Effects of Removal of GABAergic tone in Dorsomedial Hypothalamic Nucleus on Peripheral Metabolic Responses

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Abstract: Dorsomedial hypothalamus (DMH) plays a key role in the organization of functional responses to stress. Microinjection of $GABA_A$ antagonist bicuculline methiodide (BMI) into DMH evokes cardiovascular and neuroendocrine changes typically seen in experimental emotional stress. The aim of the present study was to investigate the peripheral metabolism of glucose following microinjection of BMI, (10pmol / 100nl) or vehicle (NaCl 0.9% - 100nl) into DMH. Male wistar rats were anesthetized and a guide cannula was set into DMH. The jejunal loop was isolated and perfused with Tyrode solution. Blood samples were taken 15 and 40 minutes after the beginning of experimental period. When compared to vehicle, BMI into DMH increased plasma insulin (0.22±0.04 vs. 0.34±0.04 ng/ml; $P<0.05$), decreased glucose-6-phosphate in the liver (0.012±0.003 *vs.* 0.004±0.002 μmol/g tissue; $P<0.05$) and increased this same metabolite in muscle (0.14±0.05 *vs.* 0.43±0.10 μmol/g tissue; $P<0.05$). We found that DMH activation caused changes in plasma insulin and intermediate metabolite of glycolitic pathways.

Keywords: Digestion, parasympathetic, autonomic, intestine.

INTRODUCTION

The hypothalamus plays a key role in maintaining physiological homeostasis by regulating autonomic, neuroendocrine and intestinal functions [1, 2]. The gut and brain are closely integrated, with a bi-directional pathway through the autonomic nervous system [1].

Studies have shown that the region of dorsomedial hypothalamus (DMH) is a key nucleus in the organization of the cardiovascular response to emotional stress [3]. Neurons in the DMH are under powerful GABAergic synaptic input [4,5]. Removal of this inhibitory input through microinjection of $GABA_A$ antagonist, bicuculline methiodide (BMI), evokes responses typically seen in emotional stress [3]. Injections of BMI into the compact region of DMH provokes dose-dependent cardiovascular responses [5], increases in locomotor activity, and ACTH plasma levels, the latter is a hallmark of the neuroendocrine response to stress [6]. Recently, studies have shown that chronic inhibition of GABA synthesis in the DMH elicits panic-like responses in rats [7, 8]. On the other hand, microinjection of the $GABA_A$ agonist muscimol into the DMH attenuates the increase in blood pressure and tachycardia evoked by exposure to air jet stress [9]. Altogether, these findings highlight the key role of the DMH in the organization of the physiological responses to stress.

Disinhibition of the DMH also produces important changes in the gastrointestinal tract. Greenwood & DiMicco (1995) found that the chemical disinhibition of the DMH increased jejunal and colonic motility [10]. Recently, Xavier and colleagues demonstrated that microinjection of BMI into the DMH induced pronounced falls (~50%) in mesenteric blood flow [11]. The effects of BMI injection into the DMH results in activation of vagal cholinergic pathways, involved in controlling intestinal motility, and sympathetic pathways, which are mainly responsible for modulating cardiovascular changes [10, 12, 13].

Stress situations may modify the absorptive function of the intestine. For example, opposite effects were described in the expression of GLUT 2 (an important glucose transporter in the brush border membrane) in different kinds of stress situations [14, 15]. Complementary studies have also found a reduction in Na^+, Cl^- and water absorption in individuals submitted to psychological [16] or physical stresses [17].

The similarity between the effects evoked by the injection of BMI into the DMH in rats and the response to acute stress in this species allows speculating that the same mechanism is involved in both phenomena. For this reason, the injection of BMI into the DMH has been extensively used to investigate the central pathways involved in physiological changes brought upon by acute stress [3, 12, 13, 18, 19]. Therefore, using the approach of BMI injection into the DMH, the present study was undertaken to investigate the effect produced by the removal of GABAergic tone on DMH neurons in the jejunal absorption and peripheral metabolism of glucose.

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METHODS

Animals

Adult male Wistar rats from the main breeding stock of the Institute of Biological Sciences, weighing between 270-300 g were housed under standard laboratory conditions of a 12:12-h light–dark cycle and 23 ± 2°C. The animals were housed individually and fasted for 12 h before the experimental procedures, but water was offered ad libitum. All experiments were in agreement with the International Ethical Principles in Animal Experimentation, and were approved by our local Ethics Committee in Animal Experimentation (protocol n° 21/05).

General Procedures

Under tribromoethanol anesthesia (250 mg/kg i.p.), rats were instrumented with unilateral stainless steel guide cannulas (22 gauge, 16 mm length), targeted to DMH. The adequacy of anesthesia was verified by the absence of a withdrawal response to nociceptive stimulation of a hind paw. Supplemental doses of tribromoethanol (15 mg/kg i.v.) were given when necessary. Animals were positioned in a stereotaxic frame (Stoelting, IL, USA) with the tooth bar fixed 3.3 mm below the level of the interaural line. The guide cannula was positioned using bregma as reference point and according coordinates determined by the atlas of Paxinos and Watson, 1986: 3.2 mm posterior, 0.6 mm lateral, 8.0 mm ventral [20]. The guide cannula was fixed with dental acrylic cement anchored by two screws placed in the skull. After surgical procedures, animals were allowed to recover in their home cages.

After two days of recovery, animals were anesthetized again with tribromoethanol, a small incision was made in the inguinal region, and the femoral artery was exposed. A polyethylene catheter (Clay Adams, 0.0 11 I.D.) filled with heparinized saline and sealed with a stylet was inserted in the abdominal aorta through the femoral artery (~4 cm) for recording of blood pressure and heart rate. The catheter was also routed subcutaneously to the nape of the neck where it was exteriorized and secured. All incisions were closed with small sutures. Rats were then allowed to recover in their home cages for at least 24 h before experiments began. All animals for which data were reported remained in good health conditions throughout the course of surgical procedures and experimental protocol as assessed by appearance, behavior, and maintenance of body weight.

Experimental Procedures

After 12 hours of fasting, each animal was subjected to the following protocols. For the duration of each experiment, heart rate and blood pressure were recorded continuously (model MP100 A-CE, Biopac Systems, CA, USA) and the microinjection procedures commenced only after a stable baseline heart rate and blood pressure had been established for ~15 min. Microinjections of BMI (10pmol / 100nl) or vehicle (NaCl 0.9% - 100nl; control group) were performed in different groups of rats. Injections into DMH were made on freely moving animals with a 30-gauge injection needle (17 mm length), connected to a polyethylene tubing (Norton, 0.010 I.D.) coupled to a 5-µl Hamilton syringe filled with distilled water. The polyethylene tubing was filled with the drug, and between the two liquids, there was a small air bubble. The movement of the air bubble demonstrated successful injection [21]. Heart rate and blood pressure were sampled at 15 min intervals after the microinjection.

Jejunal Perfusion

Immediately after the end of the recording of cardiovascular parameters, rats were anesthetized (Thiopentax, Cristalia, Brazil) (40 mg kg^{-1} i.p.) and the abdominal cavity was opened through a median xypho-pubic laparotomy. A 20-cm segment of jejunum was isolated, preserving nerves and the vascular pedicle and two cannulae were introduced into each extremity of the jejunal loop, one for perfusion and the other to drain the fluids. The abdominal wall was then closed in order to avoid tissue dehydration. Both cannulae were exteriorized through the extremities of the abdominal suture. Tyrode solution (137 mM NaCl, 2.7 mM KCl, 1.36 mM $CaCl_2$, 0.49 mM $MgCl_2$, 11.9 mM $NaHCO_3$, and 5mM D-glucose), at 37°C, pH 8.0 (buffered by HCO_3^-), was perfused at a rate of 0.5 mL min^{-1} for 15 min in order to equilibrate the fluids into jejunal lumen [22]. Jejunal perfusion was then continued with a Tyrode solution containing twice the normal concentrations of glucose, sodium, and potassium for the 40-min experimental period. It was started after 50-min of BMI microinjection into DMH. The effluents were collected separately into tubes every 10-min intervals and maintained on ice. Blood samples were collected in the beginning, 15-min intervals and in the end of Tyrode perfusion. After 40-min perfusion, tissue samples of liver and gastrocnemius muscle were collected and snap frozen in liquid nitrogen and kept frozen at –80° C, until biochemical analysis. The rats were euthanatized by overdose of anesthesia.

Biochemical Determinations

The effluent glucose and the plasma glucose were determined by an enzymatic method based on glucose oxidase (Glucose God-Ana, Labtest, Brazil), using a standard curve of glucose. Glucose perfusate results were expressed by the difference between influx and efflux of glucose (µmol/min). Plasma insulin was measured only on time 15 min after Tyrode perfusion by radioimmunoassay technique (Linco Research, St. Charles, MO, USA). The HOMA index, an insulin sensitivity test, was used to calculate the whole-body insulin resistance [insulin (µU/mL) x glucose (mmol/L)/22.5] [23].

Glycogen in frozen liver (1 g) or gastrocnemius muscle (1 g) was extracted by homogenization of tissue in 3 mL of 6% (w/v) ice-cold $HClO_4$. The homogenate was centrifuged at 2900xg for five minutes. The supernatant volume was measured and neutralized with 10% (w/v) KOH. The extract was hydrolysed by anthrone reagent (Merck, Darmstadt, Germany) (Hassid; Abraham, 1957) and analysed for free glucose spectrophotometrically at 620 nm. The concentration of glycogen was estimated from a standard curve of glucose. The metabolites glucose-6-phosphate, fructose-6-phosphate, and ATP were analysed in the supernatants of the tissue homogenates as previously described [24].

Histology

At the end of experiments, it was microinjected 2% Alcian Blue dye (100 nl) into injection sites for subsequent histological confirmation. The brain was removed and stored in 4% paraformaldehyde for 24 h. After this period, the

brains were placed in 20% sucrose solution for at least 2 days. Subsequently, 100-µm thick coronal sections in the region of the hypothalamus were cut on a freezing microtome. Sections were mounted on slides and then counterstained with Neutral Red for histological confirmation of the injection sites in the DMH. The Atlas of Paxinos and Watson was used as reference [20].

Data Analysis

The baseline values of mean arterial pressure (MAP) and heart rate (HR) were measured as the average values of these variables for the 5-min period immediately preceding microinjection of the drug. Changes in MAP and HR were sampled at 5-min following microinjections into DMH, calculated from the average of 1-min selected period. Comparisons between responses evoked by microinjections of BMI into the DMH and control group were determined by Student's t-test. Significance was taken at $P<0.05$. Data are expressed as mean ± SEM.

RESULTS

Microinjection of BMI into DMH (n = 6) markedly increased blood pressure (BP) (Δ MAP = 14 ± 5 *vs.* 2 ± 1 mmHg; $P<0.05$) and heart rate (HR) (Δ HR = 150 ± 22 *vs.* 2 ± 1 bpm; $P<0.05$) when compared to results from control group (n = 6), which received microinjection of vehicle into the same region. These peak changes were found about 5 minutes following DMH disinhibition.

Injection of BMI into DMH did not alter the glucose absorption (n = 6 each) (Fig. **1A**) and plasma glucose levels when compared to control group (n = 5) (Fig. **1B**). However, plasma insulin levels were significantly increased by BMI (n = 6) into the DMH compared to saline group (n = 5) only 15 minutes after perfusion (*$P < 0.05$) (Fig. **1C**).

There were no changes in the glycogen content measured from liver or muscle in both experimental groups (n = 6 each) (Fig. **2A** and **B**, respectively).

Figure 3 shows the content of glucose-6-phosphate, fructose-6-phosphate, and ATP in the liver (n = 4-6) (Fig. **3A, C, E**) and gastrocnemius muscle (n = 4-6) (Fig. **3B**, **D**, **F**). Compared to vehicle, microinjection of BMI into DMH significantly decreased liver glucose-6-phosphate levels (*$P < 0.05$) (Fig. **3A**). The other metabolites, fructose-6- phosphate and ATP were not altered (n = 4-6). The muscular glucose-6-phosphate content was significantly increased after microinjection of BMI into DMH when compared to control group (*$P < 0.05$) (Fig. **3B**). Fructose-6-phosphate and ATP were not changed by microinjection of BMI into DMH.

DISCUSSION

The experiments in the present study reveal that removal of GABAergic tone, which controls DMH neuronal activity, increase plasma insulin concentration and glucose-6-phosphate in muscle, and decrease the last one in liver. Previous studies have demonstrated that the removal of the tonic inhibitory input to DMH with BMI evokes increases in HR, MAP, renal [12] and cardiac sympathetic nerve activity [13]. In conscious rats, the activation of DMH neurons increases HR, MAP [25], which is in agreement with the cardiovascular changes observed in the present study. Cardiovascular changes resulting from BMI injection into the DMH are accompanied by other characteristic effects of the defense reaction typically seen in stress, such as an increase in plasma ACTH [6] and "fight or flight" behavioral responses [3]. The particular advantage to use physiologically awake animals instead of anesthetized is to avoid depression of SNC. Autonomic functions are significantly affected by anesthetics, and

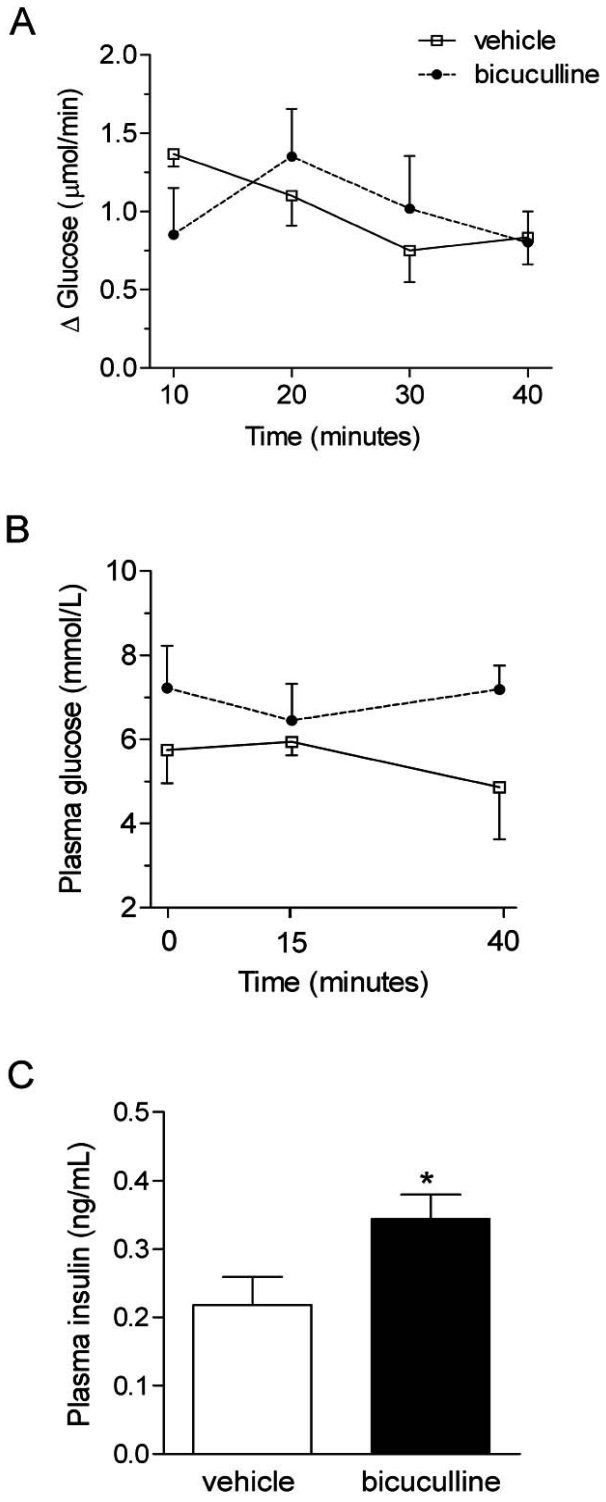

Fig. (1). Intestinal glucose absorption in the isolated jejunum (A) plasma glucose (B) and plasma insulin (C) from rats that received BMI (filled circles) (n = 5-6) and vehicle (open squares) (n = 5) into DMH (*$P < 0.05$).

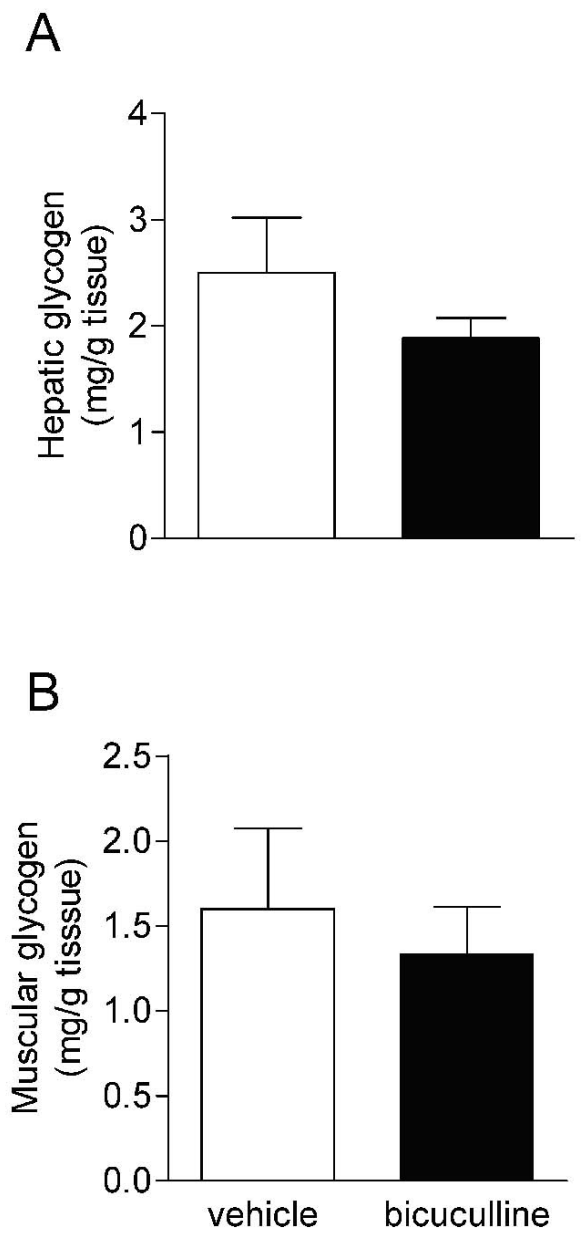

Fig. (2). Glycogen content of liver (A) and gastrocnemius muscle (B) after injection of BMI (n = 6) and saline (n = 6) into DMH.

responses to certain interventions are quite different between anesthetized and awake animals [26, 27]. For example, it is well known that GABA receptor function is allosterically enhanced by a wide range anesthetic compounds [28, 29].

It could be argued that the effect of BMI is due to a peripheral action more than on receptors located in the DMH. This seems unlikely, however, taking into account that the site of action for BMI in the DMH is very restricted. Microinjections of BMI only one mm distant from DMH results in very small and transient cardiovascular responses [30, 31], whereas injections of BMI specifically into DMH provokes large increases in mean arterial pressure, heart rate and sympathetic activity [5, 12, 13].

In the present study, no changes in jejunal glucose absorption were observed. It has been reported that microinjection of BMI into the DMH increase jejunal motility [10]. Additionally, microinjections of BMI into the same nucleus caused reductions in mesenteric blood flow [11]. These finds associated would be decreasing the chance of glucose absorption. Although these changes occur during the DMH disinhibition, in the current study these factors were not able to cause decrease in glucose absorption, probably due to differences between experimental protocols.

In the present study, an increase in plasma insulin (at 15 min after perfusion) levels was detected although no differences in plasma glucose concentrations were found in the animals submitted to microinjection of BMI into the DMH. Zaia and colleagues [32] found that DMH stimulation by noradrenalin evoked an increase in glucose plasma levels 5 and 10 minutes after the microinjection, although no change was detected 20 minutes after the noradrenalin microinjection. Likewise, animals submitted to a chronic-stress model (30 days) exhibited an increase in plasma glucose levels on Day 15 [33]. The absence of a hyperglycemic response may be due to differences between the experimental protocols. Another possibility is that the higher insulin levels, probably caused by autonomic influences directly in the pancreas

[34, 35], could facilitate the uptake of glucose by tissues, including muscle (that demonstrate an increase in glucose-6-phosphate). Taking together these considerations could explain unchanged plasma glucose.

No changes in hepatic glycogen were observed, which is in agreement with the results described by Yamada and coworkers [36]. However, the animals that were submitted to microinjection of BMI into the DMH exhibited a reduction in hepatic glucose-6-phosphate. This change could be due to a glycogenolysis in the liver, which may have caused glucose to be released into the bloodstream and consequent glucose-6-phosphate decrease. This result may traduce the catabolic state produced by the microinjection of BMI into DMH, in order to maintain energetic status to the organism.

The present study did not find any change in muscle glycogen content. However, there was a significant increase in muscle glucose-6-phosphate. Conversely to the liver, in the skeletal muscle glycolysis is mainly involved with the regulation of energy production, which is restricted to this organ [37]; therefore the increased in glucose-6-phosphate content also may play a role in restoring the glycogen pool that would be depleted in response to stress stimulation. In addition, it is possible that the increase in this metabolite in the muscle might result from blood flow redistribution evoked by a fight-or-flight response coordinated by DMH neurons. In this regard, it is known that BMI injection into the DMH induces pronounced dilatation of vessels serving skeletal muscle [38] at the same time that it causes a marked reduction in mesenteric flow [11, 38]. These alterations in blood flow replicate the pattern of hemodynamic changes resulting from acute emotional stress in several animals [38].

CONCLUSION

The activation of DMH neurons evokes changes in plasma insulin concentration, an important hormone for the glucose metabolism homeostasis. In addition, activation of DMH also changes hepatic and muscular glucose-6-phosphate, a key metabolite in glucose and glycogen metabolism. The present study supports and extends previous

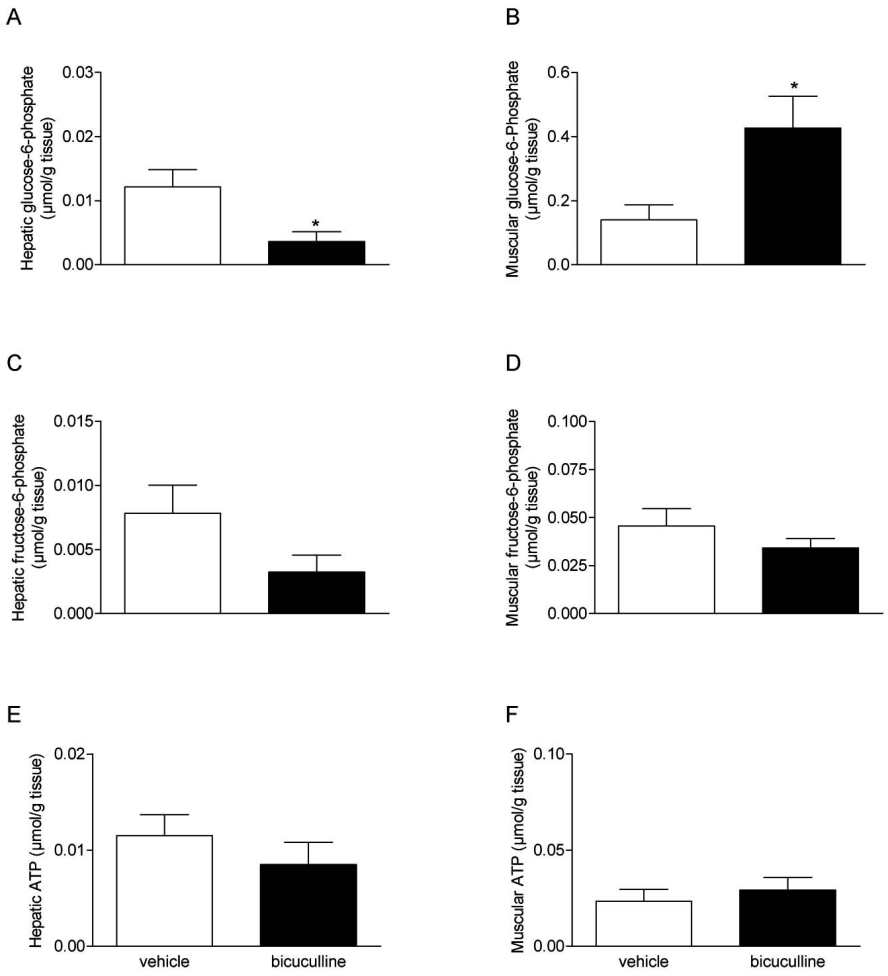

Fig. (3). Intermediate metabolites of the glycolytic pathway sampled from the liver (A, C, E) and gastrocnemius muscle (B, D and F) in rats submitted to injection of BMI (n = 4-6) and saline (n = 4-6) into DMH (*$P < 0.05$).

findings demonstrating the key role of the DMH in integrating physiological responses to emotional stress.

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