Effects of low degree of high ambient temperature on neural pathways from median preoptic nucleus to arcuate nucleus in related to food intake.

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ผลของอุณหภูมิแวดล้อมสูงระดับต่ำต่อวิถีประสาทจาก มีเดียล พรีออปติค นิวเคลียส ไปยังอาร์คูเอตนิวเคลียสที่มีความเกี่ยวข้องต่อการกินอาหาร

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พรศิริ สุวรรณภาพร : ผลของอุณหภูมิแวดล้อมสูงระดับต่ำต่อวิถีประสาทจาก มีเดียล พรีออปติคนิวเคลียส ไปยังอาร์คูเอตนิวเคลียสที่มีความเกี่ยวข้องต่อการกินอาหาร. (Effects of low degree of high ambient temperature on neural pathways from median preoptic nucleus to arcuate nucleus in related to food intake.) อ.ที่ปรึกษาหลัก : รศ. น.สพ.ดร.สัมพันธ์ ธรรมเจริญ, อ.ที่ปรึกษาร่วม : ผศ. ดร.อารี วนสุนทรวงศ์

อุณหภูมิแวดล้อมสูงเป็นอีกหนึ่งปัจจัยสำคัญที่มีผลลดการกินอาหาร เป็นที่ยอมรับกันดีว่าอุณหภูมิแวดล้อมสูงระดับสูงที่ทำให้เกิดความเครียดลดการกินอาหารผ่านสารสื่อประสาท คอร์ติโคโทรปินรีลิสซิ่งแฟคเตอร์ (corticotropin-releasing factor) โดยแกนสมองไฮโปทาลามัส ต่อมพิทูอิทารี และต่อมอะดรีนัล (hypothalamic-pituitary-adrenal axis) อย่างไรก็ตามการเผชิญอุณหภูมิแวดล้อมสูงระดับต่ำช่วงระยะสั้น แสดงให้เห็นว่าการกินอาหารที่ลดลงเกิดขึ้นก่อนการตอบสนองทางด้านสรีรวิทยาและความเครียดจากความร้อน การกินอาหารที่ลดลงภายใต้อุณหภูมิดังกล่าวมีความสัมพันธ์กับวิถีประสาท มีเดียน พรีออปติก นิวเคลียส (median preoptic nucleus, MnPO) และ อาร์คูเอท นิวเคลียส (arcuate nucleus, Arc) ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาผลของอุณหภูมิแวดล้อมสูงระดับต่ำต่อการทำงานของสมองบริเวณ Arc ที่เกี่ยวข้องกับการกินอาหาร และความสัมพันธ์ของวิถีประสาทที่สมองบริเวณ MnPO และ Arc โดยมีสมมุติฐานแรกคือ การการกินอาหารที่ลดลงภายใต้การเผชิญของอุณหภูมิแวดล้อมสูงระดับต่ำมีความเกี่ยวข้องกับเซลล์ประสาทโพรออฟพิโอเมลาโนคอร์ทิน (proopiomelanocortin, POMC) ที่สมองบริเวณ Arc สมมุติฐานที่สองคือสัมพันธ์กับวิถีประสาทระหว่างสมองบริเวณ MnPO และ Arc (MnPO-Arc) เพื่อทดสอบสมมุติฐานดังกล่าว การศึกษาในครั้งนี้ได้ปรับเงื่อนไขของอุณหภูมิแวดล้อมสูงระดับต่ำ แสดงให้เห็นถึงการเผชิญอุณหภูมิแวดล้อมสูงระดับต่ำระยะสั้น (90 นาที) จากอุณหภูมิแวดล้อมควบคุมที่ 23 องศาเซลเซียส (°C) อุณหภูมิแวดล้อมสูงที่ 30°C ช่วงระดับอุณหภูมิแวดล้อมควบคุมและอุณหภูมิแวดล้อมสูงต่างกัน 7°C (ΔT = 7°C) ผลการศึกษาพบว่าหนูแรทเผชิญอุณหภูมิแวดล้อมสูงระดับต่ำที่ช่วงอุณหภูมิดังกล่าวลดการกินอาหาร โดยไม่พบการเปลี่ยนแปลงของอุณหภูมิกาย และฮีมาโตคริต ผลการเผชิญอุณหภูมิแวดล้อมสูงระดับต่ำต่อสมองบริเวณ Arc สามารถเพิ่มจำนวนโปรตีนซีฟอส เซลล์ประสาท POMC และเซลล์ประสาท POMC ที่ถูกกระตุ้นที่สมองบริเวณ Arc ภายใต้เงื่อนไขการกินอาหาร นอกจากนี้เมื่อฉีดสารต้านการจับกันของตัวรับเซลล์ประสาท POMC (SHU9119) เข้าไปที่โพรงสมองด้านข้างของหนูแรท พบว่าสามารถผันกลับการกินอาหารที่ลดลงจากผลการเผชิญอุณหภูมิแวดล้อมสูงระดับต่ำได้ การศึกษาลำดับสุดท้ายเพื่อพิสูจน์ว่าวิถีประสาท MnPO-Arc มีความสัมพันธ์กับอุณหภูมิแวดล้อมสูงระดับต่ำ สารติดตามวิถีประสาทย้อนกลับฟลูออโรโกลด์ (Fluorogold, FG) ถูกฉีดเข้าไปที่สมองบริเวณ Arc หลังจากเจ็ดวันฉีดสาร FG เข้าไปที่สมองบริเวณ Arc พบเซลล์ประสาทติดสาร FG ที่สมองบริเวณ MnPO การพบเซลล์ประสาทติดสาร FG ที่สมองบริเวณ MnPO ชี้ให้เห็นว่าสมองบริเวณ Arc สามารถรับสัญญาณขาเข้าจากสมองบริเวณ MnPO ได้ การศึกษาในครั้งนี้จึงสรุปว่าเซลล์ประสาท POMC ที่บริเวณ Arc คือนิวเคลียสทีสำคัญยิ่งของสมองบริเวณไฮโปทาลามัสต่อการกินอาหารภายใต้ผลของการเผชิญอุณหภูมิแวดล้อมสูงระดับต่ำ ผลการเผชิญอุณหภูมิแวดล้อมสูงระดับต่ำต่อการกินอาหารที่ลดลงนั่นส่วนหนึ่งเกี่ยวข้องกับการทำงานของเซลล์ประสาท POMC ที่บริเวณ Arc และมีความสัมพันธ์กับวิถีประสาท MnPO-Arc

สาขาวิชา	สรีรวิทยา (สหสาขาวิชา)	ลายมือชื่อนิสิต .....
ปีการศึกษา	2564	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
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Pornsiri Suwannapaporn : Effects of low degree of high ambient temperature on neural pathways from median preoptic nucleus to arcuate nucleus in related to food intake.. Advisor: Assoc. Prof. Dr. SUMPUN THAMMACHAROEN Co-advisor: Asst. Prof. Dr. AREE WANASUNTRONWONG

High ambient temperature (HTa) is one important environmental factor that decreases food intake (FI). It is well accepted that high degree HTa induced heat stress decreased FI by the activation of corticotropin-releasing factor (CRF) via hypothalamic pituitary adrenal axis. However, short-term low-degree HTa exposure has been demonstrated to decrease FI earlier than activated physiological and stress responses. This effect was related to the neuronal connection between the median preoptic nucleus (MnPO) and arcuate nucleus (Arc). Therefore, the present study aimed to investigate the effect of short-term low-degree HTa exposure on the activity of hypothalamic Arc in regarding to FI, and the involvement of neuronal connection between the MnPO and Arc. It was firstly hypothesized that proopiomelanocortin (POMC) neurons in the Arc can mediate the reduction of FI under the effect of short-term low-degree HTa exposure. Second, the neuronal pathway from MnPO to Arc may related with this effect. In order to test the hypothesis, the present study refined the condition of short-term low-degree HTa. This study demonstrated that the short-term low-degree HTa exposure (90 min) based on the control ambient temperature at 23°C (CTa = 23°C) and the HTa at 30°C. The bout of CTa and HTa conditions is 7°C temperature difference (ΔT = 7 °C). The results showed that rats exposed to this condition decreased FI without changing in rectal temperature and hematocrit. The effect of short-term low-degree HTa exposure on the Arc could enhanced the number of c-Fos expression, POMC positive neurons and POMC neurons containing c-Fos positive nuclei in the Arc under feeding condition. Moreover, the injection with POMC receptor antagonist (SHU9119) into the lateral ventricle in the rat brain could reverse the effect of short-term low-degree HTa exposure on FI. Finally, to prove the neuronal connection of MnPO-Arc in related with the effect of short-term low-degree HTa exposure. The fluorogold (FG) retrograde tracer was injected to the Arc. Seven days after FG injection found FG-retrograde labeled neurons in the MnPO. The present of FG-labeled neurons in the MnPO imply that the Arc can receive the afferent signal from the MnPO. In conclusion, this study concludes that the Arc POMC neurons are a crucial hypothalamic nucleus on FI under the effect of low-degree HTa exposure. The effect of low degree HTa exposure in reduction on FI is mediated in part by activation of Arc POMC neurons and may relate with the MnPO-Arc pathway.

Field of Study:	Physiology	Student's Signature .....
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		Co-advisor's Signature .....

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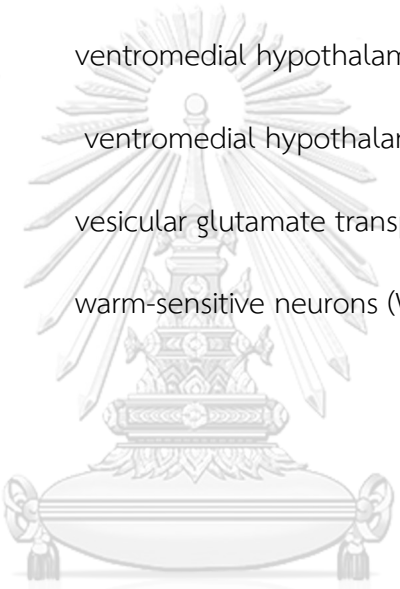
LISTS OF ABBREVIATION

°C	degree Celsius
µl	microliter
µm	micrometer
α-MSH	alpha-melanocyte stimulating hormone
ΔT	Ta difference
Aco	anterior cortical amygdaloid nucleus
AH	anterior hypohalamus
Arc	arcuate nucleus
BM	basolateral of amygdala
BMI	body mass index
BW	body weight
CRF	corticotropin-releasing factor
CTa	control ambient tempeature
DMH	dorsomedial hypothalamic nucleus
DRG	dorsal root ganglia
FG	retrograde neuronal tracer fluorogold
FI	food intake
g	gram
GABA	gamma-aminobutyric acid (GABA)

H_2O_2	hydrogen peroxide
h	hour
Hct	hematocrit
HTa	high ambient temperature
HPA axis	Hypothalamo-pituitary adrenal axis
ICV	intracerebroventricular
IHC	immunohistochemistry
ip	intraperitoneal injection
ir	immunoreactivity
kg	kilogram
LH	lateral hypothalamus
LPB	lateral parabrachial nucleus
MC3/4R	melanocortin 3/4 receptor
Mg	milligram
MeA	medial nucleus f amygdala
Min	minute
mm	millimeter
MnPO	median preoptic nucleus
MPO	medial preoptic nucleus
MPB	phosphate buffer
ng	nanogram

nl	nonoliter
nM	nanomole
NDS	normal donkey serum
NGS	normal goat serum
NPY	neuropeptide
NTS	nucleus tractus of the solitary tract
PB Triton-X	0.1 MPB + 0.3% Triton-X
PFA-0.1 MPB	4% paraformaldehyde in 0.1 MPB
POA	preoptic area
POMC	proopiomelanocortin
PVN	paraventricular nucleus
SCN	suprachiasmatic nucleus
SHU9119	melanocortin 3/4 receptor antagonist
SON	supraoptic nucleus
Ta	ambient temperature
Tb	body temperature
Tc	body core temperature
Tr	rectal temperature
TRP	transient receptor potential
TRPV1	transient receptor potential cation channel subfamily V member 1

TRPV2	transient receptor potential cation channel subfamily V member 2
TRPV3	transient receptor potential cation channel subfamily V member 3
TRPV4	transient receptor potential cation channel subfamily V member 4
VMH	ventromedial hypothalamic nucleus
VMHL	ventromedial hypothalamic nucleus, ventrolateral part
vGlut2	vesicular glutamate transporter 2
W-S	warm-sensitive neurons (W-S)



CHAPTER I
INTRODUCTION

Eating behavior is a fundamental behavior that requires for survival in animal and human. An excessive food intake (FI) and reduction in physical activity are the two most common reasons causing obesity. However, internal factors are not only contributed to the increasing prevalence of obesity (1). Ambient temperature (Ta) is one of an external factor that influences on obesity. The high prevalence of obesity was observed in countries with average Ta around 18°C (2-4). Another study also demonstrated that body mass index (BMI) of people residing in Ta above 23°C were lower than those living in Ta below 19°C (5). In addition, the prevalence of obesity was related with time living under thermal comfort zone (6). These evidences imply a close relation between Ta and obesity. Therefore, understanding the regulation of eating behavior under Ta may be a key element to pathophysiology of obesity.

The mechanisms controlling FI are not only sensitive to endogenous homeostatic signals, but also to emotion, food properties and environmental conditions (7-10). High ambient temperature (HTa) is one of influential environmental factor on FI. Several previous studies showed that high-degree HTa induced heat stress significantly decreased FI (11-14). The activation of corticotropin-releasing factor (CRF) via hypothalamic pituitary adrenal axis (HPA axis) has been shown to involve with the reduction of FI under the effect of high-degree HTa (9, 12, 15, 16). The effect of short

term low-degree HTa exposure is other HTa condition that can decrease FI. The study about short-term low-degree HTa exposure with temperature difference at 10°C based on control temperature at 20°C (ΔT = 10°C, HTa = 30°C) decreased FI without changing in CRF concentration, ACTH level, body temperature (Tb) and hematocrit (Hct) (13). These studies indicated that short-term low-degree HTa exposure can decrease FI earlier than stress axis activation.

The arcuate nucleus (Arc) in the mediobasal hypothalamus is a key brain region in controlling FI. The feeding-control action of Arc is regulated by two set of neurons: orexicgenic neuropeptide Y (NPY) neurons and anorexigenic pro-opiomelanocortin (POMC) neurons (7, 17). The activation of the Arc POMC neurons elevate the releasing of anorexicgenic neuropeptide alpha-melanocyte stimulating hormone (α-MSH). The α-MSH decreases FI by binding with melanocortin receptors, especially melanocortin 3/4 receptor (MC3/4R) (7, 18). The MC3/4R are broadly distributed at the second order neurons in hypothalamic nucleus including dorsomedial hypothalamic nucleus (DMH), ventromedial hypothalamic nucleus (VMH), lateral hypothalamus (LH), paraventricular nucleus (PVN) and nucleus tractus of the solitary tract (NTS) (17, 19, 20). Interestingly, short-term low-degree HTa exposure could enhance c-Fos expression in the Arc, DMH, VMH and NTS under feeding condition (13). These nuclei has been reported to be the second order projections of Arc POMC neurons in controlling FI (7, 21). Thus, the Arc POMC neuron may be important neuron in mediated FI under the effect of low-degree HTa exposure.

The hypothalamic preoptic area (POA) is considered to be an important thermoregulatory brain center. The subregion area called median preoptic nucleus (MnPO) is the main integrated area receiving sensory afferent neuronal pathways from the skin thermoreceptor (22, 23). The neuronal connection involved with the effect of low-degree HTa are apparently mediated by the MnPO and Arc (13). In rats, low-degree HTa exposure significantly increased c-Fos expression at the MnPO and Arc without in medial preoptic nucleus (MPO) and PVN (13) as in heat stress condition (16, 24). Interestingly, the exposure of low-degree HTa on eating induced c-Fos were enhanced in the MnPO and Arc (13). The MnPO and Arc may be a critical brain area involved with low-degree HTa exposure. The neuronal connection between the MnPO and Arc may be a significant pathway involved with the effect of short-term low-degree HTa exposure.

In this study was designed to demonstrate the effect of short-term low-degree HTa exposure at ΔTa = 7°C based on control ambient temperature (CTa) at 23 (HTa = 30°C) could decrease FI without changing in Tb and Hct. In addition, this study aimed to investigate the effect of short-term low-degree HTa exposure on Arc POMC neuron in mediated FI. The activation of Arc POMC neurons was observed by using immunofluorescence technique and injection with the antagonist of MC3/4 receptor (SHU9119) in the lateral ventricle of the rat brain. Finally, the present study was designed to map a neuronal connection of MnPO-Arc pathway in related with short-term low-degree HTa exposure by retrograde neuronal tracer injection.

Research questions

The research questions of the present study were:

1. Does MnPO pathway involve with eating-related Arc activity under the low degree of HTa?
2. Does Arc receive afferent input from MnPO pathway in related to FI under the low-degree of HTa?
3. Does POMC neurons play an important role in FI suppression under the low-degree HTa?

Hypothesis

The hypotheses of the present study were:

1. The Arc receives input from the MnPO in related to FI suppression under the low-degree HTa.
2. The POMC neurons play an important role in FI suppression under the low-degree HTa.

Objective

The objectives of the present study composed:

1. To investigate MnPO pathway on eating-related Arc activity under the low-degree HTa.
2. To investigate the afferent neural input to the Arc in related to FI under the low-degree HTa.
3. To investigate the involvement of POMC neuron in FI suppression under the low-degree HTa.

The conceptual framework of the present study was presented as followed:

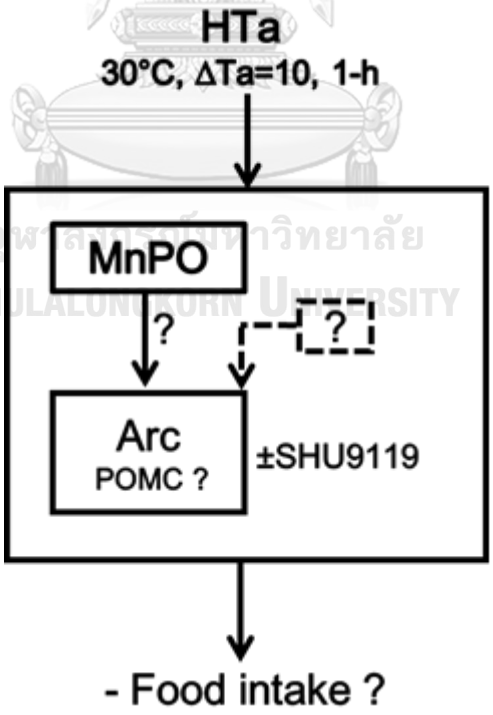


Figure 1 Conceptual framework

CHAPTER II
LITERATURE REVIEWS

2.1 Central control of body temperature

Both animal and human need maintain Tb during environmental temperature challenges, inflammatory responses, and declining energy homeostasis to protect cellular function and organism. Defending Tb against Ta is one of the most fundamental homeostasis to produce an optimal body core temperature (Tc) for cellular function (23). Maintenance of Tb during the Ta challenges is a critical brain function accomplished by complex neural circuits. The central neural circuits organize the behavioral and autonomic responses sensed by thermoreceptors at the skin. The POA is located in rostral pole of the hypothalamus provided as the center of thermoregulation. The POA receives thermosensory signals from cutaneous thermoreceptors, and provides efferent signals to regulate autonomic effector responses such as cutaneous vasodilation, sweating in human, panting in dog or salivation in rodent during HTa exposure (25, 26).

2.1.1 Thermosensory signal from skin thermoreceptor

The thermoregulation consists of sensory afferent part, integration part, and efferent part. To maintain thermal homeostasis from Ta challenges, the POA receives thermosensory afferent signals from skin thermoreceptors (27). One group of molecular entity of skin thermoreceptors called Transient Receptor Potential (TRP) channel plays

as a major role in mediated Ta stimuli (28). Among the TRP channel, TRVP4 is activated by innocuous warm temperature with Ta threshold of 25–34°C. TRPV3 is activated with Ta threshold between 33°C to 39°C. TRPV3 and TRPV4 are primarily expressed in keratinocytes of skin epidermis. TRPV1 and TRPV2 are activated by a noxious hot temperature. TRPV1 can activate at temperature above 42°C. TRPV2 is activated with Ta above 52°C (29, 30).

The environmental warming detected by skin thermoreceptors is transmitted via primary somatosensory signal to dorsal root ganglia (DRG). The DRG relays and sends information to thermal sensory neurons in dorsal horn (DH), in which laminar I neurons receive most thermosensory signals from skin. The ascending pathway of DH is then sent to lateral parabrachial nucleus (LPB) of the pons (22, 27). The MnPO region of the POA is a major site that receives a thermosenosory afferent signal from the LPB (22, 23, 27, 30).

2.1.2 The MnPO signal on ambient temperature

The MnPO can receives the input signals from fore-, mid- and hindbrain. The cutaneous thermoreceptors provide the afferent fibers to central thermoregulatory center at the POA (27). The MnPO is a primary site that receives the thermal sensory information from cutaneous thermosensory signals via the LPB. The dorsal subregion of LPB (LPBd) is the key area receiving the signal from cutaneous warming (27). The activation of LPBd-MnPO pathway drives to elicit heat-defensive responses. These

responses are mostly elicited through autonomic responses including skin vasodilation, sweating in human, panting in dog and salivation in rat (25, 31).

The efferent thermoregulatory signals from the MnPO activate warm-sensitive neurons (W-S) in the MPO that can enhance the descending inhibition signal to inhibit the motor outputs. The inhibition of motor output causes to cutaneous vasodilation and inhibited thermogenesis (23, 27). The activation of W-S neurons induces heat loss (facilitate salivary secretion in rats), suppresses heat production (shivering thermogenesis) and dilates the blood vessels in tail rat (non-evaporative heat loss). Efferent pathways from the W-S neurons also send descending signals to periqueductral gray to participate the tail vasodilation in rat (31, 32). The sympathetic premotor neurons in medullary raphe also receive inhibitory neurons in controlling skin blood flow (27). The MnPO can provide the efferent pathway to the supraoptic nucleus and PVN to regulate vasopressin release. The neural outputs from the MnPO to cortical sites could drive thirst and water intake (33, 34). The neuronal inputs from subfornical organ and organum vasculosum laminae terminalis can regulate the hypertonicity and circulating angiotensin II (33, 35).

2.1.3 Behavioral responses under high ambient temperature exposure

The homoeothermic animals have autonomic and behavioral responses in maintaining core body temperature (Tc). The behavioral responses to defend Tb during thermal environmental challenges are mostly chosen first and effective. During thermal environmental challenges, the animal seeks preferable environment or change posture to maintain Tb. The behavioral processes can activated before increasing of Tc. The skin thermosensory signal implies to be important process (31). The reduction of FI is one of the important behavioral responses during HTa exposure (13, 36).

2.2 Eating behavior

Eating behavior is an important for survival that controlled by the processes of energy and nutrient. Eating behavior is an important behavior that influences on obesity. Obesity is a result of positive energy balance in which occurred by excessive energy intake than energy expenditure (37). Food intake is provided as the behavioral contributor to maintain energy balance. An excessive FI is primary cause to develop obesity. The factors influenced on FI are not only sensitive to internal factors (endogenous homeostatic- or biological signals), but also to external factors such as emotion (mood, stress, and anxiety), properties of food (taste, smell and texture), environments (cultural, social and Ta) and sex differences (8-10, 13).

2.2.1 Food intake regulation

Food intake regulation has a well-ordered sequences related to getting and consuming food. Food intake starts by hungry that drives the human or animal to forage for food. Food intake occurs in periodic bouts or meals. The meal onset are begun when an energy (blood glucose or lipid availability) falls to a threshold value (7), the time of day, the size of previous meal, and memory of eating in the past (38-40). The meal termination can be influenced by combination of several signals such as food palatability, gastric distention, calories consumed and gastrointestinal peptides (39, 41). The total daily FI is the product of the average size and number of meal consumed (38).

The hypothalamic nuclei are demonstrated to play a key role in regulating FI. Classical studies suggested that LH were described as the “appetite-increasing center (or orexigenic)”, whereas the VMH is defined as the “appetite-suppressing center (or anorexigenic)” (42, 43). Later studies by the development of mouse genetics, electrophysiology, optogenetics, and chemogenetics has led to gain more detailed information on the identity of specific neurons that regulate feeding behavior. The hypothalamic arcuate nucleus is one of the best-studied brain region to identify the neuronal control of feeding behavior. Because, the Arc contains two important neuronal populations to control the feeding behavior: anorexigenic POMC neurons and orexigenic NPY neurons. In addition, the Arc POMC- and NPY neurons can receive information from peripheral organ and multiple part of central nervous system to

regulate eating behavior. (7, 44). The Arc is a good hypothalamic area in integrated peripheral and central inputs to produce a central signal for feeding behavior.

2.3 Role of arcuate nucleus in controlling food intake

The Arc is implied as “first-order neurons” that provides efferent signal to second-order neurons at other hypothalamic nuclei and NTS to control FI (7). The strongest neural inputs to the Arc are from periventricular nuclei, PVN and medial zone nuclei of the hypothalamus. In addition, the Arc can receive direct extra-hypothalamic inputs such as from the medial amygdala, bed nucleus of the stria terminalis, somatosensory cortex and brainstem including the LPB and NTS (21, 45).

2.3.1 Anorexigenic- and orexigenic neurons of arcuate nucleus in the regulation of food intake

The Arc has two important set of neurons that oppositely regulate FI: anorexigenic- and orexigenic neurons. The anorexigenic POMC neurons inhibit FI and promote weight loss. The orexigenic NPY neurons stimulate FI, food seeking and food hoarding. The Arc POMC and NPY neurons activation are modulated by multiple neurotransmitters and/or hormones to regulate feeding behavior.

Anorexigenic pro-opiomelanocortin neurons in the arcuate nucleus

The Arc POMC neurons are essential first-order neurons in maintaining eating behavior and energy metabolism. Mice with arcuate Pomc knockout were hyperphagia, that developed early-onset extreme obesity even if consuming a standard low-fat

chow (46). The POMC neurons of rodents are most located in the anterior and medial part of the Arc. The Arc POMC neuron is regulated by several hormones, neuropeptides, neurotransmitters and nutrients. The Arc POMC neurons encode a precursor polypeptide which posttranslational processing generates to α-melanocyte stimulating hormone (α-MSH). The POMC precursor protein is synthesized in the endoplasmic reticulum. Then it moves to the Golgi complex and sorted to deliver at secretory granules (47, 48).

The α-MSH induces anorexia upon stimulation of G_s-coupled MC3R and MC4R to activate adenylyl cyclase, resulting in elevation of intracellular cAMP (17). Within the brain, the MC4R is more widely expressed in the hypothalamic nuclei (Arc, PVN, DMH, LH, VMH), amygdala, thalamus, cortex, NTS of the brainstem and peripheral nervous system. The MC3R has more limited expression that found mainly in the brain, including the Arc, VMH, DMH, PVN and also in thalamus (17). The studies about the effect of α-MSH on FI shown that intracerebroventricular (ICV) injection of melanotan II (MT-II), a cyclic analog of α-MSH or MC3/4R agonist, could suppress FI and increase energy expenditure in rodents (49, 50). Conversely, this inhibitory effect can be blocked by co-injection with MC3/4R antagonist, SHU9119 (51). The excitement of Arc POMC neurons were suppressed during fasting, and stimulated by increasing of energy store (48). The food presentation of fasted mice or ad-libitum mice showed an immediate activation of POMC neurons (52).

The Arc POMC neurons receives major inputs from the hypothalamic nuclei including anterior hypothalamus (AH), MPO, LH, PVN, DMH, VMH and NTS. In addition, the Arc POMC neurons can receive afferent signals from the amygdala, thalamus, hippocampus, midbrain, pons, and medulla. (53). However, these extra-hypothalamic inputs does not imply that the Arc POMC neurons have a broad array of anatomical connections or multiple physiological functions.

Orexigenic neuropeptide Y neurons in the arcuate nucleus

NPY neurons also called hunger neurons that stimulate FI with a preferential effect on carbohydrate intake. The NPY neurons play a critical role in decrease latency to eat, increase motivation to eat and delay satiety by augmenting meal size (54). ICV injection of NPY could stimulate FI in a dose-dependent manner. The action sites of NPY are wildly distributed in the hypothalamus, which most highly express in the Arc. The Arc NPY neurons synthesize orexigenic neuropeptide Y within the endoplasmic reticulum and mostly stored in the dense core of large vesicles through axonal transport (7, 54). The releasing of orexigenic NPY can enhance immediately prior to the onset of feeding, and decreased gradually if FI continues. Increasing expressions of Arc NPY are directly correlated with food deprivation, and reversed by food consumption (high-carbohydrate diet) (54).

Hypothalamic areas including the PVN, DMH, VMH and LH are major projection of Arc NPY neurons (7, 55). Biological actions of orexigenic NPY are mediated by six receptors called Y1–Y8. However, the effects of Arc NPY neurons are mediated through

Y1 and Y5 receptors. The Y1 receptors are widely distributed in the Arc, PVN, VMH and DMH. The Y5 receptors are located mostly in the Arc, LH, and PVN. Both Y1- and Y5 receptors are mediated in orexigenic NPY. However, Y5 knockout mice does not play a key role in orexigenic effect. The Y2 receptors is also found on NPY-expressing neurons with a function of inhibitory autoreceptor (17, 54, 55). The downstream effect of Arc NPY neurons is dependent on the different levels of Y receptors that present at the time of NPY release.

The Arc NPY system is an important neural circuit where balance between anabolic activity NPY and catabolic activity α-MSH. Nerve terminals from Arc NPY neurons densely innervate neighboring Arc POMC neurons. The Arc NPY neurons can inhibit POMC neurons either by directly activate with Y receptors (Y1 and Y2) or via tonic release of GABA. The activity of Arc NPY neurons leads to membrane hyperpolarization and spike frequency inhibition of Arc POMC neurons (56, 57). Increasing levels of satiation signals such as peptide YY and CCK reduce the activity of Arc NPY neurons. Fasting increases tendency to eat and upregulation of Arc NPY neurons. The activity of Arc NPY neurons is reversed by re-feeding.

2.3.2 The feasibility of Arc neurons in mediated food intake under the low-degree HTa

Prevailing change in feeding status or nutrition status can modify the expression of orexigenic- and anorexicgenic neurons in the hypothalamic nuclei. The Arc is an

important part of hypothalamus in controlling daily energy intake. The eating behavior controlled by the Arc is regulated mainly by two types of neurons: anorexigenic POMC and orexigenic NPY. The Arc POMC is a precursor molecule of anorexignic neuropeptide α-MSH. The stimulation of POMC neurons triggers the release of α-MSH, which decreases FI by binding with MC3/4R on downstream neurons (18). The α-MSH is reported in mediating on thermoregulation. At HTa (28°C), ICV injection of α-MSH showed no rise of Tb, which was accompanied by a pronounced vasodilation (rise in heat loss) in rats. In contrast, at cool Ta (15°C), ICV injection of α-MSH enhanced Tb, which was accompanied by a continuous tail-skin vasoconstriction in rats (58). Therefore, the Arc POMC neurons makes a good candidate to mediate appetite suppression in response to low degree of HTa. The Arc NPY neurons may be another candidate neuron involved with short-term low-degree HTa exposure on FI. The Arc NPY neurons are highly expressed during fasting and onset of feeding (54). In nocturnal animals, the Arc NPY neurons are the most pronounced effect at the beginning of dark phase (18). Thus, the effect of low degree of HTa may reduce the activity of Arc NPY neurons.

The study about short-term low-degree HTa could enhance c-Fos expression only in the Arc of hypothalamic nuclei. The short-term low-degree HTa exposure on feeding-induced c-Fos expression could enhance c-Fos positive nuclei in the Arc, DMH, VMH and NTS (13). The DMH, VMH and NTS are second order neurons, which receive

first order projection from the Arc (17). Thus, the Arc can suggest to be the important hypothalamic nuclei in controlling FI under the low-degree HTa exposure. The Arc POMC may be a good candidate in mediating FI under the low-degree HTa. Interestingly, no study has yet demonstrated a role of Arc POMC neurons in regulated FI under the low-degree HTa. The short-term low-degree HTa exposure on the activity of Arc POMC neurons remains unclear at the present time.

2.4 Food intake under high ambient temperature (HTa)

Food intake can generate heat by the process of digestion and absorption, called thermogenic effect of food (TEF). Under living in heat exposure, FI should be low to prevent hyperthermia. In cold exposure where heat production need to maintain the Tb, FI should be high to against hypothermia (30, 36). The short-term HTa exposure on FI may describe by two characteristics. The high-degree HTa exposure reduces FI by heat stress. The releasing of CRF via HPA axis serves as the important function in reduction on FI under high-degree HTa exposure (12, 15). Another one is low-degree HTa exposure. This HTa condition can reduce FI earlier than activated physiological change in Tb and Hct. In addition, the short-term low-degree HTa exposure can decrease FI without the activation of HPA axis (13). However, the mechanism induced reduction on FI under the short-term low-degree HTa exposure is still unclear at the present time.

CHAPTER III

MATERIALS AND METHODS

After examined the effect of short-term low-degree HTa exposure at **Δ**T = 7°C based on the CTa at 23°C on FI, Tb and Hct (experiment 1), the next study was designed to investigate the effect of short-term low-degree HTa exposure on the activity of Arc POMC neurons by using immunofluorescence technique (experiment 2). Then, the injection of MC3/4R antagonist (SHU911) was used to determine the reverse effect of short-term low-degree HTa exposure on FI (experiment 3). Finally, this study aimed to investigate the short-term low-degree HTa exposure on neuronal connection between the MnPO and Arc by using Fluorogold (FG) retrograde neuronal tracer (experiment 4). The experimental design was shown in Figure 2.

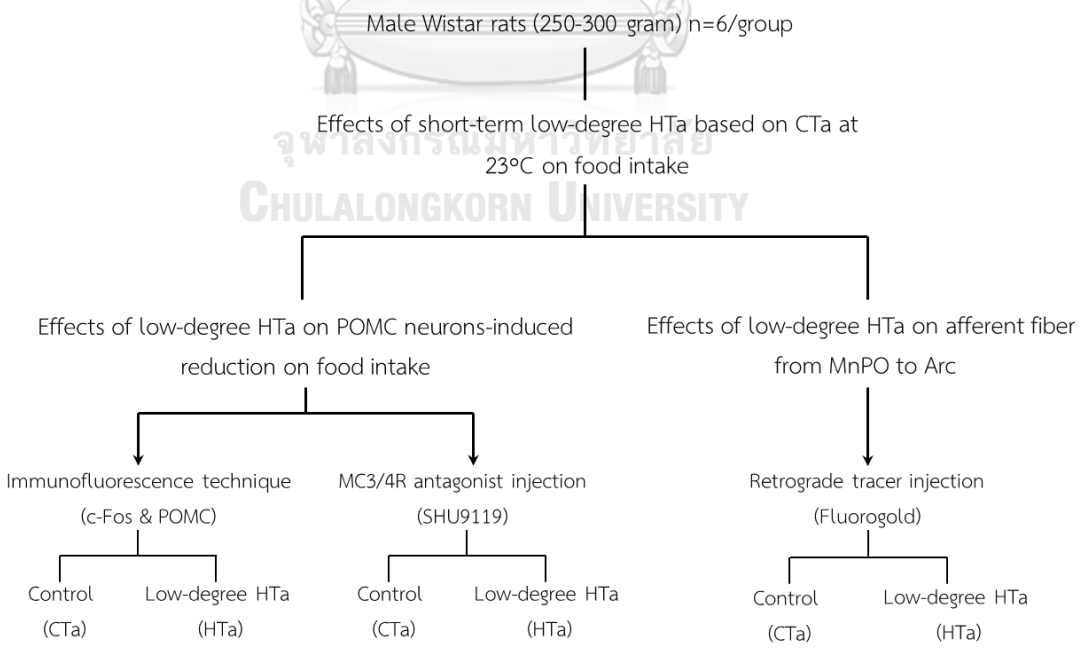


Figure 2. Summary of experiment design

3.1. Animals and housing condition

Adult male Wistar rats (250–300 g, Nomura Siam International, Bangkok, Thailand) were housed individual cage in temperature-controlled room (room#1, Ta=23±1 °C) with a 12:12 light-dark cycle (dark onset at 12:00). All rats were accessed ad libitum (#082, Perfect Companion Group Ltd., Samutprakarn, Thailand) and water. All rats were handled and habituated to the investigator at least 1 week before each experiment study. All animal procedures were approved by the Animal Used Committee, Faculty of Tropical Medicine, Mahidol University (FTM-ACUC 009/2019) and performed in accordance with the ethical principles and guidelines for the use of Laboratory Animals Science from the National Research Council of Thailand.

3.2. Training condition

At the beginning, the CTa group was moved out from room#1 to room#2, and then moved back to room#1 immediately. Another one is rats in HTa group, which were moved out from room#1 to room#2, and stayed in room#2 throughout the training period. The training procedures were conducted for 60 or 90 min exposure before dark onset. Then at the onset of dark phase, the CTa group was moved out and moved back again to room#1. The HTa group was moved back from room#2 to room#1. All rats were gave food to measure 1-h FI and 23-h FI (Figure 3). The environmental temperature of training procedure was set at 23°C in both room#1 and

room#2. The rats was trained with this program at least 7 days before each experimental start. The food cup was removed and prepared after 23-h FI recording.

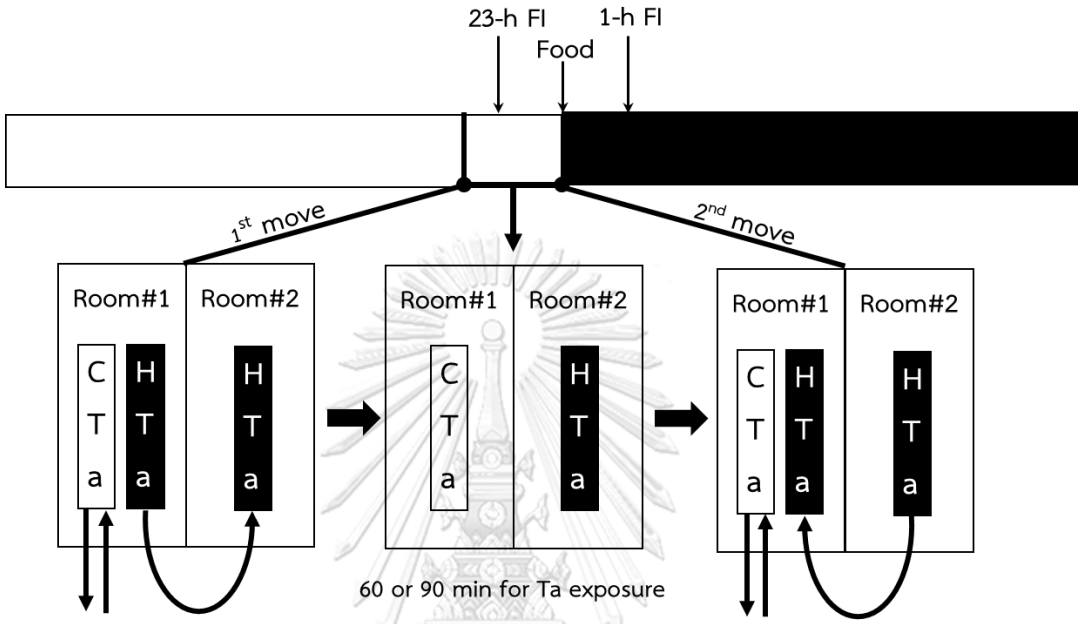


Figure 3. The training program.

Figure 3. The CTa group were moved out and back to room#1. The HTa group were moved out to room#2, and stayed throughout the training period. At the onset of dark phase, the CTa group was moved out and back to room#1. The HTa group was moved back to room #1. FI were measured at 1-h and 23-h.

3.3. Experimental protocols

3.3.1 Effects of short-term low-degree of high ambient temperature on food intake, body temperature and hematocrit.

According to the previous study demonstrated that the temperature difference at 10°C based on the CTa at 20°C (HTa = 30°C) decreased FI without changing in Tb and Hct (13). The present study aimed to refine the short-term low-degree HTa condition by selecting the basal Ta at 23°C. The HTa exposure was set with the same previous condition at 30°C. The refined HTa condition in this study was **Δ**T = 7°C based on the CTa at 23°C. To identify the short-term low-degree HTa exposure at **Δ**T = 7°C (CTa = 23°C and HTa = 30°C), the duration of HTa exposure was set to examine whether 60 min or 90 min exposure could decrease FI without changing in Tb and Hct.

The first experiment, the rats were examined for 60 min HTa exposure on FI. After 7 days of training, the room#1 was maintained at 23°C as CTa room. The room#2 was set at 30°C as HTa room. The CTa and HTa exposure were conducted before dark onset. At the onset of the dark phase after 60 min exposure, rats from both groups (n=6 in each group) were maintained in room #1. One-hour FI and daily FI (23-h FI) were measured throughout the experimental period. The second experiment aimed to investigate for 90 min HTa exposure on FI. All rats were maintained with training program at least 7 days until FI from CTa and HTa groups were similar. The rats from CTa group were assigned to HTa group and vice versa. The experimental procedure was conducted as same as 60 min HTa exposure. Another set of experiment was

designed to determine time exposure (90 min) with **Δ**T = 5°C based on CTa at 23°C on FI. The short-term low degree HTa was set at 28°C. The experimental procedures were similar to previous descriptions above. After finish the experiment, all rats were maintained at least 1 week until FI from both group was similar. The time of short-term low degree HTa exposure on FI was shown in Figure 4a.

Finally, the last experiment designed to examine the effect of short-term low-degree HTa exposure on Tr and Hct. The HTa condition was selected from the HTa exposure that had an effect on FI (**Δ**T = 7°C, 90 min HTa exposure). Immediately after 90 min exposure, rats were handled to measure Tr by using a digital thermometer (MC-246, Omron Dalian Co. LTD, Dalian, China), and collect blood from the tail vein to determine Hct. All rats were handled and habituated to the investigator at least 1 week before started experiment. The experimental procedure of short-term low degree HTa exposure on Tb and Hct was shown in Figure 4b.

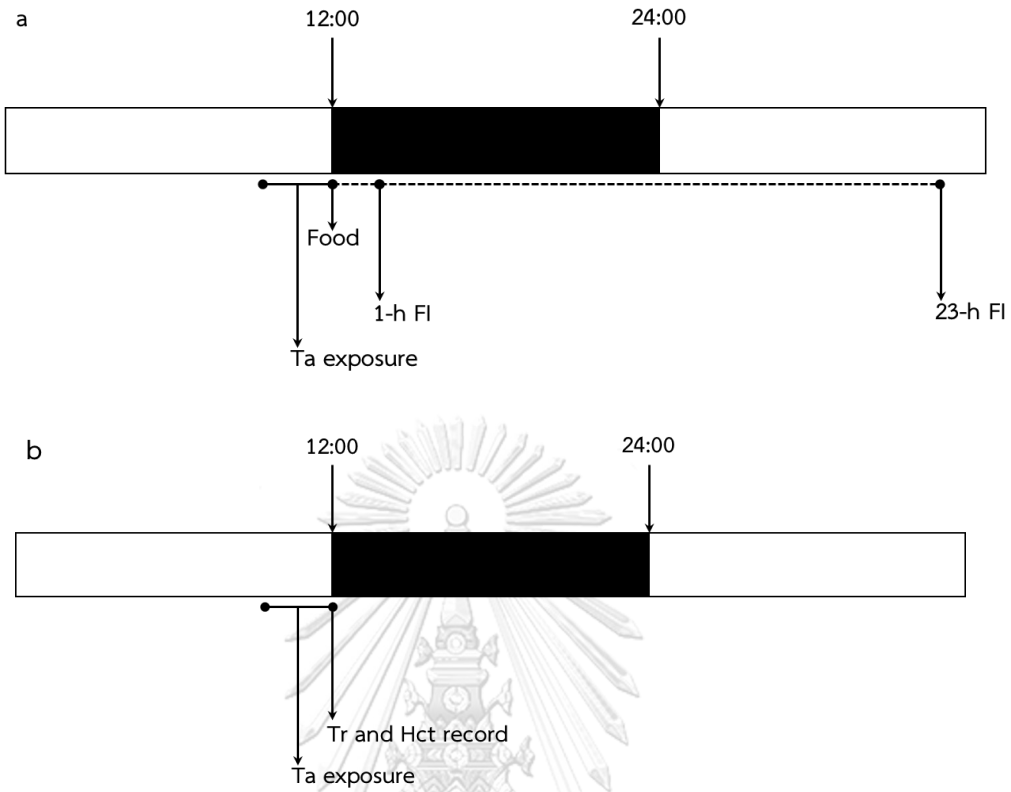


Figure 4. Time line for the first experiment.

Figure 4. One hour and daily of FI were measured after CTa and HTa exposure (a). The Tr and Hct were measured immediately after CTa and HTa exposure (b).

3.3.2. Effects of short-term low-degree of high ambient temperature on c-Fos and POMC activation in the arcuate nucleus

This experiment aimed to evaluate the effect of short term low- degree HTa exposure on c-Fos expression and Arc POMC neuron. Two experiments were performed using **Δ**T at 7°C (CTa = 23°C, HTa = 30°C, 90 min exposure). This study was designed to evaluate the short-term low-degree HTa exposure with two conitions: nonfeeding and feeding condition. The first experiment was examined under nonfeeding condition. Rats were trained with training program similar as the first experiment. Seven days after training program, the CTa group (n=6) were exposed at 23°C in room#1, and the HTa group (n=6) were exposed at 30°C in room#2. At the onset of dark phase, both groups were housed in room#1 at 23°C without food. Ninety minutes later, the rats were deeply anesthetized with pentobarbital sodium (60 mg/kg, i.p., Nembutal, Ceva Sante Animale, France). Then the rats were perfused with phosphate buffer (0.1 MPB, pH 7.4), and followed by 4% paraformaldehyde in 0.1 MPB (PFA-0.1 MPB, pH 7.4) (Figure 5a). The rat brains were removed and postfixed in PFA-0.1 MPB for 12-h at 4°C, and immersed in 20% sucrose for 48-h. The brain was cut with 40 μM-thick coronal sections (-0.20 to -3.60 mm, posterior to the bregma) by using a cryostat (HM 550, Thermo Scientific, USA). Sets of each fifth forebrain were stored at -20°C in cryoprotectant solution. The second condition, rats (n = 6 in each group) were designed to investigate the effect of short-term low-degree HTa exposure under feeding conditions. The experimental procedures were performed as described above

except allowing to access food for 1-h. Then, food were removed after 1-h FI. Ninety minutes later, the rats were deeply anesthetized and perfused (Figure 5b). The process of brain fixation and section was similar to those described above.

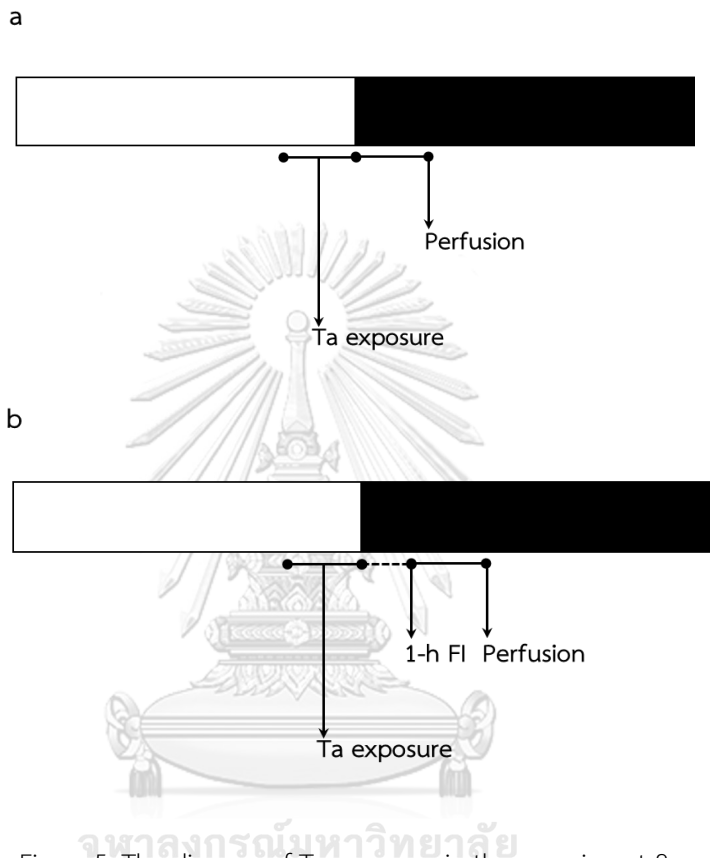


Figure 5. The diagram of Ta exposure in the experiment 2.

Figure 5. In nonfeeding condition (a), the rats from both groups were exposed temperature for 90 min before dark onset. Then, the rats were left without food for 90 min before perfusion. In feeding condition (b), the rats were allowed to access food for 1-h after 90 min exposure. Food was next removed. Then, the rats were perfused 90 minutes later.

Immunohistochemistry protocol

The effect of short-term low-degree HTa exposure on c-Fos expression, POMC positive cell and double-labeled neuron in the Arc was assessed by double-labeled fluorescent immunohistochemistry (IHC). Double-labeling of c-Fos and POMC cells was investigated the adjacent series of brain sections through the Arc. The brain sections were first reacted with c-Fos and then for POMC. After washing in 0.1 MPB, the brain sections were incubated in 1% normal goat serum (NGS; Vector Laboratories, Burlingame, CA, USA) in 0.1 MPB + 0.3% Triton-X (PB Triton-X) for 1 h. Then the brain sections were followed by primary antiserum containing a rabbit Fos antibody (1:10,000; rabbit polyclonal c-Fos antibody Ab-5; EMD Biosciences, Darmstadt, Germany) in PBTriton-X + NGS for 48-h at 4 °C. The brain sections were then incubated with Alexa Fluor 488 goat anti-rabbit (1:200, ab150077, Abcam, MA, USA) for 2-h. After washing with 0.1 MPB, the brain sections were blocked with 1% normal donkey serum (NDS; Abcam, MA, USA) in PBTriton-X for 1-h, and followed by incubation with rabbit anti-POMC antibody (1:2000, POMC (27–52), H-029–30, Phoenix Pharmaceutical, CA, USA) for 72-h and Alexa Fluor 555 donkey anti-rabbit (1:200, ab150074, Abcam, MA, USA) for 2-h. The brain sections were washed and mounted with Vectashiled Hardset mounting medium (H-1400, Vector Laboratories, CA, USA) on gelatinized microscope slides.

Table 1– Information on primary antibodies used in the second study.

Table 1 – Information on primary antibodies used in the second study.

Antibody	Species/ type	Catalog no.	Manufacturer
c-Fos	Rabbit/ Polyclonal	Ab-5	EMD Biosciences
Pro-opiomelanocortin (POMC)	Rabbit/ Polyclonal	H-029–30	Phoenix Pharmaceutical

Image analysis

The brain sections were evaluated with a fluorescence microscope (BX53, Olympus Corp., Tokyo, Japan). The number and distribution of c-Fos immunoreactivity (c-Fos-ir), POMC immunoreactivity (POMC-ir) neurons and double-labeled neurons was analyzed from three representative sections of both sides of the Arc. It was approximately 60% of the whole Arc. The four regions of the Arc were separated according to a previous description (59) using a template based on the rat brain atlas of Paxinos (60). Briefly, region I (Arc 1) was the most rostral extent of the Arc (approximately -2.12 mm posterior to the bregma). Regions II (Arc2) and III (Arc3), that represented the middle portion of the Arc, were approximately -2.56 and -3.14 mm bregma, respectively. Region IV (Arc 4) was the caudal portion of the Arc, which represented the section at approximately -3.6 mm posterior to bregma. The region of Arc 1 and Arc 2 had been demonstrated the rostral part of the Arc involved with FI regulation (61, 62). The region of Arc 3 and Arc 4 represents the caudal part in this

study, which has been shown the density of MC3-R mRNA lower than in the rostral part of the Arc (62).

Fluorescent images were acquired with a digital camera attached to BX53 microscope using a cellSens standard (Olympus Corp., Tokyo, Japan). The images of c-Fos-ir and POMC-ir were taken with same position in the slide. Each representative pictures was adjusted with contrast and brightness to optimize the image before analysis. The c-Fos-ir were counted with green fluorescence in the nucleus. The POMC-ir neurons were counted with the red fluorescence in the cytoplasm. Double-labeled neurons were counted with the yellow (or green) fluorescence in the nuclei and red fluorescent cytoplasm. The numbers of c-Fos-ir, POMC-ir and double-labeled neurons from each Arc region were averaged to represent the positive neurons.

3.3.3. Intracerebroventricular injection of SHU9119 on the effect of low-degree of high ambient temperature on food intake

To determine POMC signaling is part of the short-term low-degree HTa exposure in reduction on FI, an antagonist of MC3/4 receptor (SHU9119) was infused with subthreshold dose for feeding dose into the lateral ventricle in the rat brain. One hour FI and 23-h FI were recorded after 90 min exposure.

Intra-cerebroventricular (icv) cannulation

The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Nembutal, Ceva Sante Animale, France) and placed in a stereotaxic device (Model

900LS, David Kopf Instruments, CA, USA). A 21-gauge stainless steel guide cannula (PlasticsOne, Roanoke, USA) was implanted into the right lateral ventricle. Coordinates were relative to bregma in mm: -0.8 anterior-posterior and 1.5 lateral according to the atlas of Paxinos and Watson (60). The cannula tip was inserted down 3.5 mm below the dural surface. The cannula was anchored to the skull with stainless steel screws and dental cement. All rats were allowed to recover from surgery for 7 days. Enrofloxacin (2.5 mg/kg, Baytril, Bayer Korea Ltd., Ansan, Korea) and tramadol (12.5 mg/kg, Phamarland, Bangkok, Thailand) were given subcutaneous injection for antibacterial and analgesia, respectively. The placement of cannula was firstly assessed by ICV injection with angiotensin-II (10 ng in 1 µl, 002–12, Phoenix Pharmaceutical, CA, USA). The rats were considered to include when consumed 5 ml of water within 1-h after angiotensin-II injection.

Procedure

The rats were assigned randomly into 4 treatment groups (n = 6 per group: CTa/vehicle, CTa/SHU9119, HTa/vehicle, and HTa/SHU9119). The rats were infused either with vehicle (2 µl saline) or SHU9119 (0.1 nM in 2 µl, 043–24, Phoenix Pharmaceutical, CA, USA) via guide cannula connected to a needle injection (10 µl Hamilton syringe). Each ICV injection was given within 1 min in conscious unrestrained rat. The injection system was left in place for 1 min to allow diffusion of the injected solution. All ICV injections were conducted 1-h before Ta exposure (CTa = 23°C, HTa =

30°C, 90 min exposure). At the dark onset, the rats were allowed to access food. The food consumption was measured at 1- and 23-h (Figure 6). The rats were euthanized by pentobarbital sodium (65 mg/kg, i.p.) to verify the cannula placement using ICV injection with 2 µl Evans Blue at the end of experiment.

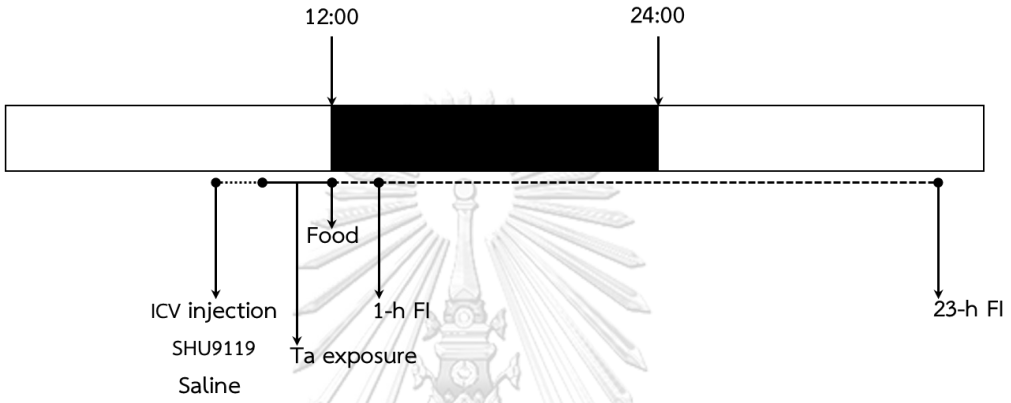


Figure 6. The diagram of SHU9119 injection.

Figure 6. ICV injection with either saline or SHU9119 was performed before 1-h Ta exposure (CTa and HTa exposure, 90 min exposure). After Ta exposure, the rats were allowed to access ad libitum food for 1-h. The food consumption was measured at 1-hFI and 23-hFI.

3.3.4. Effects of short-term low-degree HTa exposure on afferent projection to the Arc

The present study was designed to examine the neuronal projections from the MnPO to Arc after short-term low degree HTa exposure in rat. The Fluoro-Gold (FG) retrograde neuronal tracer was injected into the Arc to investigate the afferent neuronal projections to the Arc. The FG-retrograde labeled neurons was observed by immunohistochemistry with DAB.

Surgeries

The animals were anesthetized with pentobarbital sodium (50 mg/kg, i.p., Nembutal, Ceva Sante Animale, France) and placed in a stereotaxic device (Model 900LS, David Kopf Instruments, CA, USA). A 24-gauge stainless steel guide cannula (PlasticsOne, Roanoke, USA) was implanted at 2.56 mm posterior to bregma, 0.1-0.3 mm lateral to the right and 8 mm depth, according to the atlas of Paxinos and Watson (60). The guide cannula was positioned above 2 mm to the Arc with this process. The guide cannula was fixed with same procedure that described above . All rats were given subcutaneous injection for antibacterial and analgesia as described previously above.

Fluorogold retrograde tracer injection

The FG retrograde neuronal tracer was delivered to the Arc by pressure injection after rats completely recovered from the guide cannula implantation. The FG retrograde tracer (Fluorochrome, Denver, CO, USA) was administered to the Arc by pressure injection (UMP3 UltraMicroPump, World Precision Instruments (WPI), Florida, USA). A 33 gauge injection needle extended 2.0 mm beyond the tip of cannula was directly inserted through the guide cannula. The FG retrograde tracer (2% in 0.9% saline, 200 nl) was slowly delivered to the Arc via pressure injection. The injection system was left in place at least 30 min before removed the needle injection.

Experimental protocol

Seven days after FG injection, the rats were exposed at 30°C (90 min) before dark onset. After that, all rats were maintained in housing condition for 90 min. Then, the rats were anesthetized and killed by transcardiac perfusion with 0.1 MPB (pH 7.4), and followed by 4% PFA-0.1 MPB (pH 7.4) (Figure 7). The brain was removed and postfixed with 4% PFA-0.1 MPB (pH 7.4) for 12-h, and followed by 20% sucrose for 48-h. Coronal 30 µm thick brain sections (-0.20 to -3.60 mm, posterior to the bregma) were cut into a 1:5 series using a cryostat. The brain sections were stored in cryoprotectant solution and kept at -20 °C.

Immunohistochemistry protocol

For double-labeled of c-Fos and FG-labeled neurons, the brain sections were initially stained for c-Fos positive nuclei. The brain sections were rinsed three times in 0.1 MPB for 10 min, and incubated with 0.3% H_2O_2 for 30 min. The brain sections were then rinsed with 0.1 MPB and pre-incubated in 2% NDS in PB Triton-X for 1 hour. The brain sections were then incubated in rabbit polyclonal anti-c-Fos (1:5,000, ABE457, Merck Millipore, Darmstadt, Germany) in PB Triton-X + NDS at 4°C for 48-h. The brain sections were washed in 0.1 PB Triton-X and incubated in biotin-SP-conjugated donkey anti-rabbit IgG (H+L) secondary antibody (1:200, Jackson ImmunoResearch Inc.) for 90 min at room temperature. The brain sections were reacted with avidin-biotin complex (1:300 ABC, Vector laboratories, CA, USA) at room temperature for 1-h. After washing, the brain sections were stained with nickel sulfate intensified diaminobenzidine (DAB, SK-4100, Vector laboratories, CA, USA) for 10 min to produce a blue-black color of c-Fos positive nuclei. Then, the reaction was terminated by several wash with 0.1 MPB. Following c-Fos IHC, the brain sections were processed for FG-labeled neurons. The brain sections were incubated in 0.3% H_2O_2 for 20 min. The sections were incubated in 2% NDS in PB Triton-X for 1-h and followed by rabbit anti-FG primary antiserum (1:40,000 in PB Triton-X+NDS; Fluorochrome, Denver, USA) for 48 hours at 4°C. Then, sections were processed as described above for ABC Elite reagent. After washing, the FG-labelled neurons were visualized by DAB incubation (DAB, SK-4105, Vector laboratories, CA, USA) for 5 min to product a brown cytoplasm. The sections were

washed with 0.1 MPB, mounted on gelatin coated slide and coverslipped with glycerol gelatin mounting medium.

Table 2– Information on primary antibodies used in the fourth study.

Table 2 – Information on primary antibodies used in the fourth study.

Antibody	Species/ type	Catalog no.	Manufacturer
Fluorogold	Rabbit/ Polyclonal	Antibody to Fluoro-Gold	Fluorochrome
c-Fos	Rabbit/ Polyclonal	ABE457	Merck Millipore

Image analysis

The c-Fos positive nuclei were considered if cells contained blue-black color. The FG-positive neurons was analyzed when cytoplasm stained with brown color and had a visible nuclei. Double-labeled neurons were counted if a neurons contained both blue-black of c-Fos positive nuclei and brown cytoplasmic of FG-labeled neurons. The brain sections were analyzed using a microscope equipped with digital camera (DS-Ri2, Nikon, Japan). The number of c-Fos-positive nuclei, FG-labeled neurons and double-labeled neurons were counted within the hypothalamus by blinded examiner. The injection sites were evaluated by FG-tracer deposit that localized within the Arc by immunofluorescence microscope with UV excitation (DS-Ri2, Nikon, Japan).

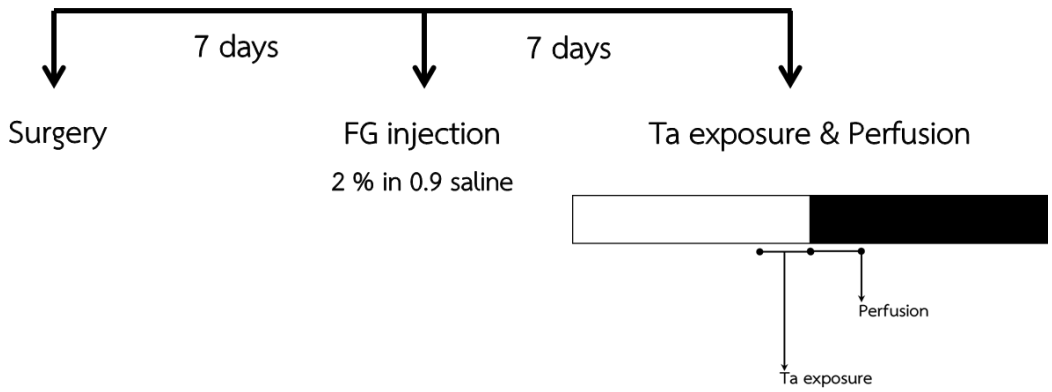


Figure 7. Time line for FG injection.

Figure 7. The FG retrograde neuronal tracer was injected in the Arc after 7 days of guide cannula implantation. Seven days after FG injection, the rats were exposed to HTa for 90 min. Then, the rats were return to housing room. Ninety minutes later, the rat were perfused to remove brain for IHC.

3.4 Statistical analysis

Statistical comparisons between the CTa an HTa groups was compared with Student's t-test. The number of c-Fos positive cells, POMC neurons and double-labeled neurons was analyzed using one-way ANOVA followed by Bonferroni post-hoc test. The effect of short-term low-degree HTa exposure after SHU9119 injection on FI was identified with two-way ANOVA followed by Bonferroni post-hoc test. All data were presented as mean of each group, means ± standard errors of the mean (SEM). The significant differences were considered at $P < 0.05$.

CHAPTER IV
RESULTS

4.1. Effects of short-term low-degree HTa exposure based on ambient temperature at 23°C on food intake

Effects of short-term low degree HTa exposure were investigated in a series of temperature differences with exposure time for 60 and 90 min, respectively. In the first experimental series, 60 min exposure of short-term low-degree HTa with **Δ**Ta = 7°C based on CTa at 23°C was no effect on 1-h FI and 23-h FI compared with CTa group (Fig. 8a, t_{10} = 0.31 and 0.54, P>0.05). In contrast to 90 min exposure, short-term low-degree HTa at **Δ**Ta = 7°C based on CTa at 23°C significantly decreased 1-h FI, but not in 23-h FI (Fig. 8c, t_{10} = 4.22, P<0.05). This low-degree HTa condition was not change on Tr and Hct (Fig. 8d and 8e, t_{10} = 1.66 and 0.08, P>0.05). Another HTa condition with 90 min exposure of **Δ**Ta = 5°C (CTa = 23°C, HTa = 28°C), the effect of short-term low-degree HTa was not significantly different compared with the CTa group (Fig.8b, t_{10} = 1.39, P>0.05).

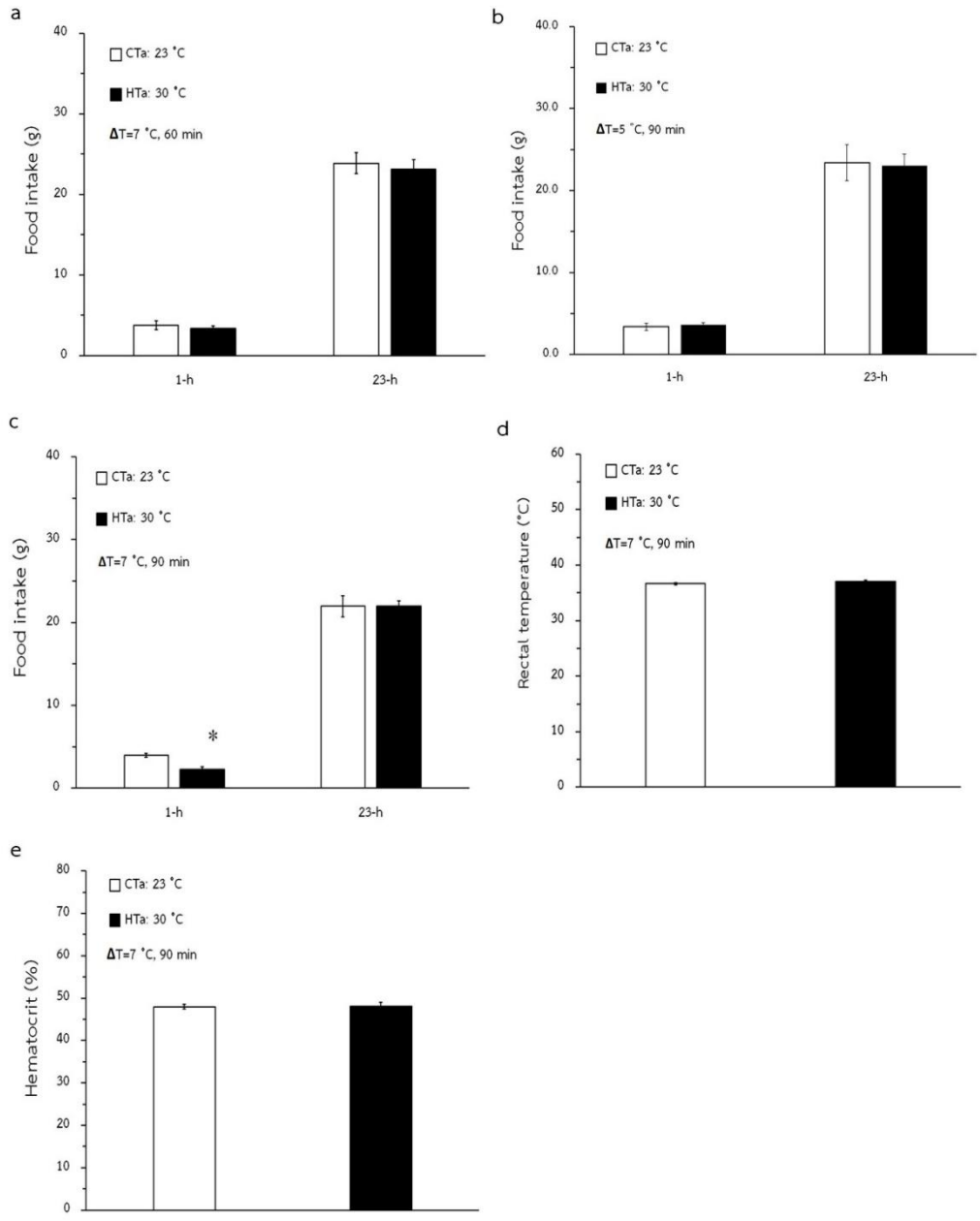


Figure 8. Short-term low-degree HTa exposure on FI, Tb and Hct.

Figure 8. (a) 60 min HTa exposure with **Δ**T= 7°C based on CTa at 23°C on 1-h FI and 23-h FI. (b) 90 min HTa exposure with **Δ**T= 5°C based on CTa at 23°C on 1-h FI and 23-h FI. (c) 90 min exposure with **Δ**T= 7°C based on CTa at 23°C on 1-h FI and 23-h FI. Ninety minute exposure with **Δ**T= 7°C based on CTa at 23°C on Tr (d) and Hct (d). * P<0.05, HTa group is significantly different from CTa group using student t-test; N=6 per each group.

4.2. Activation of POMC neurons in hypothalamic arcuate nucleus under short-term low-degree HTa exposure

The first experiment found that 90 min exposure of **Δ**T at 7°C based on CTa at 23°C (HTa = 30°C) significantly decreased 1-h FI without changing in Tr and Hct. Therefore, the activity of Arc POMC neurons was examined in both nonfeeding and feeding conditions by this low-degree HTa model.

A. Effect of short-term low-degree HTa exposure under nonfeeding condition

Under nonfeeding condition, the short-term low-degree HTa exposure significantly increased the total number of c-Fos expression, but not the POMC-ir neurons (Fig. 9a, t_9=2.33, P<0.05 and 1.30, P>0.05, respectively). The total number of c-Fos positive cells were 9±1 cells in CTa group, and HTa group were 14±1 cells. The expression of c-Fos significantly increased at the Arc2 region after short-term low-degree HTa exposure (Fig. 9b, t_9 = 2.83, P<0.05). The total number of POMC-ir neurons in CTa group was 95±6 cells, and HTa group was 128±23 cell. The number of POMC-ir neurons was not significant at the Arc2 region after short-term low-degree HTa exposure (Fig. 9c, t_9 = 1.49, P>0.05). The double-labeled neurons were not detected under nonfeeding condition.

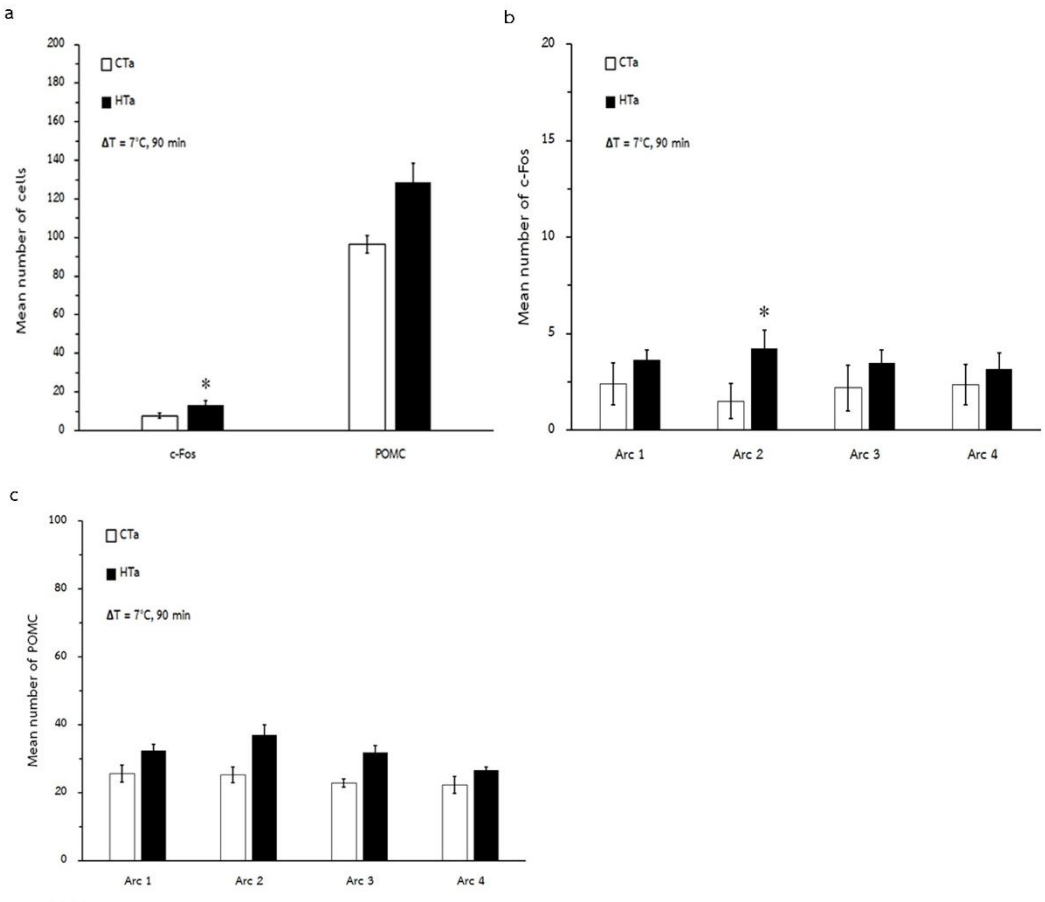


Figure 9. Neural activation in the Arc under nonfeeding conditions.

Figure 9. (a) Short-term low degree HTa exposure significantly increased the total number of c-Fos-ir, but not in POMC-ir. The number of c-Fos-ir significantly increased in Arc 2 region (b), but not in the Arc POMC-ir (c). * $P<0.05$, HTa group is significant difference from CTa group using one-way ANOVA followed by Bonferroni post-hoc test; N=6 per each group.

B. Effect of short-term low-degree HTa exposure under feeding condition

Under feeding conditions, the total number of c-Fos positive cells in HTa group (412±24 cells) was significantly higher than Cta group (242±50 cells) (Fig. 10a, t_9=3.25, P<0.05). The total number of Arc POMC-ir neurons also significantly increased in HTa group (606±24 cells) compared with the Cta group (477±53 cells) (t_9=2.36, P<0.05, Fig. 10a). Moreover, the total number of double-labeled neurons in the HTa group (186±12 cells) significantly increased after short-term low-degree HTa exposure (79±26 cells, t_9=3.99, P<0.05, Fig. 10a). The effect of short-term low-degree HTa exposure on the number of c-Fos positive cells (194±13 cells), POMC-ir neurons (216±14 cells) and double-labeled neurons (97±9 cells) significantly increased at the Arc 2 region (Fig. 10b-10d, t_8 = 4.14, 3.69 and 5.59, respectively, P < 0.05).

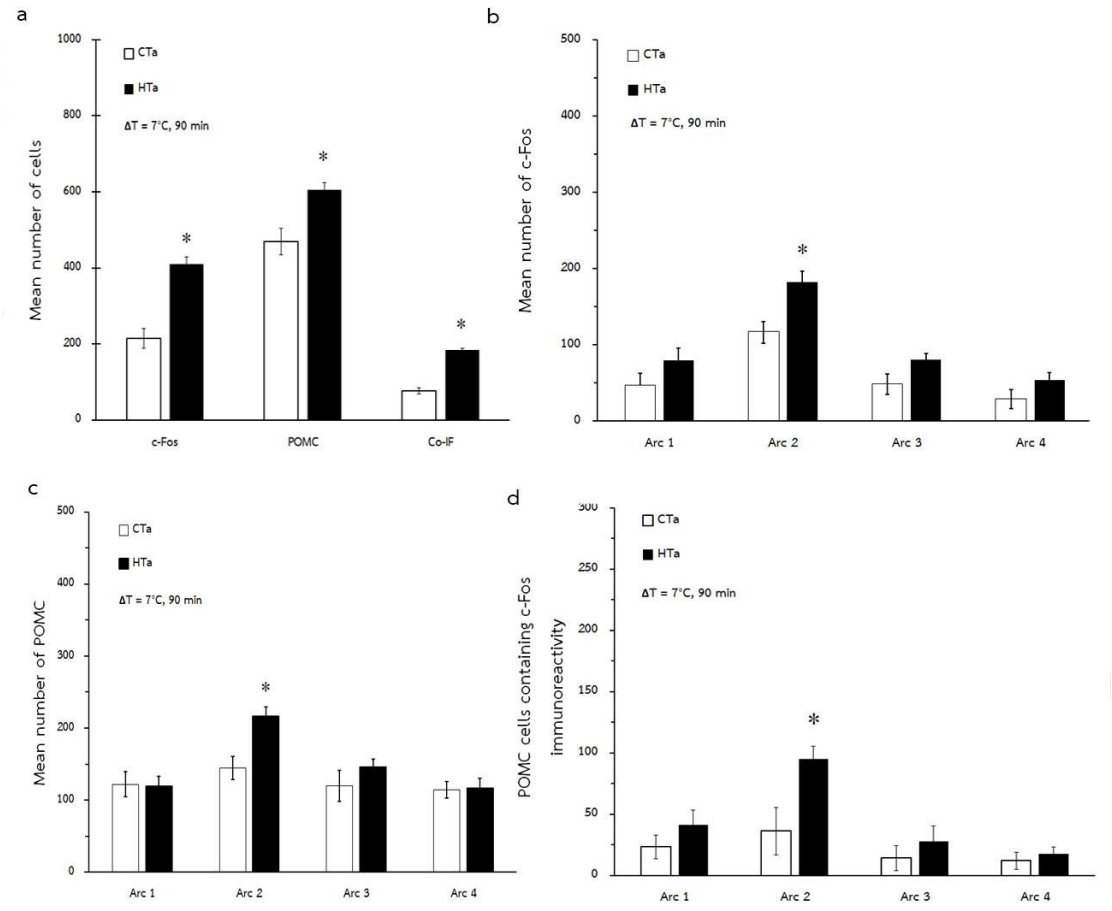


Figure 10. Neural activation in the Arc under feeding conditions.

Figure 10. Short-term low-degree HTa exposure significantly increased the total number of c-Fos-ir, POMC-ir and double-labeled neurons (a). In the region of Arc 2, the number of c-Fos-ir (b), POMC-ir (c) and double-labeled neurons (d) significantly increased in the HTa group. * P<0.05, HTa group is significant difference from CTa group using one-way ANOVA followed by Bonferroni post-hoc test; N=6 per each group.

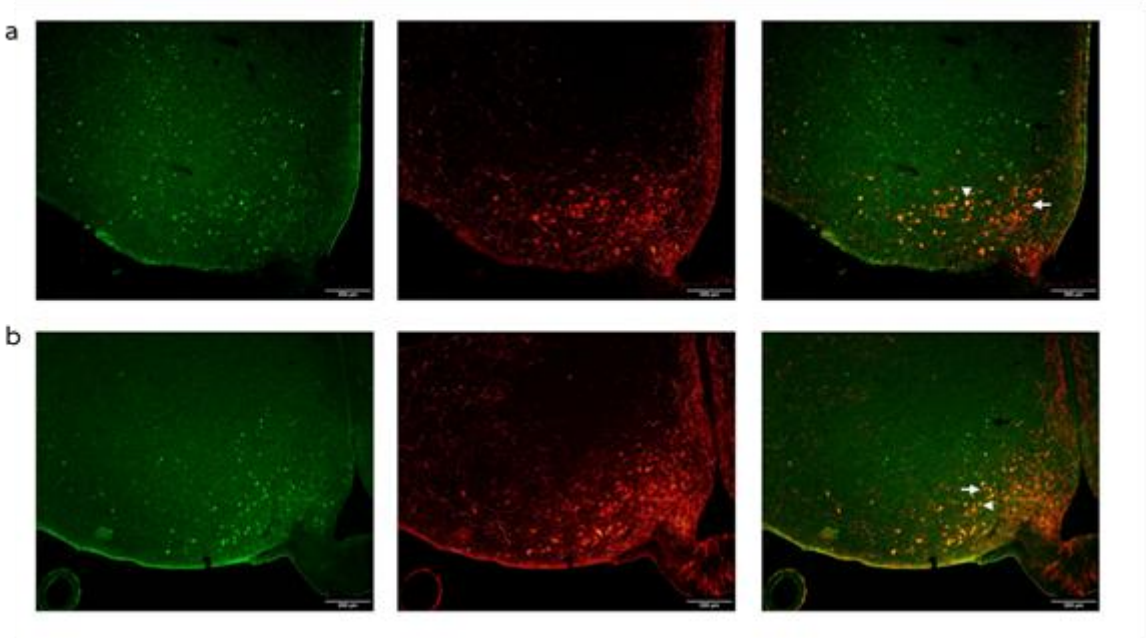


Figure 11. Representative picture of the immunofluorescence staining.

Figure 11. The picture shows c-Fos positive nuclei (left), POMC-ir (middle) and double-labeled neurons (right) in the CTa group (a) and HTa group (b). The c-Fos positive cells were shown in black arrow. The POMC positive neurons were shown in white head. The double-labeled neurons were shown in white arrowhead.

4.3. Effects of SHU9119 on the short-term low-degree HTa exposure in reduction on food intake

To examine the injection of SHU9119 could antagonize the effect of short-term low-degree HTa exposure (**Δ**T= 7°C based on CTa = 23°C and 90 min) on FI. The subthreshold dose of SHU9119 (0.1 nM in 2 µl) was injected into the lateral ventricle of the rat brain. The effect of short-term low-degree HTa exposure significantly decreased 1-h FI after vehicle infusion (Fig. 12a, t_{10} = 4.76, P<0.05). The infusion of SHU9119 could reverse the effect of short-term low-degree HTa exposure on 1-h FI that was not different 1-hFI compared with CTa group (Fig. 12a, t_{10}=1.01, P>0.05). The effect of short-term low-degree HTa exposure and SHU9119 injection had no effect on 23-h FI in both CTa group and HTa group (Fig. 12b, $F_{1,20}$=0.49 and 1.21, P>0.05).

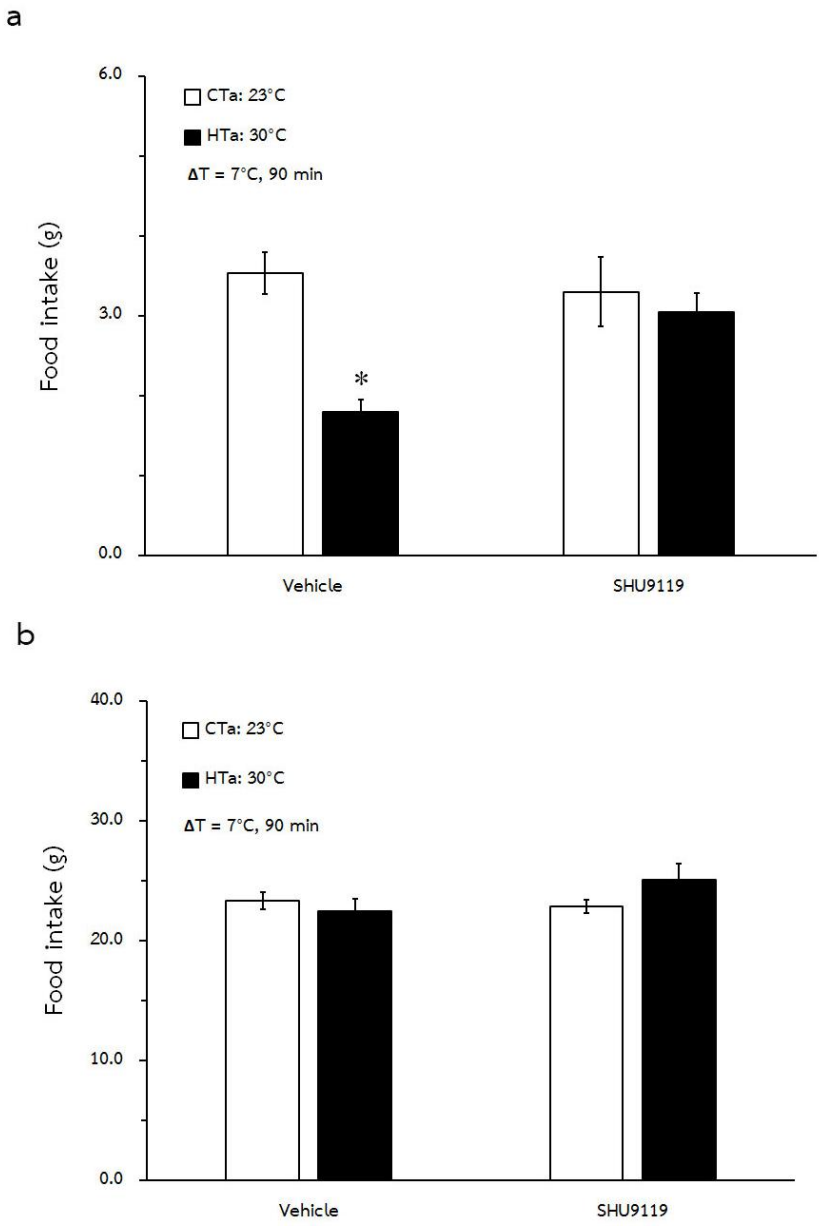


Figure 12. Effects of SHU9119 injection on HTa-induced reduction on FI.

Figure 12. The short-term low-degree HTa exposure decreased 1-h FI, and SHU9119 injection could reverse this effect (a). The effect of short-term low-degree HTa exposure and SHU9119 injection could not change 23-h FI (b). * $P<0.05$, HTa group is significantly different from CTa group using two-way ANOVA followed by Bonferroni post-hoc test; N=6 per each group.

4.4. The afferent projections to the Arc: distribution of FG-labeled neurons

To examine the involvement of MnPO pathway on eating-related Arc activity under the low-degree HTa, the present study was designed to map a neuronal connection of MnPO-Arc pathway in related with short-term low-degree HTa exposure by FG retrograde neuronal tracer injection. The distribution of FG-labeled neurons was used to investigate the afferent input from the MnPO to the Arc by IHC technique. The neuronal activity of MnPO-Arc pathway after short-term low-degree HTa exposure was identified by doubled-FG labeling with c-Fos.

A. Injection sites

Seven days after FG injection in the Arc, FG was successfully injected only two rats of the HTa group. Pressure FG injection produced FG deposits localized within the Arc. The FG injection was considered to be misplaced when FG tracer was mild spread to the VMH in this study. A representative line drawing of injection sites in the Arc is shown in Figure 13.

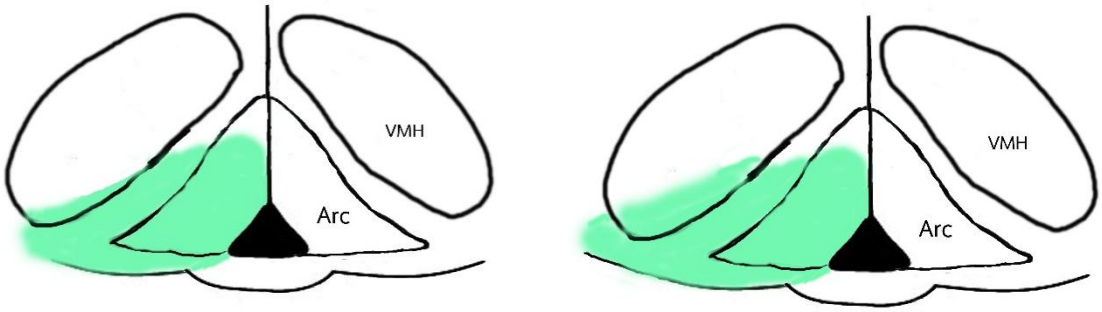
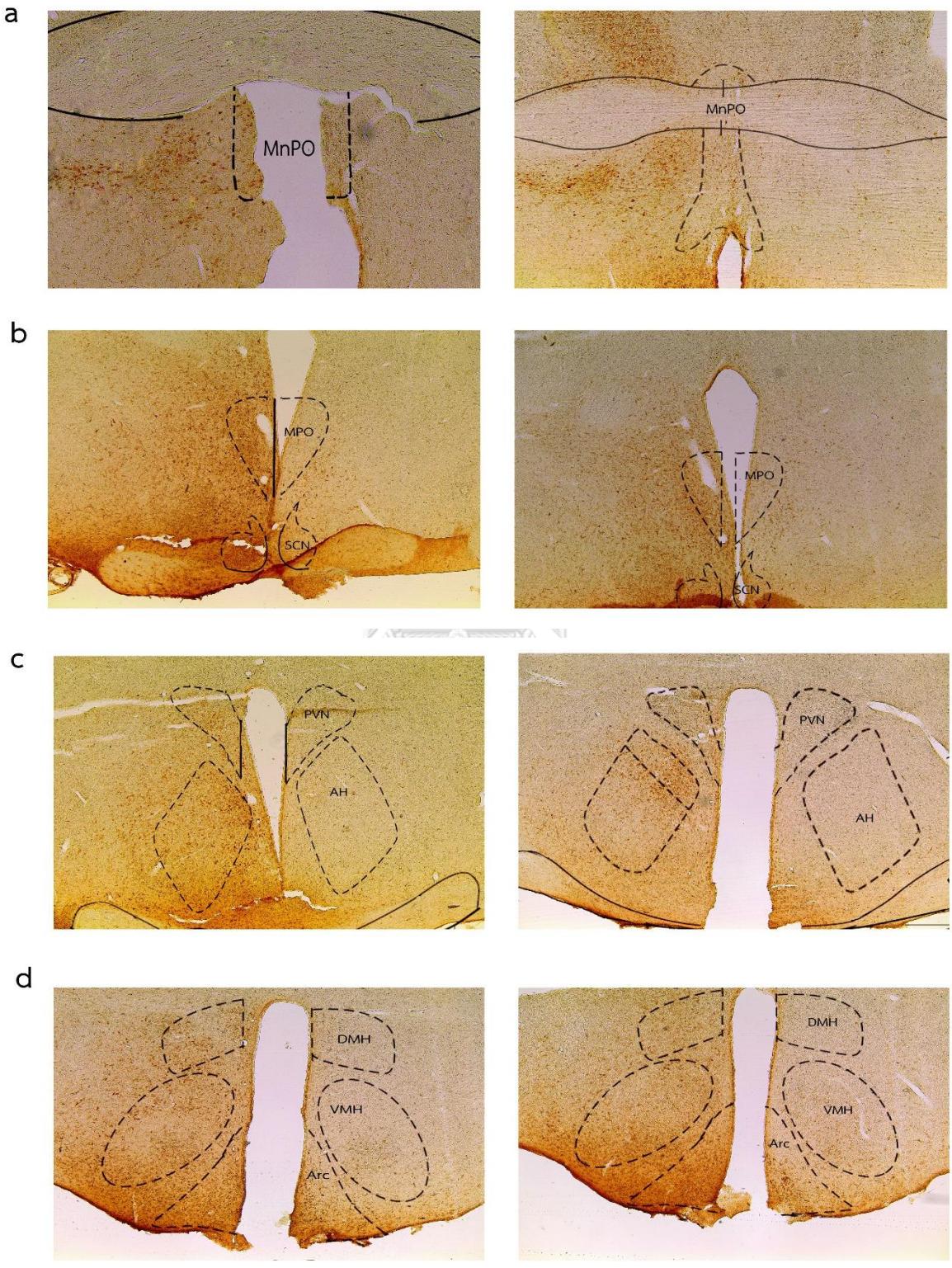


Figure 13. Schematic line drawing of FG injection sites.

Figure 13. Schematic line drawing represents a placement of pressure injection delivered FG deposits in the Arc. The FG deposit after FG injection in the Arc are illustrated by the shaded area with green color. VMH, ventromedial hypothalamic nucleus; Arc, arucuate nucleus.

B. Distribution of FG labeled neurons after short-term low degree HTa exposure

A brown cytoplasmic FG-labeled neurons were found in the MnPO (Figure. 14a) and MPO (Figure. 14b) of POA in HTa rats. The FG-labeled neurons were detected in other hypothalamic nuclei: suprachiasmatic nucleus (SCN) (Figure. 14b) and anterior hypothalamic nucleus (AH) (Figure. 14c), PVN (Figure. 14c), DMH (Figure. 14d) and VMH (Figure. 14d). Fluorogold-labeled neurons were also found in contralateral site of the Arc (Figure. 14d). In addition, the FG-labeled neurons were found in the medial amygdala (MeA) (Fig. 14e). The majority of these projections is ipsilateral to the injection site. This study did not find the expression of c-Fos positive nuclei and double-labeled neurons in any brain regions after double-labeled staining.


a
MnPO
MnPO
b
MPO
SCN
MPO
SCN
c
PVN
AH
PVN
AH
d
DMH
VMH
Arc
DMH
VMH
Arc

e

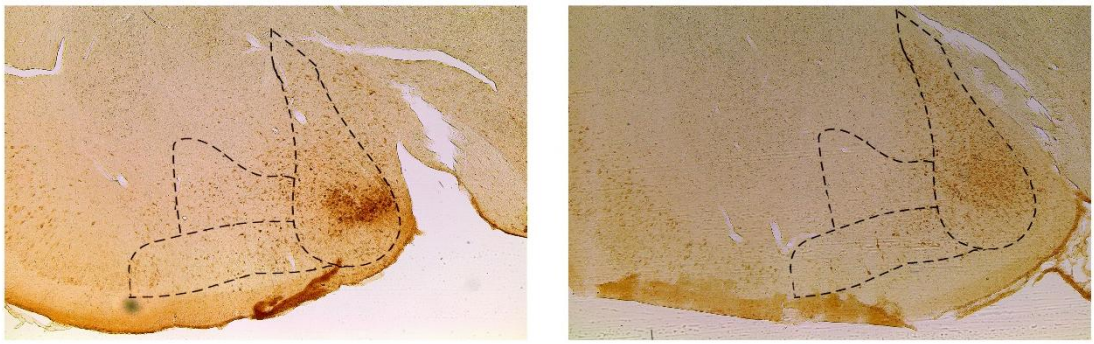


Figure 14. FG-labeled-neuron distribution in the rat brain.

Figure 14. FG-labeled neurons in the MnPO are shown in a (Bregma: -2.60 mm and -2.30 mm, respectively). FG-labeled neurons in the MPO and SCN are shown in b (Bregma: -1.30 mm). Brown cytoplasmic FG-labeled neurons in the PVN and AH are shown in C (Bregma: -1.80 mm and -1.88 mm, respectively). Figure d shows FG-labeled neurons in the DMH, VMH and Arc (Bregma: -2.56 mm). FG-labeled neurons in the amygdala are shown in e (Bregma: -2.56 mm).

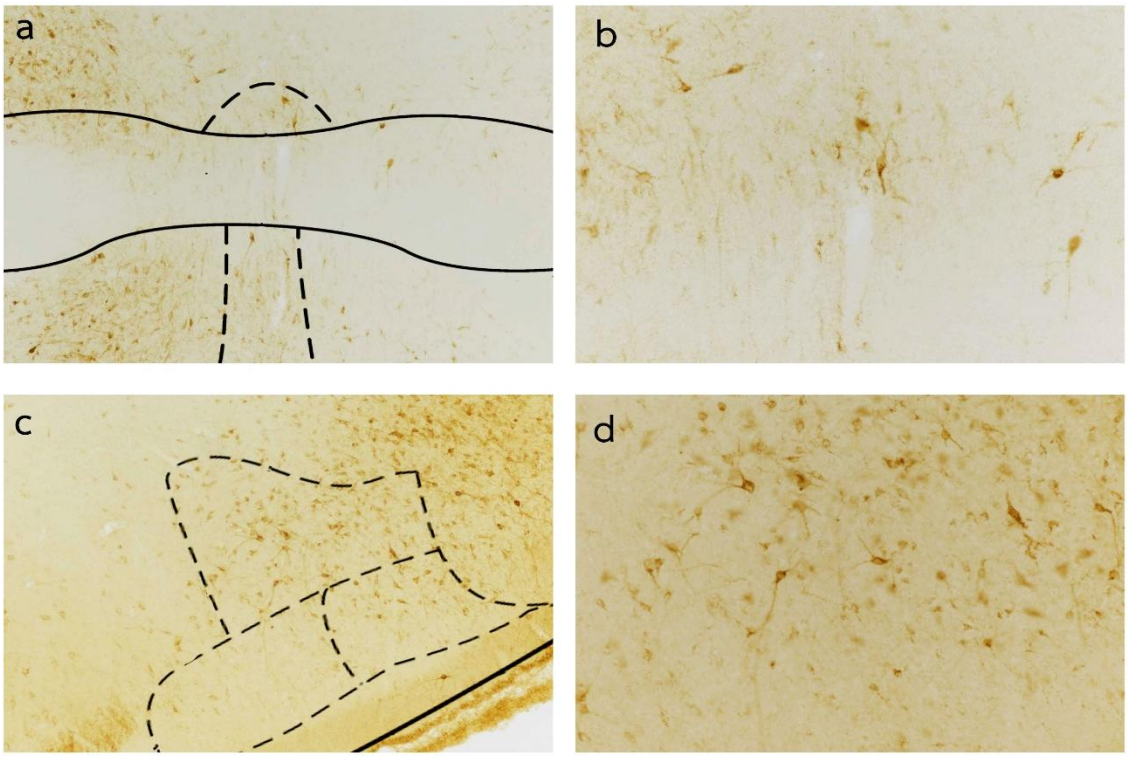


Figure 15. A representative magnification of FG-labeled neurons.

Figure 15. A representative magnification of FG-labeled neurons in the MnPO is shown in a and b. A representative magnification of FG-labeled neuron in the amygdala is shown in c and d.

C. Limitation of study

The results of FG-labeled neurons in this study have three limitations. The first limitation was the methodology of FG injection. The pressure injection of FG retrograde neuronal tracer into the Arc by pressure injection caused to excessively spread of FG tracer. The FG tracer was spread to the VMH or extended into the third ventricle. Therefore, the successful injection of this study was selected with the rats that mild spread of FG deposit in the VMH. There was successful injection only in two rats of this study. The second limitation was the IHC technique that investigated the MnPO pathway on eating-related Arc activity under the low-degree HTa by double-labeled neurons of c-Fos positive nuclei and FG labeled neurons. Unfortunately, the double-labeled staining of this study did not find c-Fos positive nuclei in the MnPO and other regions. Thus, this study could not demonstrate the activity of neuronal pathway in the MnPO after FG injection in the Arc. The third limitation was the experimental design with nonfeeding condition that could not imply directly of low degree HTa exposure in related with reduction on FI via the MnPO-Arc pathway. However, because these limitations, the findings of this study offer potential information to solve problem for further study.

CHAPTER V
DISCUSSION

The present study aimed to demonstrate the involvement of Arc POMC neurons in reduction on FI and the neuronal pathway of MnPO-Arc under the effect of short-term low degree HTa exposure. The present study hypothesized that the Arc POMC neurons can mediate the reduction of FI under the effect of short-term low-degree HTa exposure and the neuronal pathway from MnPO to Arc relates with this effect.

The refinement of short-term low-degree HTa condition on food intake, rectal temperature and hematocrit

High ambient temperature is one of important environmental factor that reduces FI. The effect of HTa exposure has been widely studied with a relatively high degree HTa on eating behavior and neuroscience. The conditions of high degree HTa mostly set with **Δ**Ta equal to or greater than 10**°**C from base of basal Ta. These HTa models had an effect on Tb, autonomic thermoregulations and stress responses (12, 63-65). However, only the short-term low-degree HTa exposure can decrease FI earlier than activated physiological and stress responses (13).

The previous study of short-term low-degree HTa demonstrated that 60 min HTa exposure at **Δ**Ta = 10°C based on CTa at 20°C (HTa = 30°C) decreased FI. With, the temperature difference at **Δ**Ta = 5°C based on CTa at 20 and 25°C (60 min exposure)

had no effect on FI (13). These results suggested in part an important role of the bout of short-term HTa exposure on FI. In the present study demonstrated that short-term low-degree HTa exposure (**Δ**Ta = 7°C, HTa = 30°C, 60 min) had no effect on 1-h FI. An extension duration of HTa exposure (90 min exposure) decreased 1-h FI without changing in Tr and Hct. In contrast with **Δ**Ta = 5°C (HTa = 28°C), 90 min HTa exposure did not decrease FI. These results support the previous HTa conditions that the short-term low-degree HTa exposure should be more than 5°C temperature difference (**Δ**Ta = 5°C) in rats (13). Interestingly, the short-term low-degree HTa condition in this study is close to the HTa level (Ta = 29°C, 120 min), in which the tail vasomotor tone was activated (66) and the core temperature was kept within normal (67). In addition, because most of control temperature used for rats in many experiments was approximately set at 23±1°C (63, 68-70). Thus, the bout of HTa exposure (**Δ**Ta = 7°C, HTa = 30°C, 90 min exposure) in this study is the appropriate short-term low-degree HTa condition on FI. Therefore, the short-term low-degree HTa condition in present study is considered to be the refinement model compared with previous report (**Δ**Ta = 10 °C from the basal Ta = 20 °C and 60 min exposure, (13).

The activity of POMC neurons in hypothalamic arcuate nucleus under the effects of short-term low-degree HTa exposure.

To better understand the effect of short-term low-degree HTa exposure on the Arc neuronal activation related with a reduction on FI, this study examined the number of c-Fos positive cells, POMC positive neurons and POMC neurons containing c-Fos in the Arc with nonfeeding and feeding conditions. Under nonfeeding condition, the effect of short-term low-degree HTa exposure was significant the total number of c-Fos positive cells in the Arc. The number of c-Fos with this effect was increase in the Arc 2 region. The number of POMC positive neurons was not change under nonfeeding condition, and the colocalization could not be detected after short-term low-degree HTa exposure. These results suggested that only a small population of Arc neurons were active in nonfeeding condition. This neuron population was apparently not POMC neurons. Under feeding condition, the c-Fos-positive cells and POMC-expressing neurons was high after short-term low-degree HTa exposure, especially in the Arc 2 region. In addition, the co-localization of c-Fos and POMC was found after short-term low-degree HTa exposure. These results interpret the effect of short-term low-degree HTa exposure into 2 phases according to eating behavior; food expectation and hunger phases and satiation phases. Under nonfeeding condition, the animals were left without food for approximately 180 min. Thus, this condition was assumed that rats

expected to get food at the dark onset. This effect was considered to the food expectation and hunger phases.

Under feeding conditions, the animals were allowed to access food for 1-h and then left without food for 90 min. The c-Fos expression was enhanced in the Arc in this condition. The c-Fos expression is well known that can activate by many stimuli. The food removing of this study can be the one major stimulus. However, with the physiology and psychology process of meal during this time frame, rats expect to complete their first meal (37). Thus, this effect considered that rats were at the satiation state from the first meal under feeding condition. This conclusion is another support in part by the rhythmic c-Fos expression in Arc. The effect of circadian control on Arc c-Fos expression was prominent at Zeitgeber time 22 (ZT22), but not at the border of the dark-light cycle. In addition, the feeding induced-c-Fos expression of the Arc was clearly at ZT14 compared with fasted rats (71, 72). The number of Arc c-Fos expression significantly increased after meals compared before meals (73). The Arc neuronal subpopulations activated before and after meals are NPY and POMC neurons, respectively (74). Although, this study did not compare the c-Fos expression between nonfeeding and feeding condition. The number of c-Fos expression in feeding condition was higher than the nonfeeding condition. These results were in line with an experiment to investigate the activation of neuronal sequence during feeding at the hypothalamus and brainstem in rats.

Under nonfeeding condition, the c-Fos expression significantly increased in the Arc 2. This result suggested that this neuronal activation should be in part the mechanism related with the short-term low-degree HTa effect on eating behavior, and perhaps decreased in hunger. These c-Fos nuclei did not colocalize with POMC neurons. It is possible for the first priority that these neuronal activation were NPY neurons. However, the activity of NPY neurons is well known to increase during fasting compared with ad libitum rat and mouse models (54, 75, 76). Furthermore, it is more than likely if this study could detect the lower c-Fos expression and lower NPY fiber in HTa group under nonfeeding condition. However, this study found the higher c-Fos expression after short-term low-degree HTa exposure in nonfeeding condition. Thus, this results could not imply the evidence related with NPY neuron deactivation. However, the effect of short-term low-degree HTa exposure should inhibit downstream of NPY signaling. Interestingly, the staining of NPY fiber was lower in the HTa group than the CTa group under nonfeeding condition (Appendix A). Therefore, the possible conclusion is the subpopulation neurons that may participate to decrease NPY fibers and signaling (77). This results suggested that short-term low-degree HTa exposure increased neuronal activation, and may decrease NPY fiber signaling at the Arc under nonfeeding condition in rat.

Under feeding condition, short-term low-degree HTa exposure on c-Fos expression and POMC-expressing neurons was prominent especially in the Arc 2 region. The number of POMC neurons containing c-Fos positive nuclei was increase in the Arc

after short-term low-degree HTa exposure. These results suggested that short-term low-degree HTa exposure could increase satiation signaling of POMC neurons in the Arc. It has been demonstrated that Arc POMC neurons has the plasticity properties regarding with eating behavior (52, 78). The interpretation of feeding related with the activation of Arc POMC should be done together with physiological and psychological processes, especially sensory detection of food (52). However, the POMC neuron activation may probably explain in part by the cannabinoid system during pre-absorptive (78). However, this study performed the neuronal activation of Arc POMC during satiation period. Therefore, to test this hypothesis, the effect of subthreshold dose of SHU9119 was investigated on FI after short-term low-degree HTa exposure.

The antagonist of MC3/4 receptor (SHU9119) on the effect of short-term low-degree HTa exposure on food intake.

The pre-infusion with subthreshold dose of SHU9119 did not influence on 1-hr FI and 23-hr FI, but the same dose of SHU9119 could reverse the effect of short-term low-degree HTa on FI. The present results indicate that the short-term low-degree HTa exposure appears to exert its function on FI by acting through POMC signaling and melanocortin receptors. In the central nervous system, the melanocortin receptors highly distribute at the downstream POMC signaling in the DMH, VMH, and NTS (17, 21). Interestingly, the effect of short-term low-degree HTa exposure increased c-Fos

expression in the DMH, VMH and NTS under feeding condition (13). The downstream POMC signaling from all regions is of interest for further investigation.

Collectively, these experiments demonstrated the mechanism of short-term low-degree HTa exposure in reduction of FI in rats. The signaling of Arc POMC neurons is an important mechanism that is enhanced by short-term low-degree HTa exposure.

The neuronal pathway of MnPO-Arc under the effect of short-term low-degree HTa exposure.

The effect of short-term low-degree HTa increased c-Fos positive nuclei in the MnPO and Arc. The feeding-induced c-Fos was enhanced in the MnPO and Arc after short-term low-degree HTa exposure (13). In addition, the neural connection between MnPO-Arc has been demonstrated to control the Tc related with day night rhythm (72). Thus, this study aimed to investigate the effect of short-term low-degree HTa in related with the neuronal connection between MnPO and Arc. The present study used retrograde tracing techniques to observe the neural connection of MnPO-Arc after short-term low-degree HTa exposure in rats. This study injected retrograde FG tracer in the Arc and investigated the FG-labeled neurons in the MnPO and another brain areas, especially in the hypothalamic nuclei.

The hypothalamus is the major sources of afferent connections to the Arc. Many retrogradely-labeled neurons were found in the hypothalamic nuclei after FG injection that localized FG deposition in all division of the Arc in this study. The FG-

labeled neurons were detected in the AH, PVN, DMH, VMH and Arc. These projections were reported in regulation of eating behavior and energy homeostasis (7, 17, 21, 47, 79). This study also found the FG-labeled neurons in the SCN. The SCN has been demonstrated to involve in the activity of the Arc POMC neurons by circadian rhythm (71, 80). Interestingly, the SCN is not source of afferent projection to the VMH (81), where retrograde tracer was less spread after FG injection in this study. Thus, the finding of FG-labeled neurons in the SCN can imply the anatomical patterns of FG-labeled signal in this study that were mainly from the FG deposit in the Arc. In extra-hypothalamic areas, the FG-labeled neurons were detected in the MeA of amygdala. This projection has been noted in previous study of Arc afferent (79).

Little evidence was reported for fiber inputs to the Arc from MnPO other than the MPO of POA region (79, 82). In this study, FG retrograde labeled-neurons were found in the MnPO and MPO after FG injection. This finding can be implied that the MnPO is a one of afferent connection to the Arc. However, this study could not clearly report the neuronal connectivity of MnPO-Arc involved with the effect of short-term low-degree HTa exposure. Because, no labeled of c-Fos positive nuclei and double-labeled neurons were observed in the MnPO and other brain areas in the present study. In future studies, it will be important to prove the FG-labeled neurons on the effect of short-term low-degree HTa-induced c-Fos expression.

CHAPTER VI
CONCLUSION

The effect of short-term low-degree HTa exposure at ΔTa = 7°C based on CTa at 23°C (HTa = 30°C) on neuronal activation in the Arc was demonstrated by the expression of c-Fos positive nuclei, POMC positive neurons and double-labeled neurons with immunofluorescence technique as the first step. Under nonfeeding conditions, short-term low-degree HTa could increase c-Fos-positive nuclei at the Arc without the activation of POMC neurons. Under feeding condition, the effect of short-term low-degree HTa could enhance the expression of c-Fos positive nuclei and POMC-expressing neurons at the Arc. In addition, the effect of short-term low-degree HTa significantly increased the double-labeled neurons at the Arc under feeding condition. Finally, pre-infusion with melanocortin MC3/4 receptor (SHU9119) could reverse the reduction on FI by effect of short-term low-degree HTa exposure. To demonstrate the pattern of efferent fiber from MnPO to the Arc, the FG retrograde tracer was injected in the Arc. The retrograde labeled neurons were detected in the MnPO, MPO, SCN, AH, PVN, DMH, VMH, and amygdala.

This study concluded that the effect of short-term low-degree HTa exposure on FI is mediated in part by the activation of POMC neurons at the Arc in rats. The MnPO-Arc pathway may related with the effect of short-term low-degree HTa exposure.

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APPENDIX

A. Effect of short-term low-degree HTa exposure on the density of NPY fibers in the Arc under nonfeeding condition.

Ninety minutes later of HTa exposure at the dark onset, the rats were deeply anesthetized with pentobarbital sodium intraperitoneal injection (60 mg/kg, i.p., Nembutal, Ceva Sante Animale, France), and transcardially perfused with phosphate buffer (0.1 MPB, pH 7.4), and followed by 4% PFA-0.1 MPB. The brains were removed and postfixed in 4% PFA- 0.1 MPB for 12 h at 4 °C, and then immersed in 20% sucrose for 48 h. The rat brain was cut into 40 μM-thick coronal sections (-0.20 to -3.60 mm, posterior to the bregma) to determine the effect of low-degree HTa on NPY density at the Arc.

NPY immunofluorescence staining protocol

Brain sections were washed for 10 min and incubated in 10% NDS (Abcam, MA, USA) in PBTriton-X for 1 h, followed by rabbit anti-NPY antibody (1:2000; 22940, ImmunoStar, WI, USA) for 72 h. Sections were washed in PBTriton-X for 10 min. The brain sections were then incubated in Alexa Fluor 568 donkey anti-rabbit (1:200, ab175470, Abcam, MA, USA) for 2 h. The brain sections were mounted onto a coverslip with Vectashiled Hardset mounting medium (H-1400, Vector laboratories, CA, USA).

For quantitation of fluorescence, NPY-ir densities of the Arc were photographed with BX53 microscope using cellSens software. The density of NPY-ir in the arc was processed and analyzed using ImageJ analysis software (National Institutes of Health). For the quantitative analysis of NPY-ir intensity, a binarization process was performed for each image to isolate labeled fibers from the background and to compensate for differences in fluorescence intensity. The integrated intensity was calculated from the total number of pixels for each image plane.

Results

Effect of short-term low-degree HTa exposure (**Δ**T= 7 ℃ based on CTa = 23 ℃, 90 min) on neural activation in the arcuate nucleus under nonfeeding conditions. The density of NPY fibers in the Arc from the HTa group was significantly lower than that in the CTa group. * HTa is significantly different from CTa, $P<0.05$, Figure S1. Optical micrograph of unilateral Arc sections showing the immunofluorescence staining of NPY fibers of the CTa group (left) and HTa group (right), Figure S2.

Figure S1

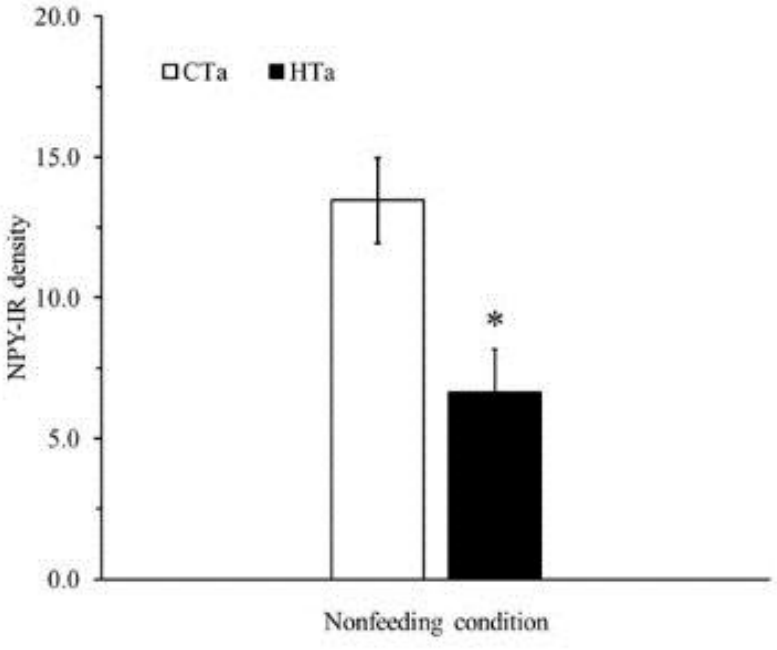

Figure S2

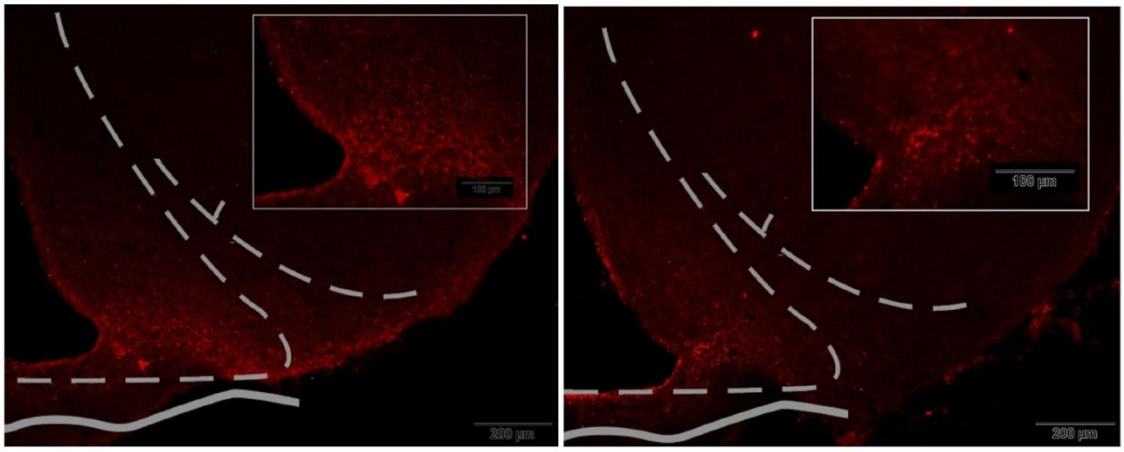

Figure A. S1: Effect of short-term low-degree HTa exposure on NPY density in the Arc from the HTa group was significantly lower than that in the CTa group. Figure S2: the immunofluorescence staining of NPY fibers of the CTa group (left) and HTa group (right). Figures came from Suwannapaporn P., et al. 2022 (60).

B. An example picture of c-Fos positive nuclei in single and double staining

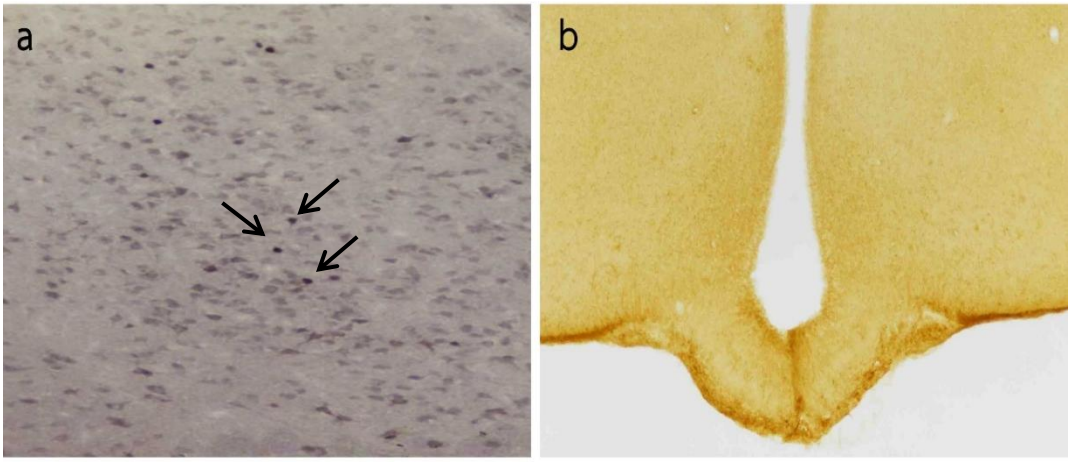

Figure B. Single staining of c-Fos positive nuclei with rabbit anti-c-Fos in is shown in a that found c-Fos positive nuclei (black arrow). Double staining of c-Fos positive nuclei is shown in b that did not find c-Fos positive nuclei. The double staining process is described with same protocol in the fourth experiment. The two brain sections is derived from rat without FG-injection.

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