General and Comparative Endocrinology 296 (2020) 113545

Contents lists available at ScienceDirect

General and Comparative Endocrinology

journal homepage: www.elsevier.com/locate/ygcen

Research paper

# Acute sleep deprivation leads to growth hormone (GH) resistance in rats

Xintong Lyu[a], Guohua Wang[b], Zhuang Pi[a], Lan Wu[a,*]

[a] Department of Pediatric Gastroenterology, The First Hospital of Jilin University, Changchun 130021, People's Republic of China
[b] Department of Neonatology, The First Hospital of Jilin University, Changchun 130021, People's Republic of China

ARTICLE INFO

*Keywords:*
Growth hormone
Signalling pathway
Acute sleep deprivation
Growth hormone receptor

ABSTRACT

Sleep is an essential physiological process that is required by all higher animals. Sleep has many important physiological functions. Previous studies have focused on the relationship between sleep and growth hormone secretion patterns. However, to date, whether sleep affects the biological activities of GH remains unclear. Here, we investigated this issue by evaluating the growth hormone receptor (GHR)-mediated intracellular signalling pathway in a sleep-deprived rat model. The results showed that GH's signalling ability is decreased in an acute sleep deprivation rat model. JAK2-STAT signalling was decreased significantly compared to that in control rats. We further analysed the possible molecular mechanism of GH signal inhibition in sleep-deprived rats. The results showed that the protein expression levels of SOCS3 (suppressors of cytokine signalling 3, which functions as the negative regulatory molecule of GH's signalling) increased; however, other negative regulatory proteins, such as protein phosphatase (PTP1B), did not change. In addition, acute sleep deprivation results in a significant increase in serum FFA (**free fatty acid**) level, which is also one of the factors contributing to GH inhibition. These findings suggest that GH signal resistance may be caused by a combination of factors. This study could serve as an important reference for related studies on the effect of sleep deprivation on endocrine systems.

## 1. Introduction

Sleep is an essential physiological process that is required by all higher animals. Sleep has many important physiological functions, such as maintaining physical and mental health (Copinschi, 2005; Garg and Kumar, 2008; Strine and Chapman, 2005). Serious health risks are posed by sleep disorders. Inadequate sleep is associated with increased mortality and reduced quality of life. In addition, sleep disturbance and short sleep duration may lead to an increased risk of multiple adverse health outcomes, such as inflammation and related disorders, including cardiovascular disease, arthritis, and diabetes mellitus (Irwin et al., 2015).

Sleep is closely associated with the endocrine system (Attal and Chanson, 2010; Copinschi, 2005). Sleep restriction and sleep deprivation may lead to cardiovascular disease, which can also lead to a cluster of chronic metabolic disorders (e.g., obesity, insulin resistance, diabetes). Many studies have consistently demonstrated that insulin sensitivity is markedly reduced in young adults following sleep loss (Broussard et al., 2015). In addition, the relationship between GH and sleep has been studied for decades (Van and Copinschi, 2000). Growth hormone (GH) is released in a pulsatile fashion from the anterior pituitary. GH secretion is closely related to the circadian rhythm (Brandenberger et al., 2000; Prinz et al., 1983). The highest levels of GH secretion occur during sleep (Brandenberger et al., 2000). Once sleep is affected (such as sleep restriction and sleep deprivation), the profile of GH secretion changes significantly. In humans, Laura et al. reported that sleep deprivation reduced nocturnal GH secretion, and the profiles of GH secretion were significantly changed (Redwine et al., 2000). Similarly, in rodent models, GH secretion in the adult rat is also correlated with the sleep-wakefulness cycle (Kimura and Tsai, 1984). Sleep deprivation reduces IGF-1 concentrations (Chennaoui et al., 2016).

Previous studies have focused on the relationship between sleep and growth hormone secretion patterns. However, to date, whether sleep deprivation affects the biological activities of GH remains unclear. Here, we investigated this issue by assessing the GH/GHR-mediated intracellular signalling pathway in an acute-sleep-deprived rat model. GH displays its physiological function mainly by activating GHR-mediated intracellular signalling pathways, such as the tyrosine kinase (Jak2) and its main substrates the signal transducer and activator of transcription (JAK2/STATs). In the current work, we study the effect of acute sleep deprivation (ASD) on GH signalling pathways in a sleep-deprived rat model. We found that the signalling capacity of GH was decreased in an acute sleep-deprived rat model. Further mechanistic

*Abbreviations:* GH, Growth hormone; GHR, growth hormone receptor; STAT, signal transducers and activators of transcription; ASD, acute sleep deprivation

* Corresponding author.
*E-mail address:* Wulanjl2019@163.com (L. Wu).

https://doi.org/10.1016/j.ygcen.2020.113545
Received 11 March 2020; Received in revised form 4 June 2020; Accepted 26 June 2020
Available online 03 July 2020

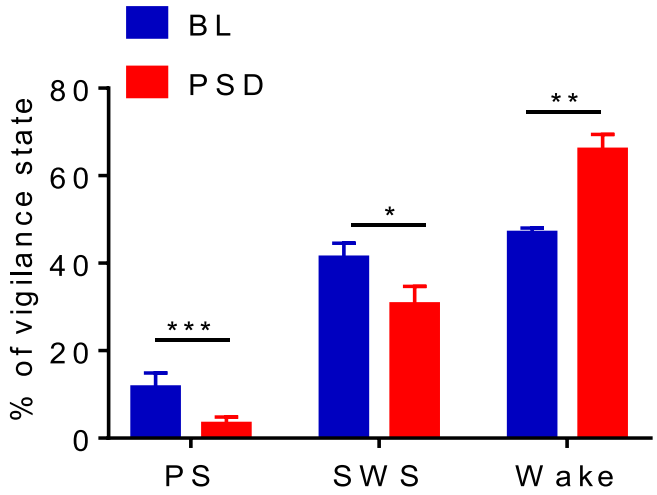



**Fig. 1.** (A) Paradoxical sleep amounts are significantly reduced during the 24 h of acute sleep deprivation. Multiple platform method is used to prepare rat sleep deprivation models. The detailed experimental process has been described in materials and methods section. *P < 0.05; **P < 0.01; ***P < 0.001.

**Table 1**
Sleep data during paradoxical sleep (PS) deprivation.

| | Paradoxical sleep | | Slow wave sleep | Wake |
|---|---|---|---|---|
| | Duration (min/24 h) | %TST | Duration (min/24 h) | |
| Baseline | 92.1 ± 3.28 | 11.8 ± 0.15 | 609.3 ± 12.45 | 737.6 ± 23.34 |
| PSD24 | 24.8 ± 2.13 | 4.36 ± 0.72 | 475.8 ± 24.21 | 940.1 ± 24.58 |

PSD: paradoxical sleep deprivation; TST: total sleep time.

studies showed that sleep deprivation increased the expression of negative regulatory proteins. In addition, we also found that the FFA level was significantly increased, which is also a negative regulator of GH signalling. In summary, current research shows that sleep deprivation can lead to GH resistance.

## 2. Materials and methods

### 2.1. Materials

The animal experiment was approved by the Institutional Animal Care and Use Committee of Jilin University (IACUC). Anti-SHC (Cat#2432), anti-IRS1 (Cat#2382), anti-IRS2 (Cat#4502), anti-phospho-specific JAK2 (Cat#3776), anti-phospho-specific STAT3 (Cat#9138), anti-phospho-specific STAT1 (Cat#8826), anti-STAT3 (Cat#9139), anti-STAT1 (Cat#14994), anti-JAK2 (Cat#3776), anti-SHP1 (Cat#ab227503), anti-PTP1B (Cat#ab245984), and anti-SOCS3 (Cat#ab75856) antibodies were obtained from Cell Signaling Technology (USA). Anti-phospho-specific-STAT5 (Cat#ab98338), anti-STAT5 (Cat#ab16276) and anti-Actin (Cat#ab179467) antibodies were obtained from Abcam (Cambridge, UK). Alexa Fluor 488/Alexa Fluor 555-labelled secondary antibodies were purchased from Sigma-Aldrich (St. Louis, USA). Anti-GHR (Cat#PA5-79309) was purchased from Thermo Fisher Scientific (USA). RIPA buffer and bicinchoninic acid assay (BCA) kits were obtained from Beyotime Biotechnology (Shanghai, China). Enhanced chemiluminescence (ECL) reagent was purchased from Pierce (Rockford, USA). Polyvinylidene fluoride (PVDF) membranes were purchased from Millipore (MA, USA). Cortisol (mouse/rat) ELISA Kit was purchased from Huacheng Biotechnology Co., Ltd (Changchun, China).

### 2.2. Animal care

Male Wistar rats aged 6 weeks and weighing ~180 g (Animal Experiment Center of Jilin University, Changchun, China) were used as animal models in the current experiments. Five animals were housed per cage in a room with controlled environmental conditions, including a temperature of 20–22 °C, a relative humidity of 55 ± 5%, and a 12-h light:12-h dark (12 L:12D) cycle (light onset at 7 a.m.). The animals were provided ad libitum access to food and water. The Institutional Animal Care and Use Committee of Jilin University (IACUC) approved the animal experiment.

### 2.3. Sleep restriction protocol

Sleep deprivation experiments are theoretically based on muscle relaxation. In the current work, sleep deprivation experiments of rats were conducted using the platform technique (Argeri et al., 2016; do Lago Godoi et al., 2005), which has been shown to be an effective technique for studying sleep (Argeri et al., 2016; do Lago Godoi et al., 2005; Murata et al., 2018). In brief, the rats were placed inside a tiled water tank (143 × 41 × 30 cm) for 24 h. The tiled water tank contains 14 circular acrylic small platforms (diameter 6.5 cm). The tanks were filled with warm water (30°C) to within 1 cm below the upper border of the platform. Prior to the start of the experiment, the rat was placed in the water tank for an hour to adapt to avoid unnecessary falling into the water. For sleep deprivation, animals (3–5 rats) were placed on the platform and were able to move around within the circular small platforms (jumping from one platform to the other). During sleep, particularly during the paradoxical stage, the loss of muscle tone makes the rats fall into the water. Therefore, the rat will wake up and climb back on the platform, so the rats remain awake. As a control, rats were placed in an identical tiled water tank that was equipped with a larger platform (diameter, 20 cm), which permits rats to sleep normally. In addition, rats were placed in the normal environment as a control to assess whether exposure to the large platform caused any adverse effects. The rats were deprived of sleep for 2 h.

### 2.4. Experimental design and surgery

After sleep deprivation, the rats were anaesthetized with 2, 2, 2, tribromoethanol (250 mg/kg, ip), and anaesthesia was ensured by the loss of pedal reflexes and corneal reflexes. The abdominal cavity was opened, and the portal vein was exposed. Then, rats were injected with vehicle or GH at a dose of 1.5 mg/kg BW, which represents a maximally effective GH dose in rats (Chow et al., 1996). At the indicated time points after the GH injection, the animals were sacrificed by decapitation. Livers were collected and immediately snap-frozen in liquid nitrogen. Then, liver tissues were stored at −80 °C for subsequent analysis. The growth hormone injection experiment was completed in the morning (7–9 am) because the endogenous growth hormone level is very low during this period (Beauloye et al., 2002). Although sleep deprivation can change GH secretion profiles, the endogenous GH level remains very low in this period (7–9 am) (Spiegel et al., 2000).

### 2.5. Determination of blood routine and liver function

Blood routine examination was performed by the automatic hematology analyzer, and blood biochemical parameters (liver function) were determined by Beckman LX20 automatic biochemical analyzer.

### 2.6. Immunoprecipitation

Immunoprecipitation was performed by incubating the protein lysates with the indicated antibodies at 4 °C overnight. Normal mouse or rabbit IgG was used as the negative control. Protein A/G-Sepharose beads were used to capture immunoglobulin-bound proteins according

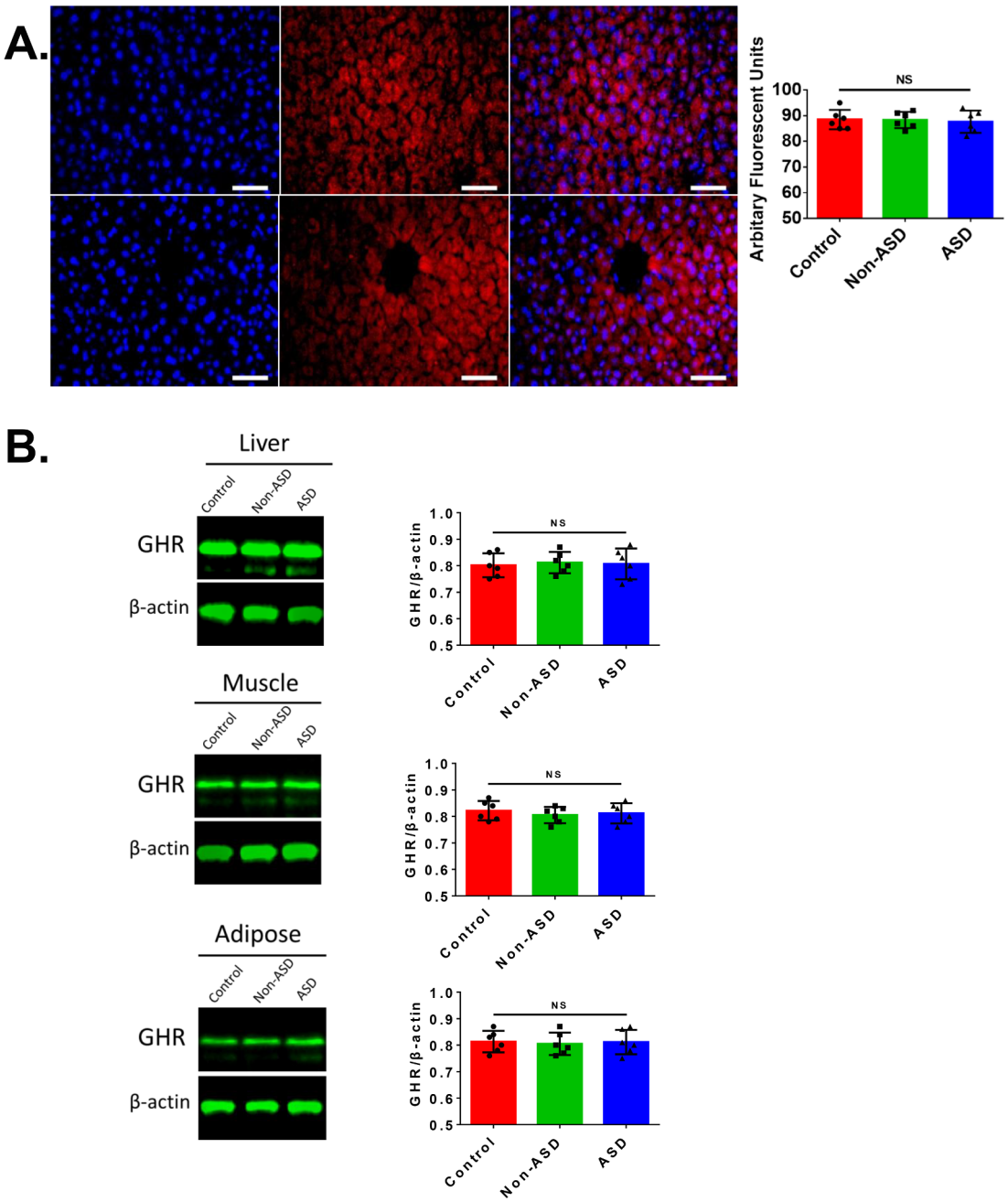



**Fig. 2.** (A) GHR expression patterns in liver from control rats and ASD rats as assessed by immunohistochemistry analyses (Bar = 50 nm). (B) GHR expression levels in liver, muscle and adipose tissues from control rats and ASD rats. Equal amounts of proteins from liver, muscle and adipose tissues of control rats or ASD rats were subjected to 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to Immun-Blot® low fluorescence (LF) PVDF membranes. After washing, the membranes were blocked for 2 h in PBS containing 5% skim milk at 37 °C. After three washes, the membranes were incubated with the indicated anti-GHR antibodies. The membranes were then incubated with the secondary antibody conjugated with Alexa Fluor 488 (green fluorescence). Fluorescent blots were imaged with the ChemiDoc TM MP imager (Bio-Rad). The bands were quantified by fluorescence densitometry using ImageJ software. Histograms represent the mean value ± s.e.m. of three independent experiments.

to the manufacturer's instructions. The immunoprecipitated samples were then subjected to Western blot analysis as described below.

### 2.7. Western blot analysis

Equal amounts of whole protein, cytoplasmic protein or nuclear protein were subjected to 10% sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and transferred to an Immun-Blot® low fluorescence (LF) PVDF membrane (BioRad, Hercules, CA, USA). After three washes, the membranes were blocked for 2 h in PBS containing 5% skim milk at 37 °C. After washing, the membranes were incubated with indicated primary antibodies. After washing three times, the membranes were incubated with rabbit anti-mouse or goat anti-rabbit immunoglobulin G conjugated with fluorescent molecules to visualize the bands. The fluorescent blots were imaged using the ChemiDoc TM MP imager (Bio-Rad). The bands were quantified by fluorescence densitometry using ImageJ software.

### 2.8. Indirect immunofluorescence assay (IFA)

Liver tissues from Male Wistar rats were cut into approximately 1-$cm^2$ pieces. Tissue samples were then fixed in 4% paraformaldehyde.

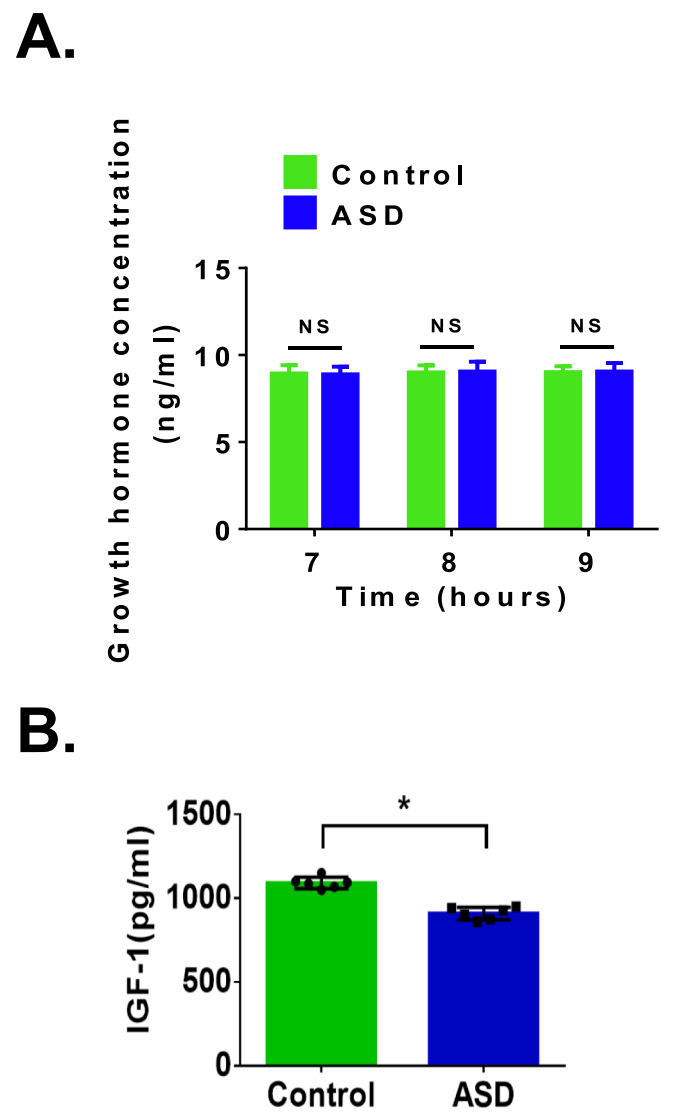



**Fig. 3.** (A) Determination of GH circulating levels. GH concentrations (from 7 am to 9 am) in serum were determined using an ELISA kit according to the manufacturer's instruction. The data represent the mean ± s.e.m. of three independent experiments (n = 6) (B) IGF-1 concentrations were determined by ELISA assay kits according to the manufacturer's instructions. The data represent the mean ± s.e.m. of three independent experiments (n = 6). A p-value of $< 0.05$ was considered statistically significant.

Then, 5 μm thick liver sections were obtained with a microtome (Leica RM2255). The sections were then blocked with 5% bovine serum albumin (BSA) for 60 min. After washing three times, the slides were incubated with the indicated antibodies at 4 °C for 12 h after permeabilization with 0.1% Triton X-100. After washing, the slides were incubated with fluorescence-conjugated secondary antibodies for 2 h at 37 °C. After washing the sections three times, cell nuclei were stained with DAPI. Confocal laser scanning microscopy was used to detect the fluorescent signal on the sections.

### 2.9. RNA preparation and qRT-PCR

Total RNA was prepared from rat livers using an RNAiso Plus Kit (TaKaRa Bio Inc., Shiga, Japan) according to the manufacturer's protocol. DNA contamination was removed by treatment with TURBO DNase (Life Technologies, Ambion). RNA concentration and purity of total RNA samples was measured with the NanoDrop Spectrophotometer (ThermoFisher). Total RNA integrity was evaluated by electrophoretic separation on 1% agarose gel. cDNA was synthesized from total RNA using the PrimeScriptTM RT reagent Kit with Gdna Eraser according to the manufacturer's protocol. Messenger RNA levels of SOCS1 (forward primer: 5′-TTGTAGCAGCTTGTGTCTGG-3′; reverse primer: 5′-GGATATTCTGCACAGCAGAA-3′) (Zheng et al., 2018); SOCS2 (forward primer: 5′-CGTGGTTCATCTGATTGACTACTACG-3′, reverse primer: 5′-TCACATAGCTGCATTCGGTGATAC-3′) (Yu et al., 2008); SOCS3 (forward primer: 5′-GCTCCAAGAGCGAGTACCAG-3′; reverse primer: 5′-AGTACACAGTCAAAGCGGGG-3′) (Zheng et al., 2018) and CIS (forward primer: 5-CTGGAGCTGCCCGGGCCAGCC-3; reverse primer: 5-CAAGGCTGACCACATCTGGG-3) (Ji et al., 2004) and β-actin (forward primer 5′-CCGTAAAGACCTCTATGCCA-3′, reverse primer:5′-AAGAAAGGGTGTAAAACGCA-3′) (Jonsdottir et al., 2000) were measured by RT-qPCR using a CFX Connect Real-Time PCR Detection System (Bio-Rad, Hercules, USA).

### 2.10. H&E staining

The tissues from control and experimental rats were removed immediately after experiments. The tissues were fixed in 4% buffered formaldehyde, and the organ specimens were fixed in paraffin. Formalin-fixed paraffin-embedded specimens were cut into sections of 7-μm thick sections, and the sections were then routinely stained with haematoxylin and eosin (H&E).

### 2.11. Measurement of GH, IGF-1 and Cortisol

Growth hormone, IGF-1 and Cortisol concentrations in the plasma were measured using enzyme-linked immunosorbent assays (ELISAs). ELISAs were conducted according to the manufacturer's instructions. The optical density (OD) of each well was analysed using a 96-well microplate reader (Bio-Rad, USA) at a 450-nm wavelength. All measurements were performed in duplicate.

In addition, serum FFA concentrations in plasma were analysed via the colorimetric method using a commercial kit (Huangcheng Chemicals, ChangChun, China).

### 2.12. Statistical analysis

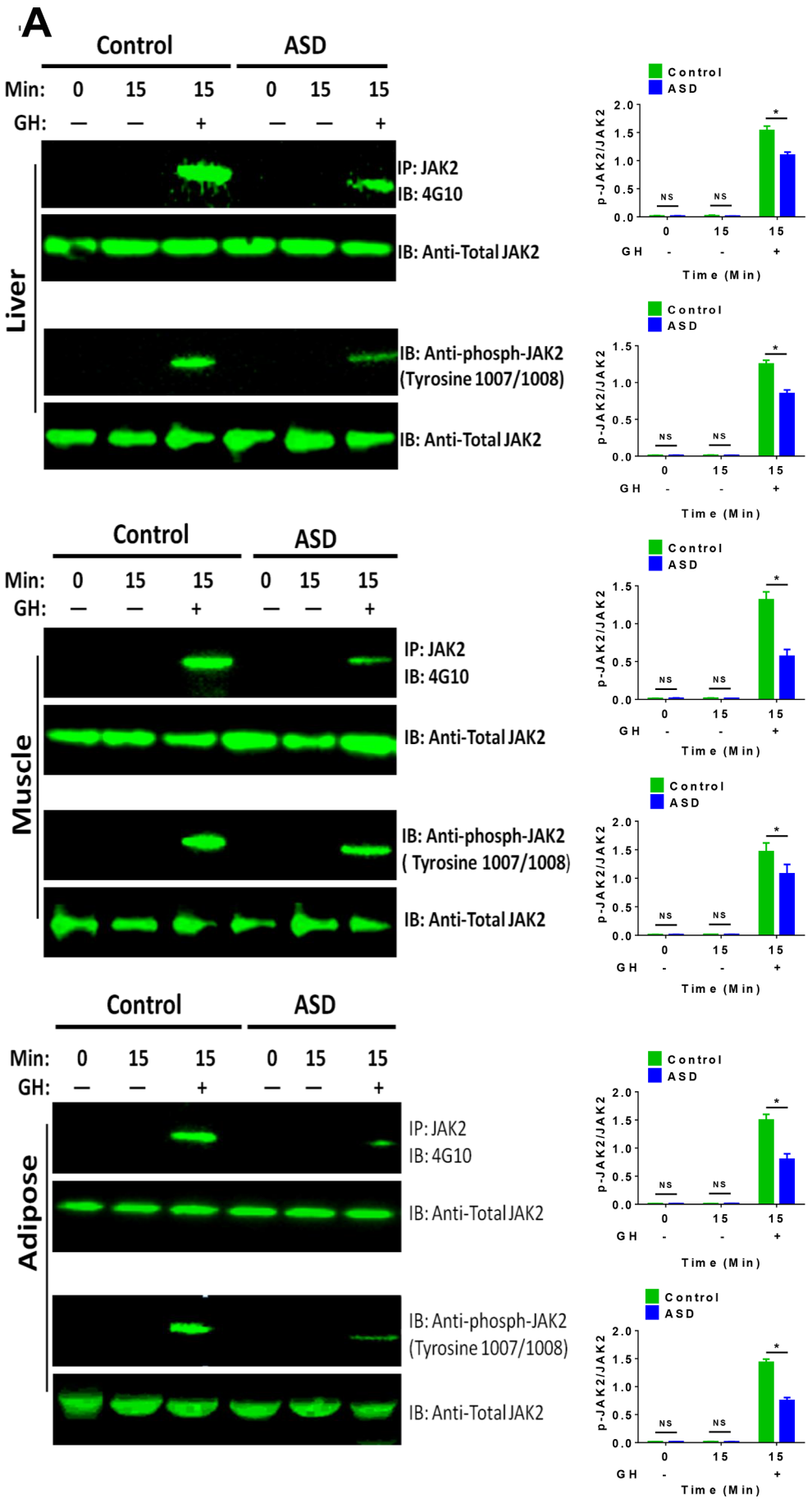
The data are reported as the mean ± standard error (S.E.). The data were analysed using a one-way analysis of variance (ANOVA). Statistical analysis was performed with SPSS software (version 20.0, SPSS Inc., Chicago, IL). A p-value of $< 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Determination of routine physiological parameters

Multiple platform method is a mature technique used to prepare rat/mice sleep deprivation models (Paradoxical Sleep Deprivation) (Rechtschaffen and Bergmann, 1995). As expected, we successfully prepared rat ASD model using multiple platform technique. As show in Fig. 1 and Table 1, in baseline conditions, rats spent ~43% of their time in SWS and ~9% in PS across the 24 h. However, in ASD group, rats spent about ~30% of their time in SWS and ~3.5% in PS across the 24 h. We also noticed that the loss of paradoxical sleep is compensated by an increase in waking (Fig. 1). In addition, Supplementary materials (Fig. 1) shows detailed sleep records in the ASD group and the control group.

Next, we analysed the effects of sleep deprivation on basic physiological parameters of control rats and acute sleep deprivation (ASD) rats, including liver function and routine blood parameters., we found that basic physiological parameters (routine blood tests and liver function analysis) exhibit no significant changes between the control




(caption on next page)

**Fig. 4.** (A) Effects of ASD on GH-induced JAK2 tyrosine phosphorylation in rat liver, muscle and adipose tissues. Liver/muscle/adipose tissue extracts from rats sacrificed at 0 or 15 min after injection of saline or GH (1.5 mg/kg) were prepared as described in the Materials and Methods section. Liver/muscle/adipose tissue extracts were immunoprecipitated with JAK2 antibody (1:500 dilutions) and immunoblotted with an antiphosphotyrosine antibody (4G10; 1:15,000 dilution). In addition, liver/muscle/adipose tissue extracts immunoprecipitated with JAK2 antibody were also analysed with anti-phospho-JAK2 (Tyr 1007/1008). After electrophoresis, membrane transfer and 5% skimmed milk powder blocking, the fluorescent blots were imaged with a ChemiDoc TM MP imager (Bio-Rad). The bands were quantified by fluorescence densitometry using ImageJ software. Histograms represent the mean value ± s.e.m. of three independent experiments (n = 6). (B) Time course of the effect of ASD on GH-stimulated JAK2 tyrosine phosphorylation. Liver/muscle/adipose extracts from control rats and ASD rats injected with GH for different time points were prepared as described in the Materials and Methods sections. Tissue extracts were subjected to SDS-PAGE and then transferred into Immun-Blot® low fluorescence (LF) PVDF membranes. After three washes, the membranes were blocked for 2 h in PBS containing 5% skim milk at 37 °C. After washing, the membranes were incubated with indicated anti-phospho antibody (Tyr1007/1008). The membranes were incubated with the rabbit anti-mouse secondary antibody conjugated with Alexa Fluor 488 (green fluorescence). The fluorescent blots were imaged with the ChemiDoc TM MP imager (Bio-Rad). The bands were quantified by fluorescence densitometry using ImageJ software. Histograms represent the mean value ± s.e.m. of three independent experiments (n = 6). A p-value of < 0.05 was considered statistically significant.

group and the sleep deprivation group (please see Supplementary materials, Table 1). In addition, we also evaluated the effect of sleep deprivation on tissue morphology. Histological examination with H&E showed no significant changes between control groups and the ASD group (please see Supplementary materials, Fig. 2).

### 3.2. Effects of acute sleep deprivation (ASD) on GHR expression

We first detected the expression pattern of liver GHR using immunohistochemistry in control rats and ASD rats. As shown in Fig. 2A, we can see that GHR mainly expressed in cytoplasm and membrane, and we did not find any significant alterations in GHR expression patterns between control rats and ASD rats. Furthermore, we further analysed the expression level of GHR by Western blot and found no significant change in GHR expression levels between control rats and ASD rats (Fig. 2B).

### 3.3. The effect of acute sleep deprivation (ASD) on JAK2 activation induced by GH injection

The above studies demonstrated that GHR expression levels did not change between control rats and ASD rats. To further exclude the effect of endogenous GH on GHR-mediated intracellular signalling, we first measured the GH concentration in plasma before GH injection, and the

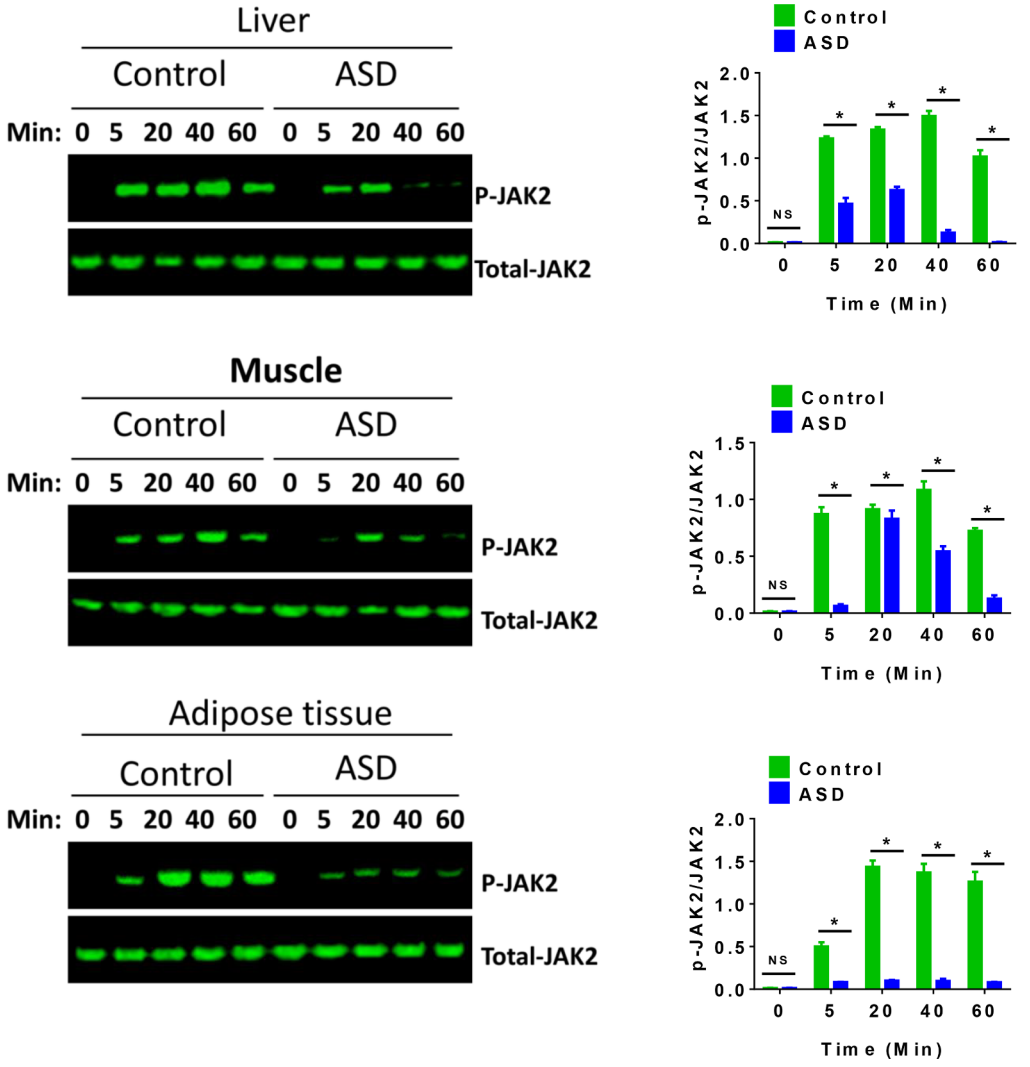



**Fig. 4.** (*continued*)

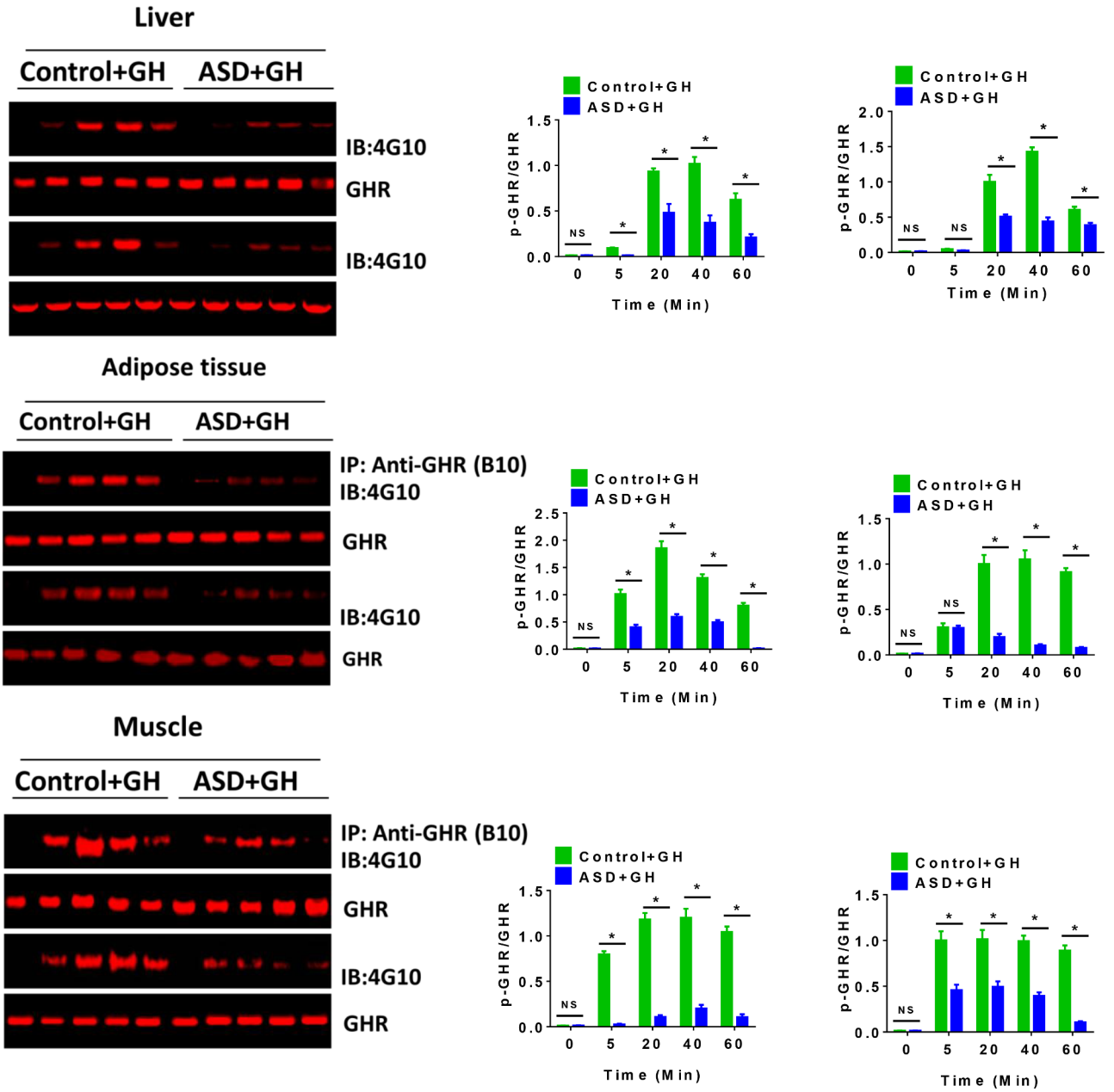



**Fig. 5.** (A) Effects of acute sleep deprivation on GH-stimulated GHR tyrosine phosphorylation in rat liver, muscle and adipose tissues. Liver/muscle/adipose tissue extracts were immunoprecipitated with anti-GHR (B10) or anti-GHR (Mab263). The immunoprecipitated proteins were resolved in SDS-PAGE and subjected to immunoblotting using an antiphosphotyrosine antibody (4G10; 1:15,000 dilution). The membranes were incubated with the rabbit anti-mouse secondary antibody conjugated with Alexa Fluor 555 (red fluorescence). The fluorescent blots were imaged with the ChemiDoc TM MP imager (Bio-Rad). The bands were quantified by fluorescence densitometry using ImageJ software. Data are represented as the mean ± s.e.m. of three independent experiments. (B) Time course of the effect of ASD on GH-stimulated GHR tyrosine phosphorylation. Data are represented as the mean ± s.e.m. of three independent experiments. A p-value of $< 0.05$ was considered statistically significant.

results showed that the endogenous GH concentrations from control rats or ASD rats were very low at 7 am to 9 am (we initiated the GH injection experiment at this time). These results were similar to those in Edén's report (1979), and the difference between control rats and ASD rats was not significant (Fig. 3A). Furthermore, we assessed IGF-1 concentrations in serum, and the results showed that the circulating levels of IGF-1 in ASD rats are significantly decreased compared with that of control rats (Fig. 3B), suggesting that GH resistance occurred in ASD rats.

Liver, muscle and adipose tissues are three important target organs of GH (Chow et al., 1996). To determine the effects of acute sleep deprivation (ASD) on GH signalling in vivo, we first explored the effects of ASD on GH-induced tyrosine phosphorylation of JAK2 (because JAK2 plays a central role in mediating GH signalling). Liver/muscle/adipose tissue samples were analysed using anti-JAK2 immunoprecipitation (IP) followed by antiphosphotyrosine immunoblotting (4G10, this antibody recognizes all tyrosine phosphorylation sites). Furthermore, we also analysed JAK2 activation using anti-phospho JAK2 (Tyr 1007/1008). As indicated in Fig. 4A, no JAK2 tyrosine phosphorylation was detected after saline injection (control). In contrast, GH injection induced tyrosine phosphorylation of JAK2 in both control rats and ASD rats. However, JAK2 phosphorylation levels in ASD rats are markedly reduced compared with control rats, and total JAK2 protein levels were comparable in control rats and ASD rats. Densitometric analyses

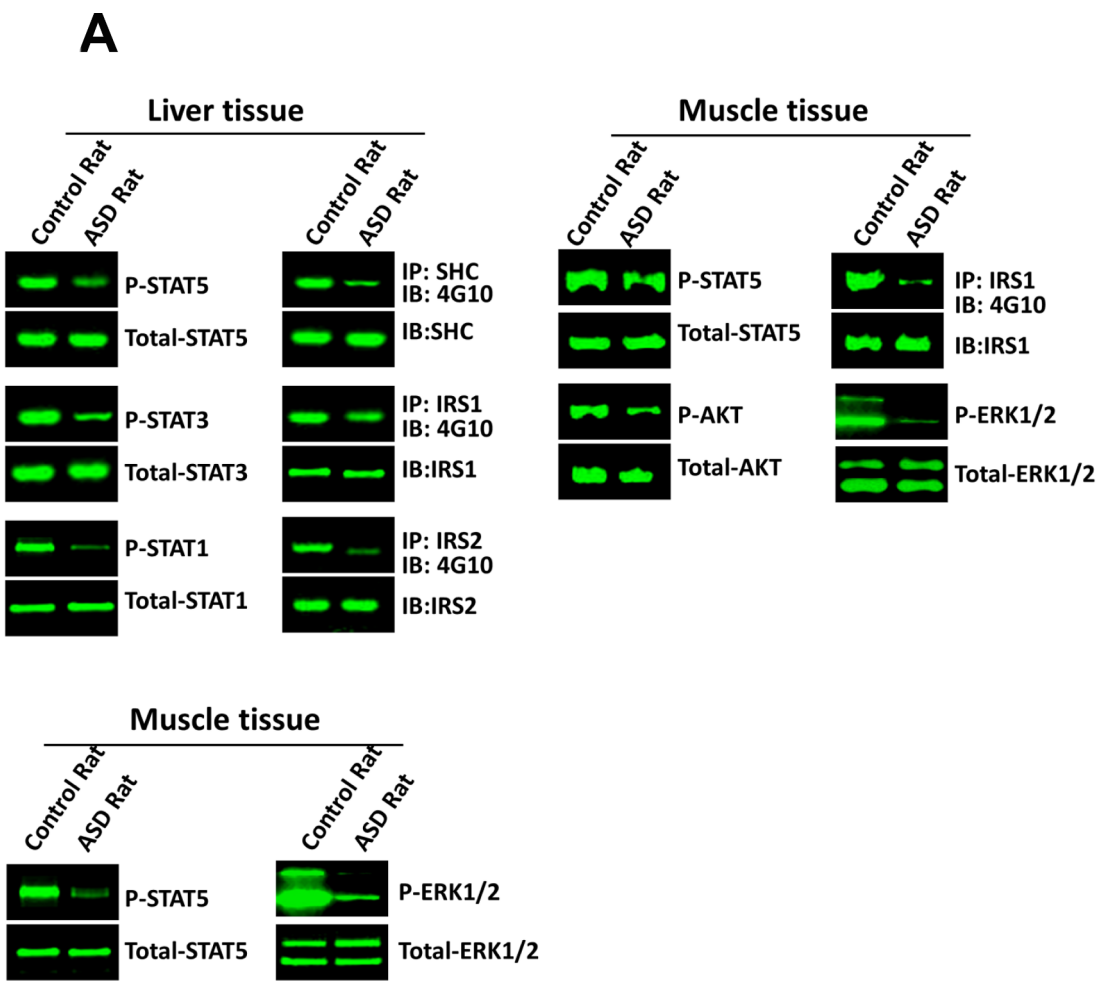



**Fig. 6.** (A) Effects of ASD on GH-stimulated signalling molecules tyrosine phosphorylation in different GH target tissues (liver, muscle and adipose tissues). (B-C) Time course of the effect of ASD on GH-stimulated signalling molecules phosphorylation. Liver/muscle/adipose extracts from control rats and ASD rats injected with GH for different time points were prepared as described in Materials and Methods. Tissue extracts were subjected with SDS-PAGE and then transferred into Immun-Blot® low fluorescence (LF) PVDF membranes. After three washes, the membranes were blocked for 2 h in PBS containing 5% skim milk at 37 °C. After washing, the membranes were incubated with the indicated primary antibodies. The membranes were then incubated with secondary antibody conjugated with Alexa Fluor 488 (green fluorescence). The fluorescent blots were imaged with the ChemiDoc TM MP imager (Bio-Rad). The bands were quantified by fluorescence densitometry using Image J software. Histograms represent the mean value ± s.e.m. of three independent experiments. (D) GH-induced IGF-1 mRNA expression in the ASD group is significantly lower than that of control group. A p-value of $< 0.05$ was considered statistically significant.

showed a significant decrease in GH-stimulated JAK2 phosphorylation in ASD rats compared with control rats (please see histogram). Furthermore, we also used anti-phosph-JAK2 (1007/1008) to detect JAK2 phosphorylation, which displayed similar results to that of 4G10. Furthermore, we analysed the GH-induced activation kinetics of JAK2 in control rats and ASD rats, as indicated in Fig. 4B. Although both control and ASD rats showed time-dependent JAK2 activation, JAK2 phosphorylation in ASD rats was significantly reduced compared with control rats.

Next, we further assessed the functional significance of the reduction in JAK2 tyrosine phosphorylation by acute sleeps deprivation (ASD). GHR is activated by JAK2; therefore, the effects of ASD on GHR phosphorylation were evaluated. Immunoprecipitation (IP) experiments were performed in tissues (liver, muscle and adipose) using two different anti-GHR antibodies (B10 (Cat#sc-137185, Santa-Cruze), which recognizes the intracellular domain of GHR), and Mab263 (Cat#sc-57161, Santa-Cruze), which recognizes the extracellular domain of GHR). Immunoprecipitated proteins were then analysed by Western blotting with an antiphosphotyrosine antibody (4G10). In control rats, robust GHR phosphorylation (a single dominant approximately 120-kDa protein band, which is the expected size for rat GHR previously described by Véronique et al (2002)) is detected after GH treatment (Fig. 5). The two antibodies that were used for immunoprecipitation (B10 and Mab263) exhibited the same expected band. In time-course analyses of GHR activation, the phosphorylation level of GHR in ASD was obviously decreased compared with that of control rats; however, GH-stimulated GHR phosphorylation also exhibited a time-dependent trend in ASD rats. In addition, we did not detect a difference in basic GHR expression between the two groups.

### *3.4. The effects of ASD on STATs, AKT, ERK1/2, IRS, and SHC tyrosine phosphorylation in liver/muscle/adipose tissues*

STATs/SHC/IRS are downstream signalling molecules mediated by JAK2/GHR, and they are also the most important signalling molecules that mediate GH action. We further analysed the activation of STATs/SHC/IRS (liver), STAT5/ERK1/2 (muscle), and STAT5/IRS/ERK1/2 (adipose tissue) in response to GH stimulation. Tyrosine phosphorylation of the abovementioned signalling molecules (liver, muscle and adipose tissue) was analysed in control and ASD rats after GH stimulation. Fig. 6A showed that ASD rats only exhibited a low level of STATs/SHC/IRS phosphorylation (in liver), a low level of STAT5/ERK1/2 (in muscle) and low level of STAT5/AKT/IRS/ERK1/2 (in adipose tissue) tyrosine phosphorylation compared to control rats.

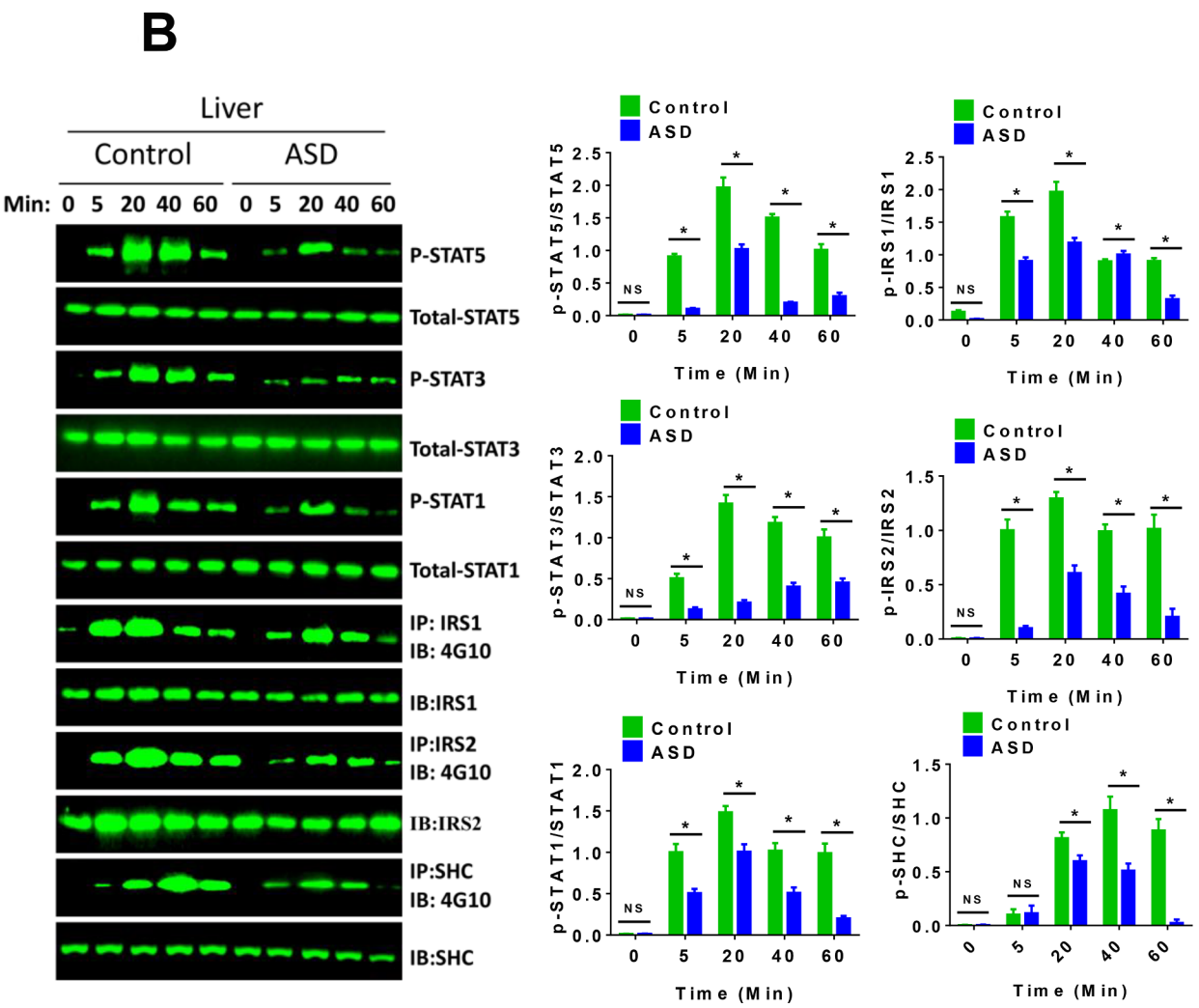



**Fig. 6.** (*continued*)

Next, we analysed the time course of signal responses in GH target tissues, including liver, muscle and adipose. As shown in Fig. 6B, the effect of acute sleeps deprivation (ASD) on STAT5/3/1, SHC and IRS1/2 phosphorylation in liver was detectable as early as 5 min after GH injection. GH-activated STAT5/3/1, SHC and IRS1/2 in liver reach maximal values at the 20- to 40-min intervals and then start to decline. However, at each time point, GH-induced activation in ASD rats was significantly attenuated compared with ASD rats. In muscle, GH-induced STAT5/ERK1/2 phosphorylation was detectable at 5 min. STAT5 and ERK1/2 phosphorylation levels reach maximum values at 20–40 min and then start to decline. In adipose tissue, the effect of ASD on GH-induced STAT5, IRS1/2 and ERK1/2 phosphorylation was detectable at 5 min. The tyrosine phosphorylation levels of STAT5, IRS1/2 and ERK1/2 were maximal 20–40 min after GH injection and then begin to decline. At each time point, the phosphorylation level of GH-activated signalling molecules in ASD rats was significantly attenuated compared with control rats.

Next, the above-mentioned studies imply that sleep deprivation may decrease GH activity. Here, we further determine the direct GH activity by detecting IGF-1 expression, because a key function of GH is to stimulate production of IGF-1 by the liver (Der Spoel et al., 2016). The results indicated that, GH-induced IGF-1 mRNA expression in the ASD group is significantly lower than that of control group (Fig. 6D).

### 3.5. Preliminary study on the mechanism of GH resistance induced by ASD

Suppressors of cytokine signalling, including SOCS1, SOCS2, SOCS3 and CIS, are the GH-induced suppressors of cytokine signalling in liver. At the nucleic acid level (mRNA level), we evaluated the mRNA expression levels of negative regulatory molecules. As shown in Fig. 7A, SOCS3 expression level in ASD rats was significantly increased compared with control rats with or without GH injection. Furthermore, the SOCS3 protein expression in ASD rats was also significantly increased compared with control rats. However, we did not detect mRNA expression differences in SOCS1/2 and CIS (data not shown). Similarly, SOCS3 expression levels were significantly increased in muscle of ASD rats (Fig. 7B).

### 3.6. Phosphatase protein content

GH-activated signalling molecules require protein phosphorylation at tyrosine, serine, or threonine residues. Protein phosphatase can dephosphorylate signalling proteins, representing the mechanism of action for signalling molecule deactivation (Flores-Morales et al., 2006).

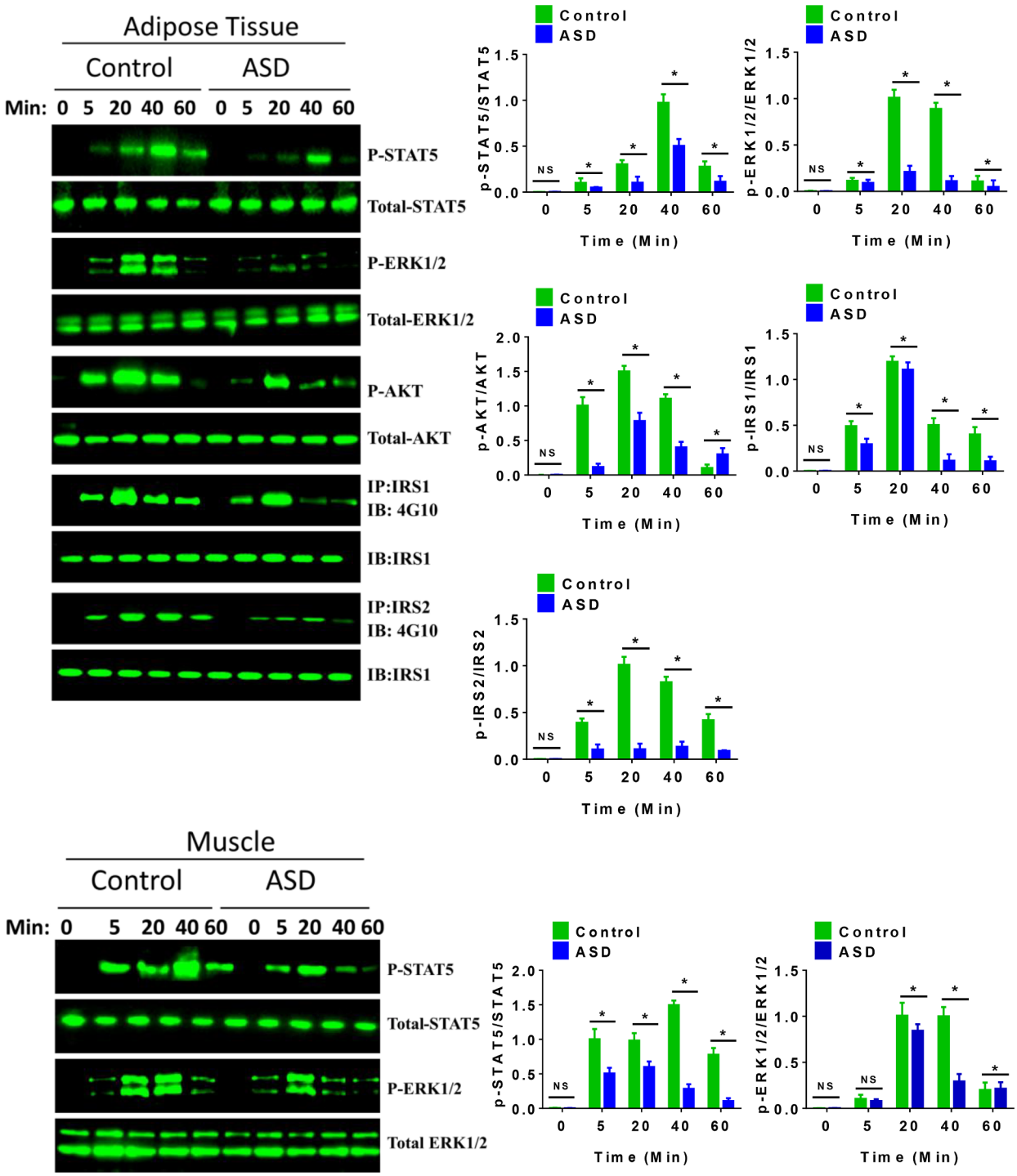



**Fig. 6.** (*continued*)

Therefore, GH's signalling abilities are also limited by dephosphorylation of activated signalling proteins by specific tyrosine phosphatises (such as PTP1B and SH2 domain-containing protein-tyrosine phosphatases (SHP/1/2)). PTP1B, SHP1 and SHP2 are involved in the specific downregulation of GH/GHR signalling. Here, we primarily assessed expression levels of PTP1B, SHP1 and SHP2 by immunoblotting using liver homogenates from control rats and ASD rats. As shown in Fig. 8, PTP1B, SHP1 and SHP2 protein expression did not significantly differ between control rats and ASD rats.

### *3.7. Free fatty acid (FFA) and Cortisol levels are significantly increased in ASD rats*

A previous study demonstrated that FFA also inhibits GH signalling.

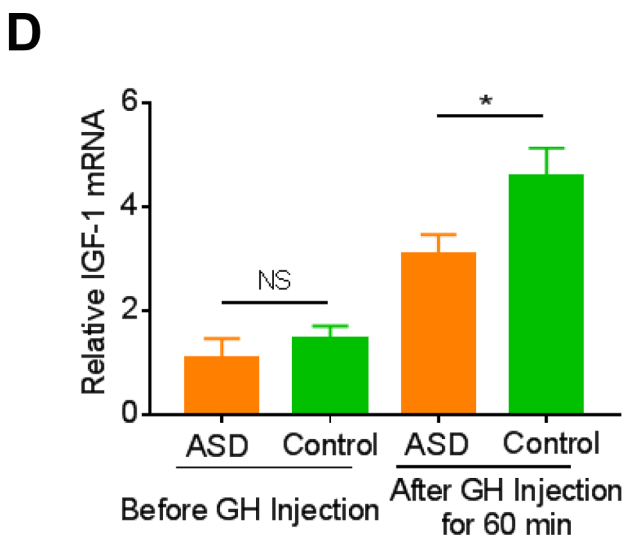



**Fig. 6.** (*continued*)

In the current study, we found that serum FFA levels in conditions of acute sleep deprivation are also significantly increased compared with control rats (Fig. 9A). In addition, we also detected the level of plasma Cortisol, and the results showed that the level of plasma Cortisol was significantly increased compared with the control group (Fig. 9B). These finding may also represent two of the mechanisms of GH resistance in ASD rats.

## 4. Discussion

Sleep is an essential physiological process that is required by all higher animals. Human beings will spend almost a third of their life sleeping (Broussard et al., 2015; Strine and Chapman, 2005). However, when encountering various sleep disorders, such as sleep apnoea and insomnia, normal sleep will be deprived. Sleep deprivation may cause a series of mental and physical health conditions. Previous studies have focused on the relationship between sleep and growth hormone secretion patterns. However, to date, whether sleep affects the biological activities of GH remains unclear. Therefore, the aim of this investigation was to explore the effect of acute sleep deprivation (ASD) on GH/GHR's biological activities by assessing the GHR-mediated intracellular signalling pathway. We found that acute sleep deprivation can lead to GH resistance.

GH's biological functions and actions are mediated by GHR expression on the surface of target organs (Lan et al., 2019). GH binding to GHR activates JAK2, which subsequently phosphorylates GHR, and multiple downstream signalling pathways are subsequently triggered, such as STATs, phosphatidylinositol 3-kinase (PI 3-kinase)/AKT and MAPK/ERK1/2 (Lan et al., 2018). These signalling pathways work together to mediate the biological function of GH. In the current work, we first evaluated GHR expression in liver/muscle/adipose tissues, and the results showed that acute sleep deprivation (ASD) did not affect the expression or distribution of GHR (Fig. 2). This finding suggests that GH resistance is not due to reduced GHR expression. In addition, it has been reported that sleep deprivation causes changes in GH secretion patterns (Brandenberger et al., 2000; Baumgartner et al., 1990); thus, we chose to start the current experiment at 7–9 am. During this period, the endogenous GH concentration was similar between the sleep deprivation group and normal control group (Fig. 3), indicating that the GH resistance status is not due to increases in endogenous GH concentrations. Next, we analyzed the GH-induced signaling profiles in ASD group and control group in Liver, muscle and adipose tissues which are three important target organs of GH. We first detected the activation of JAK2 (because JAK2 play a central role in mediating GH signalling), we found that GH-activated JAK2 phosphorylation was significantly decreased in ASD rats (Fig. 5), although GH activates JAK2 in a time-dependent manner in ASD rats model (Fig. 5). In addition to JAK2, GHR also plays a central role in mediating GH signaling; therefore we also observed that GH-induced GHR phosphorylation levels in ASD rats are also decreased significantly. We then further analyzed the time course of signaling molecule activation. Although GH still exhibited a time course response in sleep-deprived rats, at each time point, GH-induced signalling in ASD rats was significantly attenuated compared with that in control rats (Fig. 5). Furthermore, we further analysed the activation of STATs/SHC/IRS (liver), STAT5/ERK1/2 (muscle), and STAT5/IRS/ERK1/2 (adipose tissue) in response to GH stimulation in ASD group and control group. In time-dependent experiments, ASD rats only exhibited a low level of STATs/SHC/IRS phosphorylation (in liver), a low level of STAT5/ERK1/2 (in muscle) and low level of STAT5/AKT/IRS/ERK1/2 (in adipose tissue) tyrosine phosphorylation compared to control rats, although GH activates STATs/SHC/IRS (liver), STAT5/ERK1/2 (muscle), and STAT5/IRS/ERK1/2 (adipose tissue) in a time-dependent manner in ASD rats model (Fig. 6). In summary, these experiments indicate that, in the ASD rat model, the signaling activated

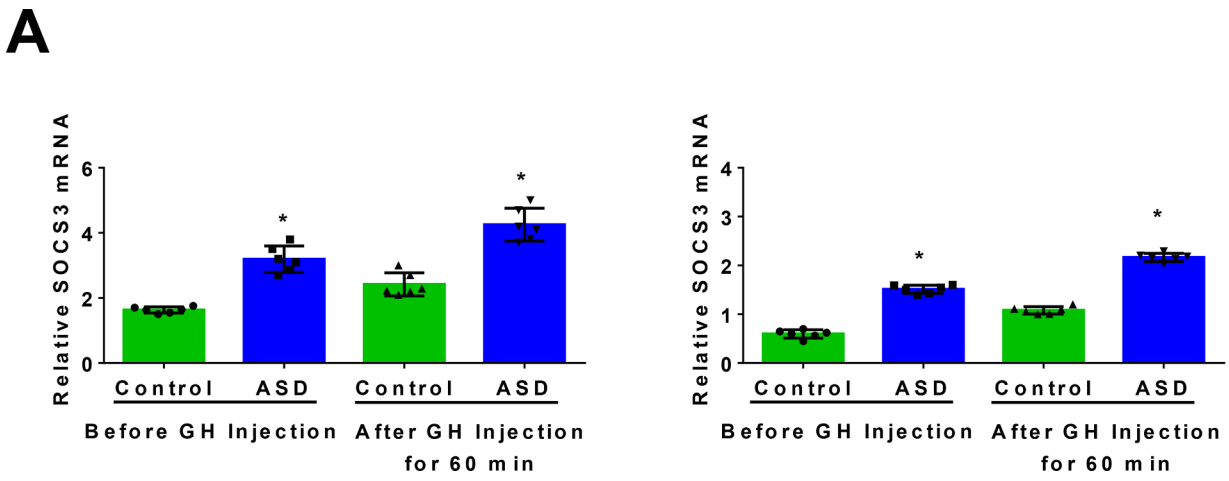



**Fig. 7.** (A) Measurement of SOCS-3 mRNA expression. Total RNA from liver and muscle tissues of control rats and ASD rats with or without GH injection was subjected to qRT-PCR with primer specific for SOCS3 to determine SOCS3 mRNA expression as described in Materials and Methods and normalized to β-actin mRNA (housekeeping gene). Bars represent means ± s.e.m. for 6 animals in each group. A p-value of $< 0.05$ was considered statistically significant. (B) SOCS-3 protein expression in liver and muscle tissue. SOCS-3 protein expression was determined by Western blot as described in the Materials and Methods section. A p-value of $< 0.05$ was considered statistically significant.

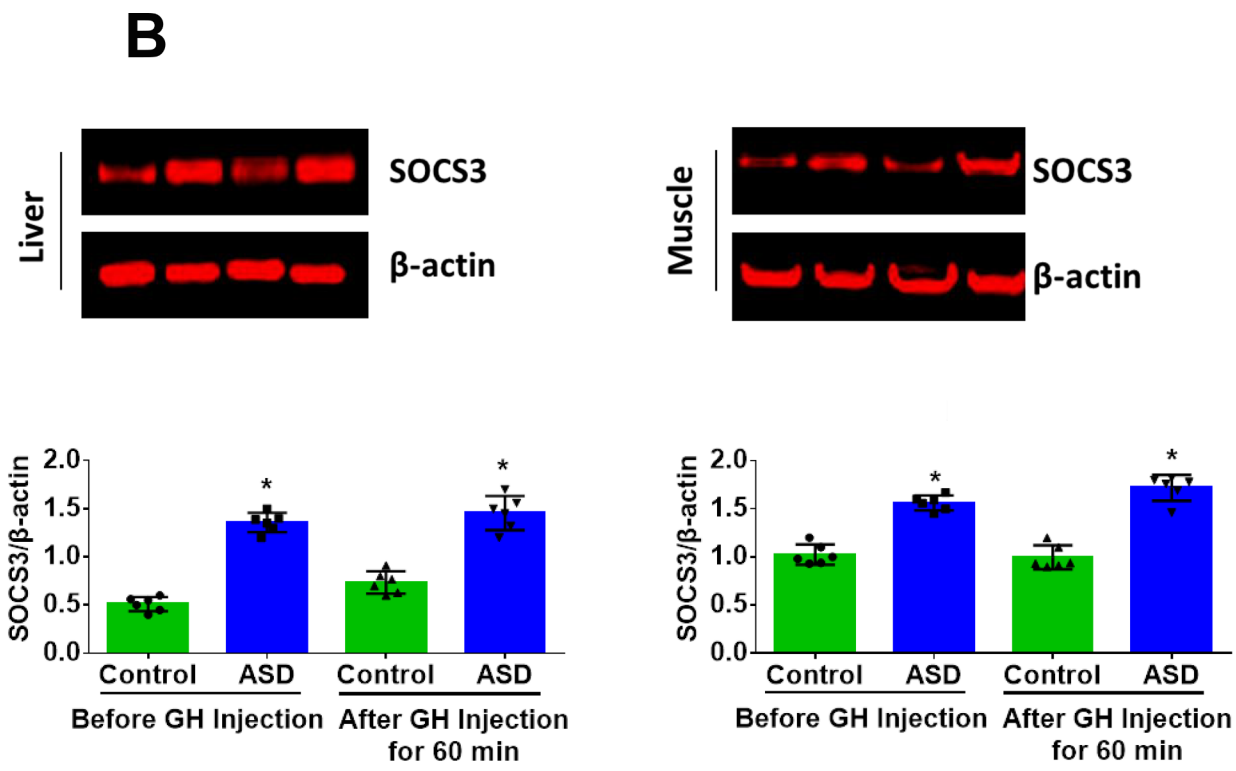



**Fig. 7.** (*continued*)

by GH is significantly decreased compared to that of control rats. Next, we further detected the IGF-1 mRNA expression (a key function of GH is to stimulate production of IGF-1 by the liver) (Der Spoel et al., 2016). As expected, we found that GH-induced IGF-1 mRNA expression in the ASD rats is significantly lower than that of control group.

The growth hormone/insulin-like growth factor (GH/IGF) axis plays important roles in growth, development and metabolism (Ian et al., 2019). The JAK2-STAT5 signalling pathway is the most important signalling pathway mediated by GH/GHR. The GH-induced JAK2-STAT5 signalling pathway is responsible for IGF-1 generation. A previous study reported that IGF-I serum levels are modulated by sleep, and IGF-1 serum levels are reduced in sleep deprivation animal models (Chennaoui et al., 2016). However, the explanation for the decrease in IGF-1 concentrations under conditions of sleep deprivation is unclear. Here, we observed that the JAK2-STAT5 signalling pathway is attenuated in response to GH. This finding may provide an explanation for low serum IGF levels caused by sleep deprivation.

Next, we assessed the mechanism by which sleep weakens GH signalling in sleep deprivation rat models. Previous studies have reported that SOCS proteins serve as negative regulators of the JAK/STAT signal cascade activated by cytokines and hormones (such as GH). The SOCS protein family consists of 8 members (CIS, and SOCS-1 to SOCS-7). In general, CIS, SOCS1, SOCS2 and SOCS3 are involved in GH's negative regulation of GH signalling (Flores-Morales et al., 2006). In this work, we analysed the expression of several molecules and found that acute sleep deprivation can be evaluated based on SOCS3 expression with or without GH treatment (Fig. 7). Another type of negative regulator of GH/GHR signalling is phosphatase. Activation of GH/GHR signalling pathways is dependent on protein phosphorylation at tyrosine, serine, or threonine residues. The mechanism of action of phosphatases is to dephosphorylate proteins, causing GHR-mediated signalling molecules to be inactivated. Phosphatases that are involved in GH/GHR signalling include SHP1, SHP2 and PTP1B. In the current work, we also evaluated the expression levels of these molecules, and we found no differences in ASD and control rats. This finding indicates that phosphatase may not be involved in GH resistance in ASD. In addition to these endogenous negative regulatory molecules regulating GH signalling, some exogenous molecules are also involved in the downregulation of GH signals, such as FFA (Møller et al., 2009). In the current study, we observed a significant increase in FFA levels, which is one of the potential factors causing GH resistance. However, the mechanism by which FFA inhibits GH signalling remains unclear, and these problems should be addressed in the future. In addition to FFA, it is well known that sleep disturbance and stress are closely related (Hirotsu et al., 2015). In the current study, we found that paradoxical sleep deprivation caused a significant increase of plasma cortisol levels compared to control rats (Fig. 9B). Studies have indicated that cortisol acts as a negative regulator of GH activity (Leproult and Van Cauter, 2010); therefore this may also be one of the factors leading to GH resistance. Taken together, these findings suggest that GH signal resistance may be caused by a combination of factors.

Taken together, we found that ASD may cause GH resistance, laying the foundation for subsequent research on the regulatory relationship between GH and sleep. This study will serve as an important reference for related studies on the effect of sleep deprivation on endocrine systems.

## CRediT authorship contribution statement

**Xintong Lyu:** Data curation, Writing - original draft. **Guohua Wang:** Visualization, Investigation. **Zhuang Pi:** Visualization, Investigation. **Lan Wu:** Conceptualization, Methodology, Supervision.

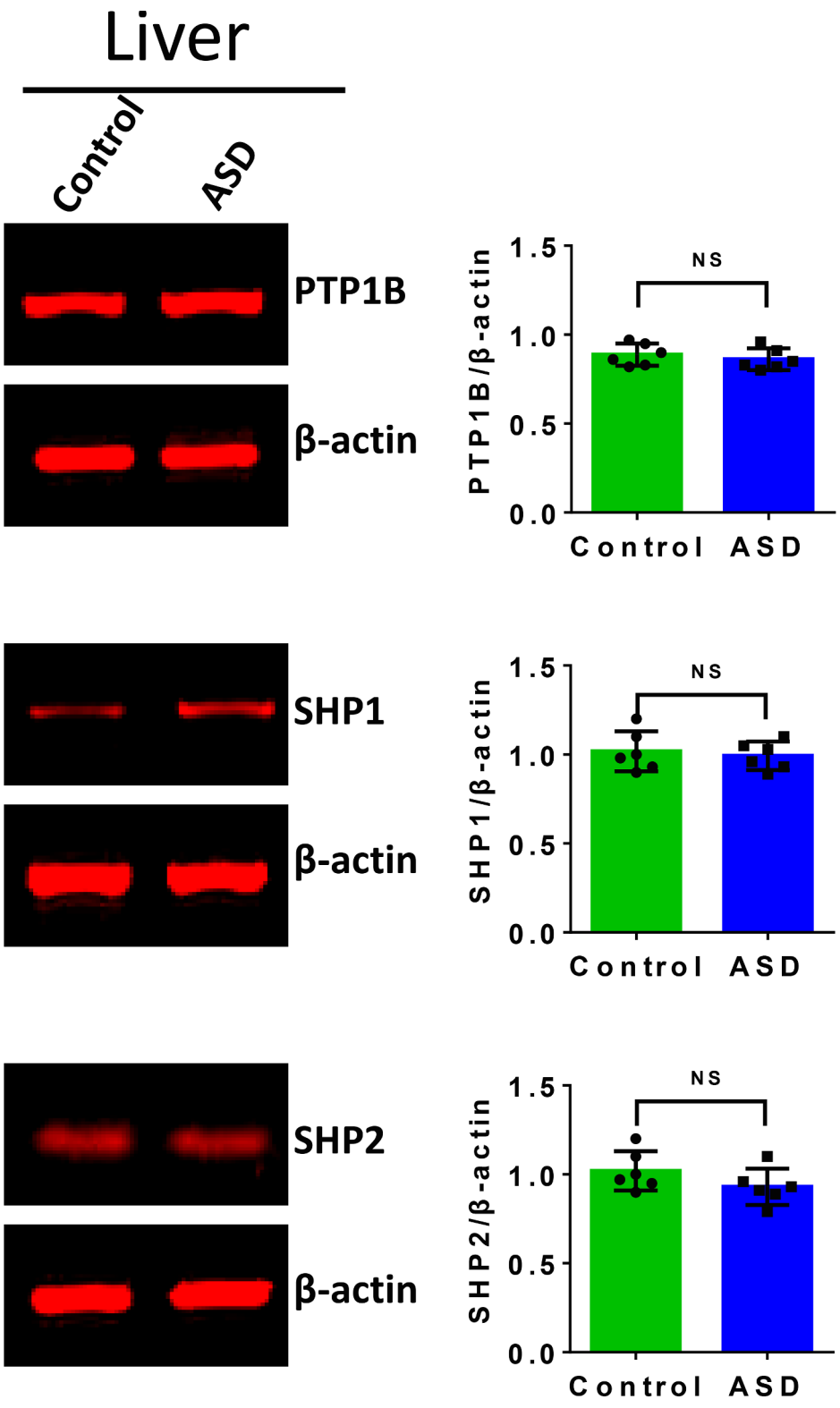



**Fig. 8.** Protein expression levels of protein phosphatases (SHP1/2 and PTP1B) in liver. Liver extracts from control rats and ASD rats were prepared as described in Materials and Methods. Liver extracts were subjected with SDS-PAGE and then transferred into Immun-Blot® low fluorescence (LF) PVDF membranes. After blocking for 2 h in PBS containing 5% skim milk at 37 °C, the membranes were incubated with the indicated antibodies. The membranes were incubated with the secondary antibody conjugated with Alexa Fluor 555 (red fluorescence). The fluorescent blots were imaged with the ChemiDoc TM MP imager (Bio-Rad). The bands were quantified by fluorescence densitometry using ImageJ software. Each picture represents a typical result of at least three separate experiments.

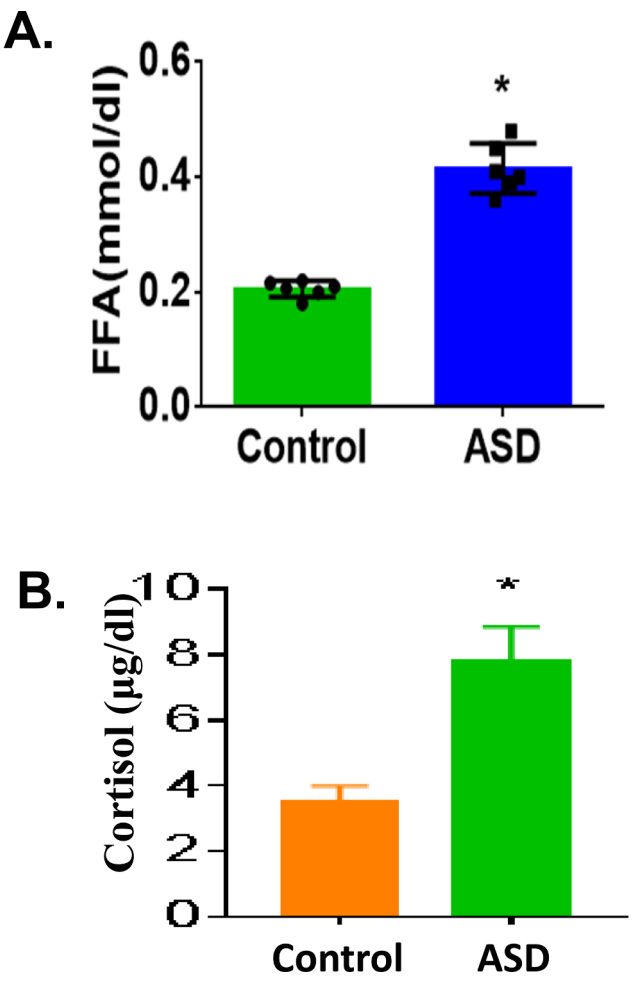



**Fig. 9.** Serum concentrations of FFA (A) and Cortisol (B) in control rats and ASD rats. Bars represent means ± s.e.m. for 6 animals in each group. A p-value of $< 0.05$ was considered statistically significant.


## Acknowledgements

This thesis is supported by the funding from the Department Of Health And Family Planning Commission Of JiLin Province (Project number: 20152036).


## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ygcen.2020.113545.

## References:


Argeri, R., Nishi, E.E., Volpini, R.A., Palma, B.D., Tufik, S., Gomes, G.N., 2016. Sleep restriction during pregnancy and its effects on blood pressure and renal function among female offspring. Physiol. Reports 4, 16.

Attal, P., Chanson, P., 2010. Endocrine aspects of obstructive sleep apnea. J. Clin. Endocr. Metab. 95, 483–495.

Baumgartner, A., Riemann, D., Berger, M., 1990. Neuroendocrinological investigations during sleep deprivation in depression II. Longitudinal measurement of thyrotropin, TH, cortisol, prolactin, GH, and LH during sleep and sleep deprivation. Biol. Psychiatry 28, 569–587.

Beauloye, V., Willems, B., de Coninck, V., Frank, S.J., Edery, M., Thissen, J.P., 2002. Impairment of liver GH receptor signaling by fasting. Endocrinology 143, 792–800.

Brandenberger, G., Gronfier, C., Chapotot, F., Simon, C., Piquard, F., 2000. Effect of sleep deprivation on overall 24 h growth-hormone secretion. The Lancet 356, 1408.

Broussard, J.L., Chapotot, F., Abraham, V., Day, A., Delebecque, F., Whitmore, H.R., Tasali, E., 2015. Sleep restriction increases free fatty acids in healthy men. Diabetologia 58, 791–798.

Chennaoui, M., Arnal, P.J., Drogou, C., Sauvet, F., Gomez-Merino, D., 2016. Sleep extension increases IGF-I concentrations before and during sleep deprivation in healthy young men. Appl. Physiol. Nutr. Me. 41, 963–970.

Copinschi, G., 2005. Metabolic and endocrine effects of sleep deprivation. Essential Psychopharmacol. 6, 341–347.

Chow, J.C., Ling, P.R., Qu, Z., Laviola, L., Ciccarone, A., Bistrian, B.R., Smith, R.J., 1996. Growth hormone stimulates tyrosine phosphorylation of JAK2 and STAT5, but not insulin receptor substrate-1 or SHC proteins in liver and skeletal muscle of normal rats in vivo. Endocrinology 137, 2880–2886.

Do Lago Godoi, F.R., Oliveira, M.G.M., Tufik, S., 2005. Effects of paradoxical sleep deprivation on the performance of rats in a model of visual attention. Behav. Brain. Res. 165, 138–145.

Der Spoel, E.V., Jansen, S.W., Akintola, A.A., Ballieux, B.E., Cobbaert, C.M., Slagboom, P.E., Van Heemst, D., 2016. Growth hormone secretion is diminished and tightly controlled in humans enriched for familial longevity. Aging Cell 15, 1126–1131.

Edén, S., 1979. Age-and sex-related differences in episodic growth hormone secretion in the rat. Endocrinology 105, 555–560.

Flores-Morales, A., Greenhalgh, C.J., Norstedt, G., Rico-Bautista, E., 2006. Negative regulation of growth hormone receptor signaling. Mol. Endocrinol. 20, 241–253.

Garg, R., Kumar, A., 2008. Possible role of citalopram and desipramine against sleep deprivation-induced anxiety like-behavior alterations and oxidative damage in mice. Indian J. Exp. Biol. 46, 770–776.

Hirotsu, C., Tufik, S., Andersen, M.L., 2015. Interactions between sleep, stress, and metabolism: From physiological to pathological conditions. Sleep Sci. 8, 143–152.

Irwin, M.R., Witarama, T., Caudill, M., Olmstead, R., Breen, E.C., 2015. Sleep loss activates cellular inflammation and signal transducer and activator of transcription (STAT) family proteins in humans. Brain Behav. Immun. 47, 86–92.

Ji, K.A., Yang, M.S., Jou, I., Shong, M.H., Joe, E.H., 2004. Thrombin induces expression of cytokine-induced SH2 protein (CIS) in rat brain astrocytes: Involvement of phospholipase A2, cyclooxygenase, and lipoxygenase. Glia 48, 102–111.

Jonsdottir, I.H., Schjerling, P., Ostrowski, K., Asp, S., Richter, E.A., Pedersen, B.K., 2000. Muscle contractions induce interleukin-6 mRNA production in rat skeletal muscles. J. Physiol. 528, 157–163.

Kimura, F., Tsai, C.W., 1984. Ultradian rhythm of growth hormone secretion and sleep in the adult male rat. J. Physio. 353, 305–315.

Lan, H.N., Li, W., Li, R., Zheng, X., Luo, G., 2019. Endocytosis and degradation of pegvisomant and a potential new mechanism that inhibits the nuclear translocation of GHR. J. Clin. Endocr. Metab. 104, 1887–1899.

Lan, H.N., Liu, H.L., Khan, M., Zheng, X., Yang, Y.J., Zhang, H., Yao, N.Q., 2018. The basic route of the nuclear translocation porcine growth hormone (GH)-growth hormone receptor (GHR) complex (pGH/GHR) in porcine hepatocytes. Gen. comp. endocr. 266, 101–109.

Leproult, R., Van Cauter, E., 2010. Role of sleep and sleep loss in hormonal release and metabolism. Endocr. Dev. 17, 11–21.

Møller, N., Gormsen, L.C., Schmitz, O., Lund, S., Jørgensen, J.O.L., Jessen, N., 2009. Free fatty acids inhibit growth hormone/signal transducer and activator of transcription-5 signaling in human muscle: a potential feedback mechanism. J. Clin. Endocr. Metab. 94, 2204–2207.

Murata, Y., Oka, A., Iseki, A., Mori, M., Ohe, K., Mine, K., Enjoji, M., 2018. Prolonged sleep deprivation decreases cell proliferation and immature newborn neurons in both dorsal and ventral hippocampus of male rats. Neurosci. Res. 131, 45–51.

Prinz, P.N., Weitzman, E.D., Cunningham, G.R., Karacan, I., 1983. Plasma growth hormone during sleep in young and aged men. J. Gerontol. 38, 519–524.

Rechtschaffen, A., Bergmann, B.M., 1995. Sleep deprivation in the rat by the disk-over-water method. Behav. Brain Res. 69, 55–63.

Redwine, L., Hauger, R.L., Gillin, J.C., Irwin, M., 2000. Effects of sleep and sleep deprivation on interleukin-6, growth hormone, cortisol, and melatonin levels in humans. J. Clin. Endocr. Metab. 85, 3597–3603.

Spiegel, K., Leproult, R., Colecchia, E.F., L'Hermite-Balériaux, M., Nie, Z., Copinschi, G., Van Cauter, E., 2000. Adaptation of the 24-h growth hormone profile to a state of sleep debt. Am. J. Physiol-Reg. I. 279, R874-R883.

Strine, T.W., Chapman, D.P., 2005. Associations of frequent sleep insufficiency with health-related quality of life and health behaviors. Sleep Med. 6, 23–27.

Van, C.E., Copinschi, G., 2000. Interrelationships between growthhormone and sleep. Growth. Horm. IGF. Res. 10, S57–S62.

Yu, J.H., Kim, K.H., Kim, H., 2008. SOCS 3 and PPAR-γ ligands inhibit the expression of IL-6 and TGF-β1 by regulating JAK2/STAT3 signaling in pancreas. Int. J. Biochem. Cell. 40, 677–688.

Zheng, G.H., Wang, Y.J., Wen, X., Han, X.R., Shen, M., Wang, S., Sun, C.H., 2018. Silencing of SOCS-1 and SOCS-3 suppresses renal interstitial fibrosis by alleviating renal tubular damage in a rat model of hydronephrosis. J. Cell. Biochem. 119, 2200–2211.