditions after defeat stress exhibited hyperinsulinemia and elevated HOMA-IR (homeostasis model assessment for insulin resistance), despite normal fasting blood glucose levels. In a pyruvate tolerance test to evaluate hepatic insulin sensitivity, chronic stress-induced abnormal glucose elevation was observed in orexin-deficient but not wild-type mice, although both types of mice were susceptible to chronic stress. In addition, insulin-induced phosphorylation of Akt in the liver was impaired in orexin-deficient but not wild-type mice after chronic stress. These results demonstrate that the central physiological actions of orexin under ad libitum-fed conditions are required for the adaptive response to chronic defeat stress, which can prevent the development of hepatic insulin resistance but not social avoidance behavior. Moreover, calorie restriction, a paradigm to strongly activate orexin neurons, appears to prevent the persistence of depression-like behavior *per se*, leading to the amelioration of impaired glucose metabolism after chronic stress; therefore, we suggest that hypothalamic orexin system is the key for inhibiting the exacerbating link between depression and type 2 diabetes.

1. Introduction

Depression and type 2 diabetes are both highly prevalent chronic diseases, and frequently comorbid (Mezuk et al., 2008; Renn et al., 2011). Furthermore, patients with comorbid depression and type 2 diabetes have been shown to have an increased risk of cardiovascular complications and a higher mortality rate than patients with diabetes alone (Nouwen et al., 2011). Although it is well established that depression and type 2 diabetes are associated, the primary cause remains unclear (Knol et al., 2006; Mezuk et al., 2008; Nouwen et al., 2011); however, increasing evidence suggests that depression is positively associated with insulin resistance as the main cause of type 2 diabetes (Timonen et al., 2006; Silva et al., 2012). Moreover, animal studies demonstrated that chronic restraint stress resulted in reduced insulin sensitivity without an increase in body weight gain (Uchida et al., 2012), and an antidepressant selective serotonin reuptake inhibitor (SSRI) restored insulin resistance in low-birth-weight rats regarded as both a pre-depressive and pre-diabetic model (Buhl et al., 2010). Therefore, blockade of the pathological link between depression and insulin resistance appears to be valuable to reduce the future risk of diabetes (Pan et al., 2012).

Chronic social defeat stress (CSDS) is a mouse model of chronic stress-induced psychiatric disorders, including major depression and anxiety (Krishnan et al., 2007). In particular, the social avoidance observed in this model is considered depression-like behavior, because it can be normalized by chronic, but not acute, treatment with SSRI (Berton et al., 2006). Furthermore, CSDS induces long-lasting metabolic abnormalities via sympathetic nervous system activation (Chuang et al., 2010b). The combination of CSDS and high-fat feeding resulted in the dysregulation of lipid metabolism (Chuang et al., 2010a); however, the influence of CSDS on glucose metabolism and adaptive mechanisms for preventing metabolic abnormalities during chronic stress have not been fully understood.

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Neuropeptides function as slow-acting neuromodulators (van den Pol, 2012). Some neuropeptides, such as neuropeptide Y and orexin, that modulate the stress response or adaptation further play crucial roles in the hypothalamic regulation for maintenance of whole-body glucose and energy homeostasis (Alldredge, 2010; Scott et al., 2011; Blouet and Schwartz, 2010). Therefore, abnormal functions of hypothalamic neuropeptides may underlie the synergistic development of depression and metabolic disorders during chronic stress.

Orexin (hypocretin) neurons producing orexin-A and -B are localized in the lateral, perifornical and posterior hypothalamus and act as an interface of systems of emotion, reward, energy/glucose homeostasis and arousal (Sakurai and Mieda, 2011; Tsuneki et al., 2008; Tsuneki et al., 2012). In depressive patients, daily oscillation of orexin A levels in the cerebrospinal fluid (CSF) is reported to be dampened (Salomon et al., 2003), and suicidal patients with major depressive disorders showed reduced CSF orexin A levels (Brundin et al., 2007). Also, mice subjected to CSDS exhibit diminished levels of orexins in the midbrain dopamine system and hypothalamus (Nocjar et al., 2012), whereas calorie restriction (CR) exerts an antidepressant-like effect by increasing the activity of orexin neurons in mice after CSDS (Lutter et al., 2008). In narcoleptic patients with orexin deficiency, depressive symptoms are strikingly overrepresented (Fortuyn et al., 2011), and the frequency of obesity and type 2 diabetes is increased (Honda et al., 1986; Schuld et al., 2000). We therefore hypothesized that orexin plays an important role in the adaptive response to chronic stress for preventing the development of both psychiatric and metabolic abnormalities in parallel.

The aim of the present study was to clarify how orexin is involved in the regulation of glucose metabolism under chronic stress conditions. To this end, we investigated the influences of orexin deficiency using preproorexin-deficient (*Orexin*$^{-/-}$) mice or activation of the orexin system induced by CR on social interaction behavior, glucose tolerance, systemic insulin sensitivity, hepatic glucose production, and insulin signaling in the liver.

2. Materials and methods

2.1. Materials

Human regular insulin Novolin R was provided by Novo Nordisk (Copenhagen, Denmark). Anti-insulin receptor substrate 1 (IRS1) antibody and anti-IRS2 antibody were purchased from Millipore (Temecula, CA). Anti-insulin receptor β subunit antibody and anti-Akt1 antibody were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (Ser^{473}) antibody and anti-β-actin monoclonal antibody were from Cell Signaling Technology (Beverly, MA). All other reagents were purchased from Sigma–Aldrich Japan (Tokyo, Japan) or Wako Pure Chemical Industries (Osaka, Japan), unless otherwise indicated.

2.2. Animals

Mice were housed at 23–25 °C under a 12:12 h light–dark cycle (lights on from 0700 to 1900 h) with free access to normal chow diet (PicoLab Rodent Diet 20; Japan SLC, Hamamatsu, Japan) and water. *Orexin*$^{-/-}$ mice were generated as described previously (Chemelli et al., 1999). *Orexin*$^{+/-}$ mice (N11 backcross to C57BL/6J) were crossed, and the *Orexin*$^{-/-}$ offspring were mated to obtain the male *Orexin*$^{-/-}$ mice used in this study. Male C57BL/6J mice were purchased from Japan SLC and used as wild-type (WT) controls. Male ICR (CD-1) mice were also from Japan SLC. All experimental procedures used in this study were approved by the Committee for Animal Experiments at the University of Toyama.

2.3. Chronic social defeat stress (CSDS)

The CSDS test was performed as shown in Fig. 1A, according to previously described methods (Krishnan et al., 2007; Chuang et al., 2010b) with slight modification. In brief, a male WT or *Orexin*$^{-/-}$ mouse of 2–3 months old was intruded into the home cage of an aggressive male ICR mouse of 2–4 months old, and exposed to defeat stress with physical attack by the resident ICR mouse for 10 min. The intruding mouse was further exposed to subsequent 24-h stress with aversive sensory stimulation in the home cage of the resident mouse, where the intruding and resident mice were separated with a transparent plastic partition plate with small holes. The CSDS stimulation was repeated for 10 consecutive days, using a different ICR mouse every day. Similar handling was applied to non-stressed controls without using ICR mice. To evaluate the behavioral consequences, the social interaction test was performed at day 11 and 26, and the social interaction ratio (percentage) of time spent in the interaction zone in the absence versus presence of unfamiliar ICR mice during 150-s of observation was calculated. Defeated mice with a social interaction ratio <100 and >100 were considered 'susceptible' and 'unsusceptible' to CSDS, respectively. Unsusceptible mice were not included in the following experiments. The amount of food intake was measured from day 14 to 16. Thereafter, mice were randomly selected and subjected to either ad libitum feeding (AL) or the CR regimen. The CR group was food restricted (70% of ad libitum intake), based on the average food intake for three days. Consequently, eight subgroups were prepared: control WT-AL group, control WT-CR group, susceptible WT-AL group, susceptible WT-CR group, control *Orexin*$^{-/-}$-AL group, control *Orexin*$^{-/-}$-CR group, susceptible *Orexin*$^{-/-}$-AL group, and susceptible *Orexin*$^{-/-}$-CR group.

2.4. Glucose tolerance test, insulin tolerance test, and pyruvate tolerance test

On experimental day 27 (Fig. 1; 17 days after the last defeat stress), mice were fasted for 5 h and then injected with either glucose (2 g/kg, i.p.), insulin (0.75 U/kg, i.p.), or pyruvate (2 g/kg, i.p.) for the respective tolerance tests. Five-hour fasting was chosen according to a recently established method (Andrikopoulos et al., 2008). Blood samples were collected from the tail vein at the indicated times. Blood glucose levels were measured using a FreeStyle Freedom glucose meter (Nipro, Osaka, Japan).

2.5. Measurements of serum parameters

On experimental day 29 (Fig. 1; 19 days after the last defeat stress), mice were fasted for 5 h and then blood samples were collected from the orbital sinus of mice at 1300 h. The samples were kept at room temperature for 1 h and then centrifuged at 900*g* for 30 min. The supernatants were obtained as serum samples and stored at −80 °C until use. Serum levels of insulin and leptin were measured by ELISA kits (Takara Bio, Shiga, Japan). Insulin sensitivity was assessed by homeostasis model assessment of insulin resistance (HOMA-IR), calculated as follows: HOMA-IR = (fasting serum glucose in mg/dl) × (fasting serum insulin in μU/ml)/405. Serum levels of corticosterone under ad libitum-fed conditions were measured using a corticosterone EIA kit (Cayman Chemical, Arbor, MI).

2.6. Western blot analysis

On experimental day 31 (Fig. 1; 21 days after the last defeat stress), mice were fasted for 5 h and then intraperitoneally (i.p.) injected with insulin (0.75 U/kg) or phosphate-buffered saline. Thirty minutes after injection, the liver tissue was rapidly isolated,

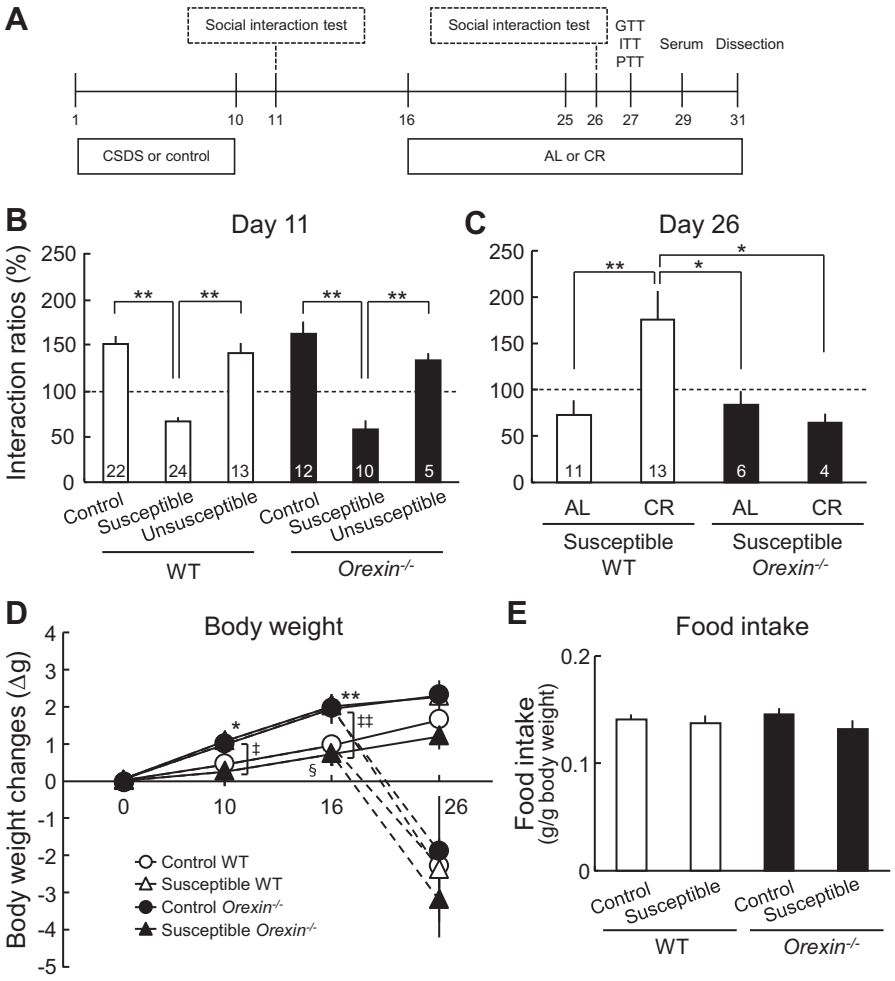

Fig. 1. Influence of chronic social defeat stress (CSDS) on social behavior, body weight, and food intake in wild-type and *Orexin*$^{-/-}$ mice. (A) Experimental design. Wild-type (WT) and *Orexin*$^{-/-}$ mice were subjected to CSDS for 10 days and then separated into susceptible and unsusceptible subpopulations. Subsequently, non-stressed control mice and susceptible mice were fed ad libitum (AL) or maintained under calorie-restricted (CR) conditions. (B) Social interaction ratios on day 11 in WT and *Orexin*$^{-/-}$ mice in the presence or absence of CSDS stimuli. Score <100 is the index of social avoidance (depression-like behavior) after CSDS. The number of mice in each data set is indicated within the column. $^{**}P < 0.01$. (C) Social interaction ratios on day 26 in susceptible WT and susceptible *Orexin*$^{-/-}$ mice under AL or CR condition. $^{*}P < 0.05$ and $^{**}P < 0.01$. (D) Changes in body weight under AL (solid lines) or CR (dotted lines between day 16 and 26) condition, $n = 3$–10. $^{*}P < 0.05$ and $^{**}P < 0.01$, control WT vs. susceptible WT mice; $^{\ddagger}P < 0.05$ and $^{\ddagger\ddagger}P < 0.01$, susceptible WT vs. susceptible *Orexin*$^{-/-}$ mice; $^{\S}P < 0.05$, control *Orexin*$^{-/-}$ vs. susceptible *Orexin*$^{-/-}$ mice. (E) Average food intake measured from day 14 to day 16 after CSDS, $n = 4$–6. Data are the means ± S.E.M.

homogenized, lysed and subjected to Western blot analysis, as previously described (Tsuneki et al., 2008).

2.7. Statistical analysis

Data are expressed as the means ± S.E.M. The significance of differences was assessed by one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test, using Statview software (SAS Institute, Cary, NC). $P < 0.05$ was considered significant.

3. Results

3.1. Influences of CSDS and orexin deficiency on whole-body glucose homeostasis in mice

We first conducted the social interaction test using WT and *Orexin*$^{-/-}$ mice after CSDS, and observed that both mice exhibited similar behavioral changes on experimental day 11, i.e., one day after the last defeat stress (Fig. 1B): 65% ($n = 24$) and 35% ($n = 13$) of WT mice and 67% ($n = 10$) and 33% ($n = 5$) of *Orexin*$^{-/-}$ mice became susceptible and unsusceptible to CSDS, respectively. These behavioral abnormalities were also observed in susceptible WT and susceptible *Orexin*$^{-/-}$ mice at day 26, i.e., 16 days after the last stress episode under ad libitum-fed conditions (Fig. 1C); however, the social avoidance phenotype was significantly ameliorated in susceptible WT ($P < 0.01$) but not susceptible *Orexin*$^{-/-}$ mice under CR conditions. Susceptible WT mice had gained more weight than non-stressed control WT mice by day 10 ($P < 0.05$) and 16 ($P < 0.01$), whereas susceptible *Orexin*$^{-/-}$ mice had gained less weight than control *Orexin*$^{-/-}$ mice by day 16 ($P < 0.05$) (Fig. 1D). More importantly, however, these changes had disappeared by day 26 both under ad libitum-fed and CR conditions. Food intake was not affected by CSDS and orexin deficiency under the present conditions (Fig. 1E).

Next, we investigated the influences of CSDS and orexin deficiency on whole-body glucose metabolism and systemic insulin sensitivity. In the glucose tolerance test in mice maintained under ad libitum-fed conditions, blood glucose levels 15 ($P < 0.05$), 30 ($P < 0.01$), and 60 min ($P < 0.05$) after glucose loading in susceptible WT mice were significantly higher than in control WT mice (Fig. 2A). In addition, blood glucose levels at 30 ($P < 0.01$), 60 ($P < 0.01$), and 120 min ($P < 0.05$) in control *Orexin*$^{-/-}$ mice were higher than in control WT mice. Furthermore, susceptible *Orex-*

in$^{-/-}$ mice exhibited significantly increased glucose levels at 15 ($P < 0.05$), 60 ($P < 0.01$), and 120 min ($P < 0.01$) compared to susceptible WT mice, and slightly increased levels at 15 min than control *Orexin*$^{-/-}$ mice ($P = 0.069$). On the other hand, CR improved the impaired glucose tolerance in susceptible WT and control *Orexin*$^{-/-}$ mice (Fig. 2B); however, blood glucose levels at 60 and 120 min in susceptible *Orexin*$^{-/-}$ mice were still higher than those in susceptible WT mice ($P < 0.05$). These abnormalities in glucose regulation were not due to obesity-related metabolic changes, since no group of mice showed abnormal weight gain on day 26, as shown in Fig. 1D. Moreover, the insulin tolerance test using mice maintained under ad libitum-fed conditions demonstrated that glucose levels at 120 min in susceptible *Orexin*$^{-/-}$ mice were significantly higher than in susceptible WT mice ($P < 0.05$), although CSDS had no direct effect on systemic insulin sensitivity in WT and *Orexin*$^{-/-}$ mice (Fig. 2C). Similar results were obtained under CR conditions (Fig. 2D). These results indicate that in the absence of endogenous orexin, CR failed to ameliorate CSDS-induced social avoidance and its associated impairment of blood glucose regulation.

3.2. Influences of CSDS and orexin deficiency on hepatic insulin sensitivity in mice

To explore the mechanism underlying the impaired glucose metabolism by CSDS and orexin deficiency, serum parameters were investigated in WT and *Orexin*$^{-/-}$ mice that were maintained under ad libitum-fed conditions after CSDS and fasted for 5 h. CSDS did not affect fasting blood glucose levels in WT and *Orexin*$^{-/-}$ mice, although a slight increase ($P = 0.070$) was observed in susceptible *Orexin*$^{-/-}$ mice compared to susceptible WT mice (Fig. 3A). Serum insulin levels were not altered by CSDS in WT mice; however, insulin levels in susceptible *Orexin*$^{-/-}$ mice were significantly higher ($P < 0.01$) than in control WT and susceptible WT mice, and slightly higher ($P = 0.066$) than in control *Orexin*$^{-/-}$ mice (Fig. 3B). Importantly, HOMA-IR, an index of hepatic insulin resistance (Abdul-Ghani et al., 2006), was exclusively increased in susceptible *Orexin*$^{-/-}$ mice (Fig. 3C). Moreover, serum leptin levels in susceptible *Orexin*$^{-/-}$ mice were significantly higher ($P < 0.01$) than in susceptible WT mice, and slightly higher ($P = 0.062$) than in control *Orexin*$^{-/-}$ mice (Fig. 3D). Neither CSDS nor orexin deficiency affected serum corticosterone levels in mice (Fig. 3E).

Since hepatic insulin resistance contributes to the promotion of hepatic glucose production (DeFronzo, 2009), glucose-producing activity is anticipated to be increased in the liver of susceptible *Orexin*$^{-/-}$ mice maintained under ad libitum-fed conditions. To address this hypothesis, we conducted the pyruvate tolerance test to evaluate the ability of the liver to produce glucose from a gluconeogenic substrate pyruvate, which is negatively regulated by endogenous insulin (Yao and Nyomba, 2008). Although there was no difference in glucose elevation after pyruvate loading among control WT, susceptible WT, and control *Orexin*$^{-/-}$ mice, the glucose levels at 30 min in susceptible *Orexin*$^{-/-}$ mice were significantly higher ($P < 0.05$) than in control *Orexin*$^{-/-}$ mice (Fig. 4A). In addition, the glucose levels at 30 and 60 min in susceptible *Orexin*$^{-/-}$ mice were significantly higher ($P < 0.01$) than in susceptible WT mice.

Finally, to reveal the impact of CSDS and orexin deficiency on insulin action in the liver, we analyzed hepatic insulin signaling in mice that were maintained under ad libitum-fed conditions after CSDS and fasted for 5 h. Neither CSDS nor orexin deficiency affected the protein levels of insulin receptor, IRS1, and IRS2 (data not shown). Furthermore, similar levels of Akt phosphorylation induced by insulin were observed in the liver of control WT, susceptible WT, and control *Orexin*$^{-/-}$ mice (Fig. 4B); however, the basal levels of Akt phosphorylation in the liver of susceptible *Orexin*$^{-/-}$ mice were apparently elevated, and insulin failed to cause a significant increase in the phosphorylation of Akt. These results indicate

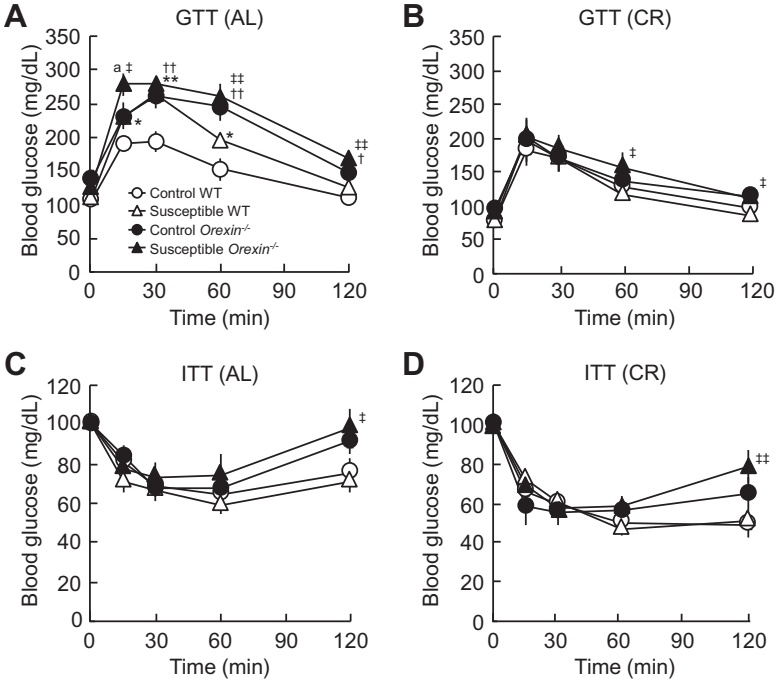

Fig. 2. CSDS-induced impairment of blood glucose regulation in WT and *Orexin*$^{-/-}$ mice. Control WT (open circles), susceptible WT (open triangles), control *Orexin*$^{-/-}$ (closed circles), and susceptible *Orexin*$^{-/-}$ mice (closed triangles) were fed ad libitum (AL) or maintained under calorie-restricted (CR) conditions. On day 27, mice were fasted for 5 h and then subjected to glucose tolerance test (GTT) under AL (A, $n = 4$–11) or CR (B, $n = 5$) condition and the insulin tolerance test (ITT) under AL (C, $n = 5$–10) or CR (D, $n = 4$–10) condition. Data are the means ± S.E.M. $^{*}P < 0.05$, control WT vs. susceptible WT mice; $^{\dagger}P < 0.05$ and $^{\dagger\dagger}P < 0.01$, control WT vs. control *Orexin*$^{-/-}$ mice; $^{\ddagger}P < 0.05$ and $^{\ddagger\ddagger}P < 0.01$, susceptible WT vs. susceptible *Orexin*$^{-/-}$ mice; $^{a}P < 0.1$, control *Orexin*$^{-/-}$ vs. susceptible *Orexin*$^{-/-}$ mice.

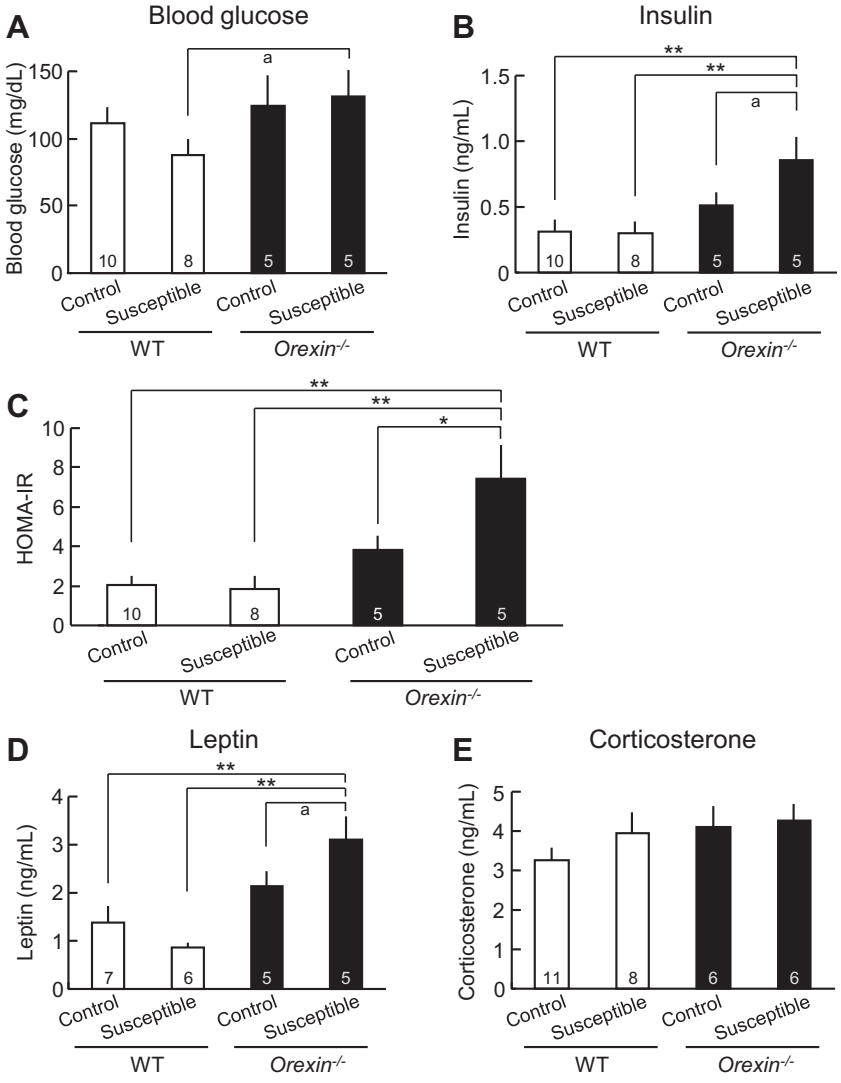

Fig. 3. Combined effects of CSDS and orexin deficiency on serum parameters in mice. On day 29, control WT, susceptible WT, control *Orexin*$^{-/-}$, and susceptible *Orexin*$^{-/-}$ mice maintained under ad libitum-fed condition were fasted for 5 h, and then serum samples were obtained. (A) Serum glucose levels; (B) Serum insulin levels; (C) HOMA-IR; (D) Serum leptin levels; (E) Serum corticosterone levels. Data are the means ± S.E.M. The number of mice in each data set is indicated within the column. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{a}P < 0.1$.

that hepatic insulin resistance was promoted by the combination of CSDS and orexin deficiency.

4. Discussion

Orexin is known to be a crucial factor for maintaining whole-body energy and glucose homeostasis under physiological conditions (Tsuneki et al., 2012); however, the functional significance of the orexin system under depressive conditions remains unknown. The present study demonstrated that CSDS, a mouse model of depression (Lutter et al., 2008), impaired glucose metabolism. In particular, hepatic glucose regulation was severely impaired by the combination of CSDS and orexin deficiency in mice. Moreover, CR strongly reversed the depressive symptoms and metabolic abnormalities via the orexin system. To our knowledge, this is the first report to demonstrate that the central actions of orexin are required for maintaining both mental and metabolic health under chronic stress conditions.

Since depression has an adverse effect on type 2 diabetes, a certain proportion of diabetes is considered to be preventable with intervention for depression (Silva et al., 2012). To develop a novel, effective antidepressant treatment, the neural mechanisms of resilience to stress, as well as the pathophysiological mechanisms of depression, need to be clarified (Krishnan and Nestler, 2008). A major finding of the present study is that the central actions of orexin are involved in the maintenance of glucose homeostasis in a depressive state. Under ad libitum-fed conditions, CSDS caused social avoidance disorder in WT and *Orexin*$^{-/-}$ mice, consistent with previous studies (Krishnan et al., 2007; Lutter et al., 2008). Nevertheless, WT mice subjected to CSDS exhibited normal HOMA-IR, an index of hepatic insulin resistance (Abdul-Ghani et al., 2006), and normal hepatic glucose production in the pyruvate tolerance test when compared to non-stressed control WT mice. In contrast, *Orexin*$^{-/-}$ mice exposed to CSDS exhibited increased HOMA-IR and increased hepatic glucose production, whereas no significant changes in these parameters were observed in non-stressed control *Orexin*$^{-/-}$ mice. Although orexin neurons are known to be activated under stress-like conditions associated with elevated levels of arousal and need for action (Berridge et al., 2010), the activity of orexin neurons under ad libitum-fed conditions is reported to

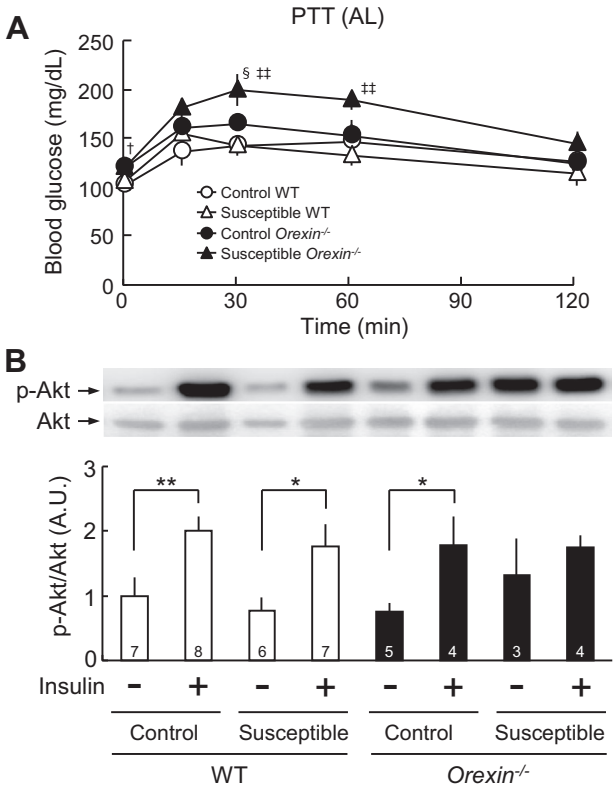

Fig. 4. Combined effects of CSDS and orexin deficiency on glucose production and insulin signaling in the liver of mice. (A) Pyruvate tolerance test (PTT) using control WT (open circles, $n = 5$), susceptible WT (open triangles, $n = 7$), control *Orexin*$^{-/-}$ (closed circles, $n = 5$), and susceptible *Orexin*$^{-/-}$ mice (closed triangles, $n = 5$), all of which were maintained under ad libitum-fed conditions until day 27 and fasted for 5 h. Data are the means ± S.E.M. $^{\dagger}P < 0.05$, control WT vs. control *Orexin*$^{-/-}$ mice; $^{\ddagger\ddagger}P < 0.01$, susceptible WT vs. susceptible *Orexin*$^{-/-}$ mice; $^{\S}P < 0.05$, control *Orexin*$^{-/-}$ vs. susceptible *Orexin*$^{-/-}$ mice. (B) Western blot analysis showing the insulin-induced phosphorylation of Akt in the liver. Mice fed ad libitum until day 31 were fasted for 5 h and then injected with insulin (0.75 U/kg, i.p.) or phosphate-buffered saline. The liver was isolated 30 min after the injection. Data are the means ± S.E.M. The number of mice in each data set is indicated within the column. $^{*}P < 0.05$ and $^{**}P < 0.01$. A.U., arbitrary unit.

be lower than that under CR conditions (Lutter et al., 2008). We consider that basal or moderately increased activity of orexin neurons during defeat stress under ad libitum-fed conditions may be sufficient to prevent the impairment of hepatic glucose regulation but not the development of depression-like behavior.

Hepatic glucose production is the main regulator of fasting blood glucose levels, and subjects with impaired fasting glucose have hepatic insulin resistance (Abdul-Ghani et al., 2006). Hepatic insulin action to inhibit glucose production is centrally regulated via the autonomic nervous system (Marino et al., 2011). Hypothalamic orexin has the ability to modulate hepatic glucose production via the sympathetic nervous system (Yi et al., 2009). In the present study, we investigated the influence of CSDS on insulin signaling through the Akt pathway in the liver, and found that insulin-induced phosphorylation of Akt remained intact in susceptible WT mice exposed to CSDS. In contrast, CSDS-susceptible *Orexin*$^{-/-}$ mice showed an increase in basal Akt phosphorylation levels and failure of insulin to increase phosphorylation levels, although control *Orexin*$^{-/-}$ mice had no such abnormalities. Susceptible *Orexin*$^{-/-}$ mice also showed marked hyperinsulinemia, which was greater than that in control WT mice. These results suggest that chronic elevation of circulating insulin induced by a combination of CSDS and orexin deficiency causes an increase in the basal levels of Akt phosphorylation and promotes the development of insulin resistance in the liver via a possible negative feedback mechanism. Thus, central actions of orexin appear to be required for preventing the rapid development of hepatic insulin resistance under chronic stress conditions. Given that CSF levels of orexin A display a circadian rhythm (Zhang et al., 2004), daily activation of orexin neurons may contribute to the maintenance of normal glucose homeostasis via rhythmic control of autonomic balance, even in the depressive state.

Hypothalamic leptin signaling has beneficial effects on glucose metabolism, including suppression of hepatic gluconeogenesis via the autonomic nervous system (Marino et al., 2011). Enhanced orexin action is reported to improve leptin sensitivity in diet-induced obese mice (Funato et al., 2009). In the present study, we found that susceptible *Orexin*$^{-/-}$ mice showed striking hyperleptinemia, indicative of leptin resistance, although their feeding behaviors were unaltered. Since the glucoregulatory actions of leptin are considered to be separated from the established anorectic effect (Coppari and Bjørbæk, 2012), we suggest that stress responses, such as a sustained increase in sympathetic tones, selectively impair the pathway to mediate the glucoregulatory actions of leptin under orexin-deficient conditions, leading to the exacerbation of hepatic insulin resistance without affecting food intake.

Another major finding of the present study is that CR completely reversed CSDS-induced glucose intolerance and insulin insensitivity as well as depression-like behavior in WT but not *Orexin*$^{-/-}$ mice. CR has been reported to cause antidepressant effects via the activation of orexin neurons without changing orexin expression in CSDS-exposed mice (Lutter et al., 2008). Central actions of orexin A are mediated by orexin receptor 1 (OX_1R) and OX_2R, which are differentially distributed in the brain (Carter et al., 2009). It has been shown that OX_1R and OX_2R signalings promote opposite effects, i.e., prodepressant- and antidepressant-like effects, respectively (Scott et al., 2011). During repeated stress, the effects of a higher dose of orexin A at OX_2R, which has ~2-fold lower affinity for orexin A than OX_1R (Sakurai et al., 1998), could prevent effects at OX_1R in the paraventricular nucleus of the thalamus of rats (Heydendael et al., 2011). Therefore, we speculated that CR-induced strong activation of orexin neurons might prevent the continuum of depressive disorders via antidepressant OX_2R signaling and eliminate the cause of impaired glucose metabolism. Further studies are required for revealing the precise mechanisms.

We consider that the present study has limitations to directly predict the therapeutic potential of orexin for the treatment of depression in humans, because the influences of orexin deficiency and CR on glucose metabolism under chronic depressive conditions were investigated only in mice exposed to CSDS. In addition, since C57BL/6J mice instead of *Orexin*$^{+/+}$ littermates were used as WT controls, subtle genetic differences might be included in the observed differences between *Orexin*$^{-/-}$ and WT mice. At present, however, only a limited number of animal methods are available for evaluating chronic depression, in contrast to the models of acute stress disorders, such as the forced-swim test (Krishnan and Nestler, 2008). Our results reinforce the importance of the CSDS model for studying the chronic metabolic changes associated with sustained constant depressive symptoms.

In conclusion, we provide evidence that the hypothalamic orexin system is required to prevent the development of hepatic insulin resistance in a depressive state and could further prevent the persistence of depression *per se* if highly activated. Thus, orexin appears to have potential to be a novel target in pharmacological intervention for preventing the depression–diabetes link.

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