Mice with nucleus tractus solitarius injury induced by chronic restraint stress present impaired ability to raise blood glucose and glucagon levels when blood glucose levels plummet

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Abstract. Chronic restraint stress (CRS) induces insulin-resistant hyperglycemia by inducing injury to the brain neurons in the nucleus tractus solitarius (NTS). However, the CRS mice did not suffer from hypoglycemia. In this study, mice of both CRS and NTS mechanical injury models were induced to investigate whether impaired glucose metabolism has changed upon the extension of the survival time after modeling. Body weight, food and water intake, fasting blood glucose, glucose tolerance, and glucose metabolism related to blood hormone levels were monitored for 12 weeks following the induction of injury. The mice were also administered with insulin intraperitoneally, and the blood glucose and glucagon levels were measured and compared to those in the control mice administered with saline. The results showed that the body weights of CRS-hyperglycemic mice were significantly higher than those in the control group, while the body weights of NTS mechanically injured mice were significantly lower than those in the control group. The food and water intake of both CRS-hyperglycemic and NTS mechanically injured mice were significantly more than those in the control groups. Although the levels of fasting blood glucose and resting serum hormone in the injured mice have returned to normal levels, the utilization of glucose and hypoglycemic counterregulation (the response that raises the blood glucose levels) was impaired in either CRS-hyperglycemic or NTS mechanically injured mice. The blood glucagon levels following insulin administration showed abnormal increase. These findings suggest that the CRS-induced NTS injury resulted not only in early insulin-resistant hyperglycemia but also impaired the ability to raise blood glucose and glucagon levels when blood glucose levels plummet in the later stage.

Key words: Nucleus tractus solitarius, Chronic restraint stress, Hypoglycemia, Glucagon, Mouse
[8-10]. However, it is unclear as to whether CRS can impair these two types of neurons. In the present study, the body weight, food and water intake, fasting blood glucose, glucose tolerance, and blood hormone levels were analyzed for 12 weeks following CRS induction in a mouse model. The impairment of PrRP and GLP-1 neurons in NTS mice was assessed. Our findings showed impairment of hypoglycemic counterregulation in CRS-hyperglycemic (CRS-H) mice. This injury is similar to that observed in the mouse model with mechanical injury of NTS. The number of PrRP and GLP-1 neurons was decreased, and subsequently the PrRP neurons were lost. The impairment of hypoglycemic counterregulation might be associated with the dysfunction of NTS neurons following CRS.

Materials and Methods

Mice

Six-weeks-old male KM mice (closed colony with heterozygous genetic background; body weight 27.9 ± 1.16 g, fasting blood glucose level was 5.5 ± 0.2 mmol/L) were purchased from the Animal Experiment Center of Sichuan University. Mice were maintained under a 12-h light/dark cycle with free access to food and water at 22 ± 2°C in a humidity of 60% ± 5%. The size of the cages was 290 mm × 180 mm × 150 mm, and 4 or 5 mice were accommodated per cage. This study was approved by the Chengdu Medical College Medical Ethics Committee. Mice were marked and randomized by body weight for CRS, surgery of NTS mechanical injury, and control groups. The body weight of the mice was monitored using an electronic balance (JA31002). To measure the food and water intake, excess amount of food and water was given daily (at 14:00), and the same was followed the next day at the same time. The average consumption of food and water was calculated by dividing the difference between the two-day food intake (drinking water) by the number of mice in the cage. The mice were subjected to fasting for 5 hours before monitoring the fasting blood glucose. The blood glucose levels were measured using a portable blood glucose meter (Lifescan, Onetouch ultra) for detecting the blood from the cut tip of the tail.

CRS modeling

The protocol for induction of CRS model was done as reported previously by Zheng et al. [2]. In brief, the mice were randomly divided into two groups: group A (N = 25) and B (N = 20). The mice in group A were monitored consecutively for 12 weeks following CRS induction and group B mice were sacrificed at the end of CRS induction for immunohistochemistry. Mice were restrained individually for 6 hours per day for 7 consecutive days and then were set free for 3 days. This 10-day cycle was repeated 4 times. Mice in pseudo-restraint control (N = 10) entered the restraint devices at the same time as that of CRS mice, but then were released and set free to move immediately. There were 25 mice in group A for CRS modeling and 10 for control. At the end of the four cycles of CRS, eight CRS mice had fasting hyperglycemia (the blood glucose level was higher than 6.8 mmol/L), and 12 CRS mice were observed to be normoglycemic. Mice (N = 5) with fasting blood glucose between 6.2 and 6.8 mmol/L were not used in the subsequent experiments. The starting point (week 0) was the end of the fourth cycle of the CRS. Both model and control mice were free to move for the next 12 weeks (Fig. 1).

Mechanical injury of NTS

Mice were divided into NTS mechanical injury (N = 10) and sham control (N = 10). The protocol for the injury and sham surgery was done as reported previously by Zheng et al. [2]. The site of surgical injury was the right NTS (coronal plane of interaural –3.76 mm, bregma –7.56 mm [11], 0.2 mm away from the midline, and vertically punctured to a depth of 4.5 mm). The needle tip of the sham operation group was present in the fourth ventricle (3.6 mm in depth). The starting point (week 0) of the experiment was on day 10 after surgery. Both NTS-injured and sham-operated mice were maintained under normal conditions until the end of week 12. The operation and brain tissue changes of NTS mechanical injury is presented in Fig. S1.

Serum hormone determination

Blood was collected (at the end of week 12, before GTT) by cutting the tip of the mouse tail, and was allowed for clotting at room temperature for 30 min, then centrifuged at 4°C, 1,000 g for 20 min. The supernatant was then taken for testing (20 mL per single test). The serum samples were treated for an additional 1 hour in 75°C water bath to determine corticosterone. Hormone concentrations were determined by double antibody sandwich (for insulin and glucagon, Cloud-Clone, China) and competitive inhibition (for corticosterone, Cloud-Clone, China) by ELISA protocol, respectively. The data were determined by measuring the absorbance at 450 nm (microplate reader, PERLONG DNM-9602).

Glucose tolerance tests (GTT)

Mice were subjected to fasting for 5 h, and the fasting blood glucose concentration was measured as described above. The mice were then injected with glucose solution (i.p. 2 g/kg), and glucose levels were monitored at
30, 60, 90, and 120 min by discarding the first drop of the blood each time.

**Insulin injection and specimen collection**

At the end of induction of either CRS or NTS mechanical injury, the mice were intraperitoneally injected with saline. After 1 hour, the blood was withdrawn from the tail tip to measure glucagon concentration. Next day, the mice were challenged with 0.6 mIU/g of human insulin (Novolin R, Novo Nordisk, Denmark) and followed the same procedure to collect the blood. The saline and insulin injections and blood collection procedures were performed again at the end of week 12 following CRS or NTS mechanical injury (for group A only). The mice were then sacrificed 1.5 hours after insulin injection by administering an overdose of sodium pentobarbital and 4% neutral buffered paraformaldehyde was used to perfuse intracardially. The brains were harvested and fixed overnight at 4°C.

**Tissue processing and immunohistochemistry**

The brain specimens were routinely embedded in paraffin and serially sectioned at 6 μm thickness. Immunohistochemistry was performed following the SABC procedure (SPN-9001, ZSGB-BIO, China). The primary antibodies against GLP-1 (glucagon-like peptide-1), rabbit polyclonal IgG, 1:450 (ab22625, Abcam, UK) and PrRP (prolactin-releasing peptide), rabbit polyclonal IgG, 1:100 (SAB1303518, Sigma-Aldrich, USA) were used for immunohistochemical staining. The results of the reaction were visualized by DAB method.

**Statistical analysis**

The results were expressed as means ± SEM. Normal distribution and equality of variances were examined using Shapiro-Wilk test and Levene’s test. The data of body weight gain, corticosterone levels, GTT, and insulin tolerance test (ITT) were compared using repeated-measures two-way ANOVA, with time as the variable factor, and treatment as fixed factors. If a significant difference was revealed by ANOVA, then post hoc Bonferroni’s test was performed to further characterize the group differences. Data at a single time point were compared using one-way ANOVA, and a post hoc Student-Newman-Keul’s test was used to further characterize the group. Data between groups were compared with independent Student’s t tests. Analyses were performed using IBM SPSS (version 22), and p < 0.01 was considered to be statistically significant, and statistical charts were drawn with GraphPad Prism (version 7.0).

**Results**

**CRS and NTS mechanical injury had different effects on body weight gain and food/water intake following induction**

CRS and NTS mechanical injury demonstrated different effects on body weight gain in mice. Compared with pseudo-restraint control mice, CRS-H mice gained weight faster followed by induction while was slightly lowered at the end of induction. Eight weeks after the induction, the body weights of mice in CRS-H group were significantly higher than those in the control group (Fig. 2A). CRS-normoglycemic (CRS-N) mice showed
no change in weight and no significant difference was observed when compared to control mice. The body weights of NTS mechanically injured mice were significantly lower than those in the sham group from week 2 to 12 (Fig. 2A), suggesting that the NTS impairment induced by CRS was different from that of direct NTS mechanical injury. The consumption of food and water of CRS-H and NTS mechanically injured mice was significantly increased when compared to those in the control groups (Fig. 2B, C). CRS-H mice showed improved feeding behavior and successfully used nutrients to rapidly increase their body weight, while NTS mechanically injured mice utilized nutrients less effectively and weight gain was much slower although there was increased food and water intake (by considering the lighter body weight, the relative increase was greater than the CRS-H group).

Blood glucose and serum hormones of injured mice returned to normal levels following injury induction

The fasting blood glucose levels of CRS-H and NTS mechanically injured mice returned to normal levels at week 12 following induction of injury (Fig. 2D). At week 0, no significant differences in serum corticosterone levels were observed between the groups (Fig. S2). Compared with pseudo-restraint control mice, the CRS mice showed elevated levels of serum insulin (Fig. S3). At the end of week 12, the serum insulin levels and corticosterone levels showed no differences between the groups (Fig. 2E, F). These results suggested maintenance of blood glucose and serum metabolism-related hormones at normal levels under conditions, and changes in the amount of food and water consumption in model mice as well as differed in weight gain, resulting in changes in the CNS or endocrine system.

GTT showed abnormal fluctuations in blood glucose levels in injured mice

The fasting blood glucose levels in CRS-H mice and NTS mechanically injured mice showed typical insulin-resistant hyperglycemia at the end of the injury induction (Fig. 3A). At the end of week 12, although the fasting blood glucose levels in mice of each group were maintained at normal levels, GTT showed impaired blood glucose stabilization mechanism of CRS-H and NTS mechanically injured mice. The common features involved in both groups were that the blood glucose levels showed a significant increase after glucose injection and were fluctuated (Fig. 3B). At 30 min after adminis-
tration of glucose, the increase in blood glucose levels was greater than that of the control group, resulting in continuous decrease in blood glucose levels. At 120 min, the blood glucose levels of CRS-H and NTS mechanically injured mice were significantly lower than those of the control groups. These results suggested that following injury, the blood glucose levels of mice whose NTS were injured by either CRS or surgery can be maintained without stimulation, but utilization of glucose when blood glucose concentration increased and the ability to raise blood glucose levels when they fall below normal levels were both impaired (Fig. 3A). The impairment of the ability to raise blood glucose levels is considered as an indirect change in the later stage.

**Injured mice showed impaired hypoglycemic glucagon secretion response following insulin challenge**

Following CRS or NTS surgery, the injured mice demonstrated less reduction in the blood glucose levels than the control after insulin administration, suggesting insulin resistance in both injury models (Fig. 4A). Following repeated administration of insulin at week 12, CRS-H and NTS mechanically injured mice had impaired glucose utilization within 60 minutes after administration as compared to the control groups. The continuous decrease in blood glucose levels was significant when compared to those in the control group at 120 minutes after administration (Fig. 4B). ELISA of blood glucagon showed that the basal levels of glucagon were increased following induction of CRS or NTS mechanical injury. Following insulin challenge, the increased blood glucagon levels in both injured groups were significantly higher when compared to the control groups (Fig. 4C). After 12 weeks of injury, the basic levels of blood glucagon returned to normal. After insulin challenge, the increase in blood glucagon levels was significantly smaller than that of control (Fig. 4D). These results suggested that following the injury, the CRS or NTS mechanically injured mice not only had impaired glucose utilization but also had impaired ability to increase blood glucose concentrations in response to low blood glucose levels.

**Neurons in NTS of CRS-H mice had secondary loss following injury**

Compared with CRS-N mice, the number of PrRP and GLP-1 immunoreactive neurons in NTS of CRS-H mice had significantly decreased at week 12 following injury induction (Fig. 5), but there were no differences in the number of PrRP neurons in CRS-H mice when compared to CRS-N mice at week 0 (Fig. 5). These results suggested that there was secondary loss of PrRP neurons in NTS of CRS-H mice at later stages.

**Discussion**

This study showed abnormal metabolic parameters and dysfunction of blood glucose metabolism in mice with CRS. After chronic stress, the phenomenon of fasting blood glucose levels gradually returning to normal state, i.e., from a high level, has been reported in rats [12]. However, the recovery of fasting blood glucose

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**Fig. 3** The GTT results of CRS-H and NTS mechanically injured mice showed different characteristics at the end of modeling and the 12th week after modeling. GTT data at week 0 showed typical insulin-resistant hyperglycemia (A). At the 12th week, the GTT data showed that the glucose utilization was still abnormal (B). In addition, the mice could not raise the blood glucose level in time. The blood glucose levels of CRS-H and NTSi-op mice were significantly lower than that of the control groups at 120 minutes after glucose injection (B). Data are mean ± SEM; Statistical test: repeated measures two-way ANOVA with Bonferroni post hoc test; * CRS-H compared with CRS-Ctrl, \( p < 0.01 \); # NTSi-op compared with NTSi-sham, \( p < 0.01 \). CRS, chronic restraint stress; CRS-Ctrl, pseudo-restraint control; CRS-N, normoglycemia after CRS; CRS-H, hyperglycemia after CRS; NTS, nucleus tractus solitarius; NTSi-sham, sham operation of the NTS; NTSi-op, surgical injury of the NTS.
levels does not reflect normal regulation of glucose metabolism. The GTT results showed that even if the fasting blood glucose levels in CRS-H mice were normal after week 10 of injury induction, then defective blood glucose utilization was observed (Figs. 3 and S4). At this time, the blood glucose levels were more dramatically increased after glucose injection. More importantly, following injury, the hypoglycemic counterregulation in CRS-H mice was also impaired. The blood glucose levels in the second half of GTT could not rise normally and also could not increase in time after insulin administration (the time required to return to normal level was much longer than in the control or CRS-N group; Fig. S5). In addition, the amount of insulin-induced glucagon secretion was decreased, suggesting that the dysfunction of glycogen breakdown produces glucose in response to decreased blood glucose levels. Similar metabolic dysregulation was observed in NTS mechanically injured mice but not in CRS-N or sham mice, suggesting the association of glucose-related NTS impairment [2] with CRS-induced changes following injury. Only mice with NTS impaired by CRS exhibited metabolic dysregulation, and the cause of NTS injury under CRS has not been fully understood. There are also some minor differences between the models of NTS mechanical injury and CRS. All NTS mechanically injured mice developed defective hypoglycemic counterregulation, while CRS-H mice showed slight abnormalities. NTS mechanically injured mice showed impairment in hypoglycemic counterregulation 2 weeks earlier than CRS-H mice. These observations suggested that the impairment of hypoglycemic counterregulation did not occur in the early stage when CRS caused NTS injury but was regarded as a secondary change following injury.

Under hypoglycemic conditions, the perception of blood glucose and counterregulation in the brain is achieved through a neural pathway that is initiated from the lateral parabrachial nucleus (LPBN) [13]. LPBN neurons project toward steroidogenic factor-1 (SF-1) neurons in the ventromedial hypothalamus (VMH) [14, 15], and SF-1 neurons project toward the bed nucleus of the stria terminalis (BNST) and other parts of the hypothalamus, participating in sympathetic activation and endocrine regulation against hypoglycemia [14, 16, 17].
Previous studies have shown that VMH plays a pivotal role in physiological response to hypoglycemia. VMH is considered to be a satiety center, which mediates the response of hypothalamus in hypoglycemia [16, 18-22]. After VMH injury, the increased blood glucagon levels and catecholamines in hypoglycemia are reduced by 50%–80% [23]. Our study showed increased blood glucagon levels in the injured mice after reduction in insulin administration, and this is consistent with the feature of obesity in mice causing by VMH injury [16, 18]. Activation of VMH increased the amplitude of blood glucose responsiveness, but fasting blood glucose levels [14], insulin levels [24], and corticosterone levels [25] were unaffected. Our study showed that the metabolic parameters such as fasting blood glucose, insulin, and corticosterone levels appeared normal in mice after week 12 of injury induction, and this was consistent with the characteristics of VMH injury. In addition, the increased food intake in injured mice was also similar to the symptoms of VMH injury. However, this is different from the dysregulation in CRS-H mice as the brain of CRS-H mice only shows neuronal impairment in NTS region and cerebral cortex, and no other impairment is observed in the hypothalamus and other parts of the brainstem [2]. Moreover, the sudden decrease in blood glucose levels caused by insulin challenge cannot activate c-Fos expression of VMH [4, 26], and the gamma aminobutyric acid (GABA) signaling of VMH plays a protective role [25], making neurons less susceptible to metabolic stress. The effect of VMH injury on blood glucose metabolism remains complex, causing no changes in glucose tolerance [27], and this was inconsistent with the results of our experiment. Similarly, LPBN showed no injury in CRS mice, and even if there were undetectable lesions, the initial performance should be a counterregulation disorder [13, 15], instead of insulin-resistant hyperglycemia at week 0 of the experiment.

The regulation of feeding, water drinking [28], and energy metabolism is done by neural pathway of LPBN→VMH→BNST, but its precise mechanism has not been fully understood. NTS is actually the first region in the brain to sense hypoglycemia and is also a key structure in CRS injury. NTS is a relay nucleus with special visceral sensation and is the first area in the brain.
to sense changes in the blood glucose levels [29, 30].
NTS is involved in glucose-excited and glucose-
inhibitory neurons and have complex connections to
other areas of the brain that are involved in energy regu-
lation [4, 20]. Therefore, when the blood glucose levels
rise or fall, NTS is the first to sense the change and then
it notifies other brain regions to initiate regulation. NTS
is also one of the earliest well-developed structures in the
circuits of feeding and nutrition regulation [31], and
this also reflects the basis and importance of NTS in
metabolic regulation. Under stress conditions, the
medulla oblongata subsides hypothalamus to control
energy metabolism [32], and PrRP and GLP-1 neurons
of NTS regulate stress and energy and are regarded as
upstream regulators of the hypothalamus [33-35]. In
addition, NTS has extensive fiber projections into other
areas of the brain [36, 37]. For example, cholecystokinin
and noradrenergic neurons of NTS in mice project into
CGRP neurons of LPBN, inhibiting the feeding behavior
[38]. Most of the GLP-1 fibers that are derived from
medulla oblongata also project and excite CGRP neurons
in LPBN [39], while the GLP-1 fibers in the brain, espe-
cially the brainstem, are almost entirely from NTS [10,
36]. In the present study, the number of GLP-1 neurons
in NTS of CRS-H mice was significantly reduced, and
the effectiveness of the aforementioned regulatory mech-
nisms is inevitably affected. It has been reported that
after chronic immobilization stress, the dendritic fields
of BNST neurons increase [40], which might be considered
as an adaptive change when the source signal weakens. It
can be seen that the NTS injury causes LPBN to lack a
perceptual signal input, and thus, the hypoglycemic
counterregulation remains to be abnormal. This meant
that when the threat of hypoglycemia occurs, the sensory
ability of the brain is then impaired, and the LPBN and
its downstream regulatory pathway cannot respond effect-
ively. Therefore, after inclusion of NTS, the “NTS→
LPBN→VMH→BNST” regulatory pathway is consid-
ered complete.

An impaired hypoglycemic counterregulation was
developed after 8 to 10 weeks after the injury (Fig. S4)
but not immediately after NTS injury (by both CRS and
surgery). This suggests that NTS injury might have initi-
ated a chain reaction. At the beginning, it is regarded as a
disorder of blood glucose regulation caused by loss of
perception, and the mechanism of raising blood glucose
level under hypoglycemia showed no impairment. After
the injury, the compensation burden or local circuit
reconstruction caused by NTS injury gradually affected
the hypoglycemic counterregulation. The results of this
study showed that PrRP neurons of NTS in CRS-H mice
have secondary loss in the later stage, which might be
related to this chain reaction. The PrRP neurons are
mainly distributed in the NTS and ventrolateral medulla
[41, 42], and the ventrolateral medulla is mainly
involved in satiety regulation and inhibits feeding [8, 9].
There were no secondary changes of PrRP neurons in
this region by CRS (data not shown), and the food intake
of the model mice was not decreased in this study. PrRP
neurons in the NTS are involved in endocrine responses
under stress conditions [43, 44]. In this experiment,
CRS-H mice have developed an impairment of hypogly-
cemic counterregulation approximately 2 weeks later
than those in the NTS mechanically injured mice. The
two-week time difference is due to the result of gradual
degeneration and decompensation of PrRP neurons. The
relationship between the secondary loss of PrRP neurons
in NTS and the impairment of hypoglycemic counter-
regulation, and as to why the above secondary changes
occur at later stage should be further studied.

There are several technical issues in this study that are
still necessary to be researched. The first is the determi-
nation of the starting point after modeling (week 0). The
week 0 of CRS-injured mice was at the end of the fourth
cycle of CRS induction, while the week 0 of NTS sur-
gery mice was on day 10 after surgery. According to our
observation from the previous study, after 4 cycles of
CRS, insulin-resistance hyperglycemia in mice has been
stabilized, and the NTS neurons no longer undergo new
apoptosis [2]. On the other hand, the fasting blood glu-
cose levels in mice increases and are stabilized at a
higher level, and the inflammatory response of the
injured tissue begins to be weakened on day 10 after
NTS surgery [2]. These two time points correspond to
the time when NTS injury processes are substantially
completed for the two models, respectively. Secondly,
there is no routine ITT after modeling in this experiment,
and this is because there is a gradual loss of hypoglyce-
ic counterregulation. The model mice cannot tolerate
repeated administration of conventional doses of insulin,
and hypoglycemia itself might further impair the regula-
tory function of the brain [6]. Thirdly, although the
impairment of hypoglycemic counterregulation was
observed in this experiment, the observation indicators
are incomplete. The acute response to hypoglycemia is
not only glucagon secretion, but also other reactions,
such as elevated adrenaline levels [20]. We did not
observe a difference in the increase in adrenaline levels
between the groups in previous experiments. This might
be due to reagent or technical reasons, or the adrenaline
response of NTS-injured mice was not affected. Specific
reasons need to be further studied. Fourthly, the “NTS→
LPBN→VMH→BNST” regulatory pathway proposed in
this paper contains many inhibitory projections. The c-
Fos immunoassay is considered ineffective or the results
are difficult to interpret. The resolution of functional
magnetic resonance also remains unsatisfactory for research on the mouse brainstem. To further study the regulation of this pathway, it is necessary to redesign the experiments using other methods.

**Conclusion**

In summary, this study demonstrated that the mice with CRS-induced NTS injury had early insulin-resistant hyperglycemia and developed impaired ability to raise the blood glucose levels in the later stage. The defective metabolic regulation might be associated with secondary impairment of NTS neurons, and this observation provides new ideas in explaining the mechanism of recurrent hypoglycemia in some metabolic diseases such as type 2 diabetes mellitus. This might in turn assist in preventing and treating hypoglycemia caused by abnormal CNS regulation in the future.

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**Conflict of Interest**

Authors declare no conflict of interest related to the study.

**Author Contributions**

W. B., X. Zheng and X. Zhou designed the experiments and analyzed data. X. Zheng and W. B. wrote the manuscript. W. B., X. Zheng and S. W. performed the experiments. X. Zhou reviewed and edited the manuscript.

**Fig. S1** Examples of NTS injury. (A) Needle route in the coronal section interaural –3.76 mm, bregma –7.56 mm; The endpoint of the arrow represents the furthest point of the mechanical injury, and the box is the area where the NTS is observed in (E)–(G). When the brain tissue was sectioned, the rostral part was close to the observer, and the injury is on the right side. (B) HE staining low magnification imaging of a NTS-injured mouse (12 weeks after surgery); ▲ Indicates the needle route; Bar = 100 μm. (C) HE staining image for the end area of the needle; Bar = 80 μm. (D) The trend of postoperative fasting blood glucose levels. After NTS mechanical injury, the fasting blood glucose increased gradually. * NTS-injury compared with sham operation, p < 0.01. (E) The NTS area at the end of the 4th cycle of CRS (from a hyperglycemic mouse); ↑ Normal neurons; △ Abnormal neurons with concentrated staining; Bar = 100 μm. (F) The NTS area 10 days after mechanical injury with few normal neurons; ▲ Pigmentation; Bar = 100 μm. (G) The NTS area 10 days after sham operation; ↑ Normal neurons; Bar = 100 μm.

Remarks: A successful surgery of NTS mechanical injury has three manifestations: 1) a gradually elevated fasting blood glucose level, 2) Normal appetite and bowel movements, and 3) Histologically confirmed lesions include NTS but do not involve dorsal nucleus of the vagus nerve.
**Fig. S2** Serum corticosterone levels. (A) Serum corticosterone levels in CRS mice increased during the first two restraint cycles compared with the pseudo-restraint control, and returned to normal after the 3rd cycle. So at the end of CRS (week 0), there was no difference of serum corticosterone levels between CRS and control mice. * CRS compared with pseudo-restraint control, \( p < 0.01 \). (B) Serum corticosterone levels before and 10 days after the NTS mechanical injury. The operation had no significant effect on serum corticosterone levels. CRS, chronic restraint stress; Ctrl, control.

**Fig. S3** Comparison of serum insulin levels on the 7th day after the end of the 4th CRS cycle. Serum insulin levels in CRS mice are significantly higher than those of controls regardless of hyperglycemia. * Compared with CRS-Ctrl, \( p < 0.01 \). CRS, chronic restraint stress; CRS-Ctrl, pseudo-restraint control; CRS-N, normoglycemia after CRS; CRS-H, hyperglycemia after CRS. This data is from our previous experimental study and has been published as part of figure 2D of the article Zheng et al., 2018.
Fig. S4 GTT data of CRS and NTS mechanically injured mice at different time points. The monitoring time range is week 0 to 10 after modeling. The fasting blood glucose of CRS-hyperglycemic mice returned to normal at the 10th week, when these mice showed a disorder of hypoglycemic counterregulation. The fasting blood glucose of NTS mechanically injured mice also returned to normal at the 10th week, but the counterregulation disorder appeared at the 8th week (a few individuals appeared on the 6th week). Data are mean ± SEM; Statistical test: repeated measures two-way ANOVA with Bonferroni post hoc test; * CRS-H compared with CRS-Ctrl, \( p < 0.01 \); # NTSi-op compared with NTSi-sham, \( p < 0.01 \). The results are pre-experimental data of mice different from that in the text.

Fig. S5 Time-extended GTT data of CRS and NTS mechanically injured mice at 6th and 10th week after modeling. At the 6th week after modeling, the blood glucose level of CRS-hyperglycemic mice could recover to a slightly hyperglycemic state at 120 min after glucose injection. The recovery time required for NTS mechanically injured mice was longer than that of the CRS group. At the 10th week, CRS-hyperglycemia and NTS mechanically injured mice showed hypoglycemia 120 min after glucose injection. The recovery time of blood glucose level of the CRS group was prolonged by about 1 hour, while the NTS mechanical injury group was delayed for a longer period of time, and one mouse died after the test. Data are mean ± SEM; Statistical test: repeated measures two-way ANOVA with Bonferroni post hoc test; * CRS-H compared with CRS-Ctrl, \( p < 0.01 \); # NTSi-op compared with NTSi-sham, \( p < 0.01 \). The results are pre-experimental data of mice different from that in the text. CRS, chronic restraint stress; CRS-Ctrl, pseudo-restraint control; CRS-N, normoglycemia after CRS; CRS-H, hyperglycemia after CRS; NTS, nucleus tractus solitarius; NTSi-sham, sham operation of the NTS; NTSi-op, surgical injury of the NTS.
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