

Original Article

Can hypoxia enhance sexual arousal?-Molecular-biological analysis of the hypothalamus in male rats placed with oestrous female rats under hypoxic conditions-

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Abstract: To clarify whether sexual arousal could be enhanced by hypoxia, and to explain the pathogenesis of autoerotic death (AED)/autoerotic asphyxiation (AEA), we investigated the molecular-biological and hormonal interactions in male rats between hypoxia (10% O₂) and sexual stimulation (placing male rats with oestrous female rats) using hypothalamus tissue and serum. Serum oxytocin concentration was significantly increased by either hypoxia or sexual stimulation without significant change of *Oxt* mRNA expression. Serum concentration of adrenocorticotrophic hormone was significantly elevated by sexual stimulation, although serum concentration of copeptin was significantly suppressed by hypoxia. Moreover, serum concentration of luteinising hormone was increased by hypoxia. These results suggested that hypoxia would complicate the response to sexual stimulation, and effect a change in sexual arousal. Moreover, sexual stimulation significantly suppressed mRNA expressions of *Hspa4* and *Hyou1*, which might indicate that sexual stimulation reduce tolerance to hypoxia/ischaemia. Accordingly, it is possible that AED/AEA would be attributed to a reduced tolerance to hypoxia/ischaemia induced by attempts to obtain sexual arousal under a hypoxic condition and would not be a simple accident.

Keywords: Oxytocin, sexual stimulation, hypoxia, autoerotic asphyxia (AEA), oxygen-regulated protein 150 (ORP150)

Introduction

Although it is known that some individuals obtain sexual arousal by oxygen deprivation resulting from chest compression, use of a noose or ligature, donning a plastic bag or mask, or chemical asphyxia, whether hypoxia can actually evoke sexual arousal and, if so, by what mechanisms, is still unclear [1-6]. However, these individuals gradually increase the severity of the acts for producing sexual arousal over time and may eventually die, and this is called autoerotic asphyxiation (AEA). AEA comprises the majority of autoerotic deaths (AED) [7-10]. Autoerotic fatalities annually account for approximately 2 to 3 deaths per million persons in the United States [2], and 0.56-0.14 deaths per million per year in other parts of the world [7, 8, 10]. From the viewpoint of forensic medicine, it is important to diagnose precisely the

manner and cause of death in suspected cases of AEA, as accidental or AED, particularly, because the activity is secretive, and the findings at the death scene are often changed by family members or friends, which may lead to homicide cases being overlooked [10-13].

A feature sometimes seen in AEA cases is the presence of a safety device, which should have function to prevent an extraordinary situation, but unfortunately did not work on this occasion [11-13]. This can be explained by rapid development of unconsciousness resulting from cerebral ischaemia before self-extrication from the compression of the neck [14-16]. However, it is possible that the occurrence of AEA would be not only accidental but also related to some morphological and/or functional alteration in organs, especially in the central nervous system. The resolution of the mecha-

nisms resulting in AEA could lead to prevention of AEA/AED.

Indeed, it is difficult to confirm whether hypoxia can induce sexual arousal in an animal experimental model. Thus, in this study, to clarify whether sexual arousal can be enhanced by hypoxia and to explain the pathogenesis of AEA/AED, we investigated the molecular-biological and hormonal interactions between hypoxia and sexual stimulation in male rats using neuropeptides and chaperones.

Material and methods

This experiment was reviewed by the Committee on the Ethics of Animal Experiments in the Faculty of Medicine, Mie University and it was carried out under the control of Guidelines for Animal Experiments in the Faculty of Medicine, Mie University and the Law (No. 105, Act on Welfare and Management of Animals) and Notification (No. 6, Standard relating to the care and management of experimental animals) of the Government of Japan. Moreover, this study followed the 'Guide for the Care and Use of Laboratory Animals', published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Procedure

Nine-week-old male Wistar rats (250-270 g, CLEA Japan, Inc., Tokyo, Japan) were purchased and housed in our darkroom, where the temperature was controlled ($22 \pm 1^\circ\text{C}$) on a 12:12 light-dark cycle with the lights on at 7 p.m. for 1 week before the experiment. For 3 days immediately before the experiment, the rats were separately reared in another darkroom under room air without female rats, on the same light-dark cycle as the previous darkroom. Food and water were available ad libitum in both darkrooms.

After the pre-experiment, these male rats were divided into two groups: the hypoxia group, which were kept in a chamber where the oxygen concentration was controlled to $10 \pm 1\%$ ($n = 16$), and the room-air group, which were kept under an oxygen concentration of 20% in a fully ventilated chamber ($n = 17$). Within the hypoxia group, the rats were subdivided into two groups: lone male rats in a hypoxia-controlled chamber for 1 h in the "single under hypoxia" group (HS;

$n = 8$), and male rats, each with three 10-week-old oestrous female Wistar rats (230-250 g, CLEA Japan, Inc. Tokyo Japan) kept together in the chamber for the same amount of time given to the single rats under hypoxia conditions in the "sexual stimulation under hypoxia" group (HX; $n = 8$). As with the hypoxia group, the room-air group was subdivided into the "single under room-air" group (RS; $n = 9$) and the "sexual stimulation under room-air" group (RX; $n = 8$). In addition, hypoxic condition was provided by mixing pure O_2 with pure N_2 in a mixing chamber, which was equipped with an O_2 monitor (OXY-1, Jikco Ltd., Tokyo, Japan) and regulated to $10 \pm 1\%$ of O_2 , and connected to the chamber housing the rats.

The oestrous female rats were selected as follows: when the back of a rat was gently rubbed by hand, if she stretched and poked her buttocks up, this was considered a lordosis and she was selected as an oestrous female rat. Moreover, because rats have a 4-day oestrous cycle, male rats were in the chamber with three oestrous female rats to increase their opportunity to encounter oestrous rats.

The experimental males were placed in a separate compartment in the lower section of the chamber, divided from the compartment above them by a wire-meshed ceiling. Oestrous females were placed in the upper compartment, which allowed the experimental male to initiate only genital contact through the ceiling. Immediately after being allowed to achieve sexual arousal for 1 h, the male rat was killed by decapitation. Next, the brain was removed and the blood was collected from the cervical stump. The hypothalamus area of the brain was trimmed (**Figure 1**) [17], and soaked in RNAlater® (Life Technologies Corp., Carlsbad, CA) for one night, and then stored at -80°C for later analysis of mRNA expression. The remainder was fixed in 4% formaldehyde for histopathological analysis with immunohistochemical staining. Serum was separated from blood cells and fibrin by centrifugation and then stored at -80°C for determination of hormones.

To eliminate chronological differences of mRNA expression and hormonal secretion, all experiments were carried out between 9 a.m. and noon local time.

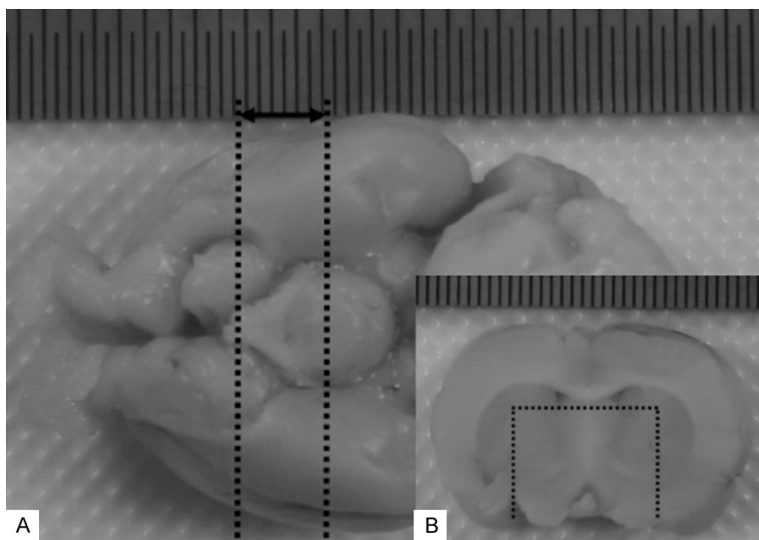


Figure 1. Macroscopic observation of the base of rat brain (fixed in 4% formaldehyde). A. The rat brain is frontally sliced according to the two dashed lines, and the frontal section between the two dashed lines is used for analysis of mRNA expression. B. In the frontal section for analysis of mRNA expression, the specimen is further trimmed according to the strong dashed line, which includes hypothalamus. A front side of the caudal residual portion after cutting off the section for analysis of mRNA expression is used for immunohistochemical analysis using anti-Hsp70 and anti-Orp150 antibodies.

Determination of hormones in serum

Serum concentrations of adrenocorticotrophic hormone (ACTH), follicle stimulating hormone (FSH), and luteinising hormone (LH) were determined by multiplex assay system (MAGPIX® System, Luminex Corp., Austin, TX), using the MILLIPIX® MAP Rat Pituitary kit (Millipore Corp., Billerica, MA). Serum concentrations of copeptin (CPP), which is a precursor of arginine-vasopressin (AVP) and has been shown to represent AVP concentrations closely [18, 19], and oxytocin (OT) were measured using enzyme-linked immunosorbent assay kits for each hormone (USCN® Life Science Inc., Houston, TX, and Enzo® Life Sciences Inc., Farmingdale, NY, respectively), according to the instructions of each manufacturer.

Quantitative reverse transcription-polymerase chain reaction analysis

In each group, mRNA was extracted from the hypothalamus using the QuickGene RNA tissue kit SII (Kurabo Industrial Ltd., Osaka, Japan) according to the manufacturer's instructions. The extracted mRNA was then used as a template, and complementary DNA (cDNA) was synthesised by using a first-strand cDNA syn-

thesis kit (ReverTra Ace® qPCR RT Master Mix, Toyobo Co.Ltd., Osaka, Japan), according to the attached instructions.

The mRNA levels of arginine vasopressin (Avp), oxytocin (Oxt), solute carrier family 6, member 4 (Slc6a4), heat shock protein 4 (Hspa4), hypoxia up-induced 1 (Hyou1), and actin beta (Actb, as internal standard) were relatively quantified with TaqMan® Gene Expression Assays (Life Technologies Corp.), using a fluorescence detection system (StepOne®, Life Technologies Corp.) by DDCT methods, and compared with the hypothalamus of a rat in the single under room-air group, in accordance with the attached instructions. The PCR mixture consisted of 0.5 µL of each

TaqMan® Probe including forward and reverse primers and 5 µL of Thunderbird® Probe qPCR Mix (Toyobo) in a final volume of 10 µL. The cycling conditions were 95°C for 20 s, followed by 45 cycles at 95°C for 1 s and 60°C for 20 s.

Immunohistochemical analysis

Hypothalamus fixed in 4% formaldehyde was embedded in paraffin, and then 4-µm-thick sections were cut and stained immunohistochemically with anti-heat shock protein (Hsp) 70 and anti-oxygen regulated protein (Orp) 150 antibodies.

Antigen retrieval was performed by microwaving sections in Histofine antigen retrieval solution, pH 9.0 (Nichirei, Tokyo, Japan). Endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide in methanol for 15 min at room temperature (RT). The sections were then washed three times with phosphate-buffered saline for 5 min at RT. As the primary antibodies, anti-Hsp70 antibody (mouse-mono, SMC-100A, 1:500, StressMarq Biosciences Inc., Victoria, Canada) and anti-Orp150 antibody (RabMAbs®, ab124884, 1:100, Abcam plc, Cambridge, UK) were applied to the hypothalamus for 24 h at 4°C. As the secondary

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Table 1. Serum concentrations of 5 hormones after exposure to hypoxia and/or sexual stimulation

Hormone	Means \pm SE (pg/mL)				<i>t</i> (<i>p</i>)		
	RS	HS	RX	HX	H	X	H*X
Oxytocin	327.4 \pm 72.4	416.1 \pm 101.2	544.4 \pm 56.7	564.9 \pm 49.2	2.12 (0.0426)	7.12 (<0.0001)	-1.33 (ns)
Copeptin	46.50 \pm 33.20	13.45 \pm 5.98	34.75 \pm 22.66	15.68 \pm 9.49	-3.16 (0.004)	-1.07 (ns)	1.43 (ns)
ACTH	3.2	3.2	6.583 \pm 8.745	8.341 \pm 5.514	0.48 (ns)	2.39 (.0234)	0.50 (ns)
LH	359.5 \pm 337.6	805.1 \pm 392.4	463.5 \pm 299.5	503.9 \pm 309.2	2.12 (0.0424)	-0.79 (ns)	-1.73 (ns)
FSH	5338.8 \pm 1413.6	4918.6 \pm 804.7	4892.1 \pm 1414.8	4405.0 \pm 1418.5	-1.00 (ns)	-1.06 (ns)	-0.07 (ns)

In this method, minimum limit of detection of ACTH was 3.2 pg/mL. Thus, if the quantitative value was less than 3.2 pg/mL, it was reluctantly considered as 3.2 pg/mL. ns, not significant. H, hypoxia; X, sexual stimulation; RS, single under room air; HS, single under hypoxia; RX, sexual stimulation under room air; HX, sexual stimulation under hypoxia. ACTH, adrenocorticotropic hormone; FSH, follicle stimulating hormone; LH, luteinizing hormone.

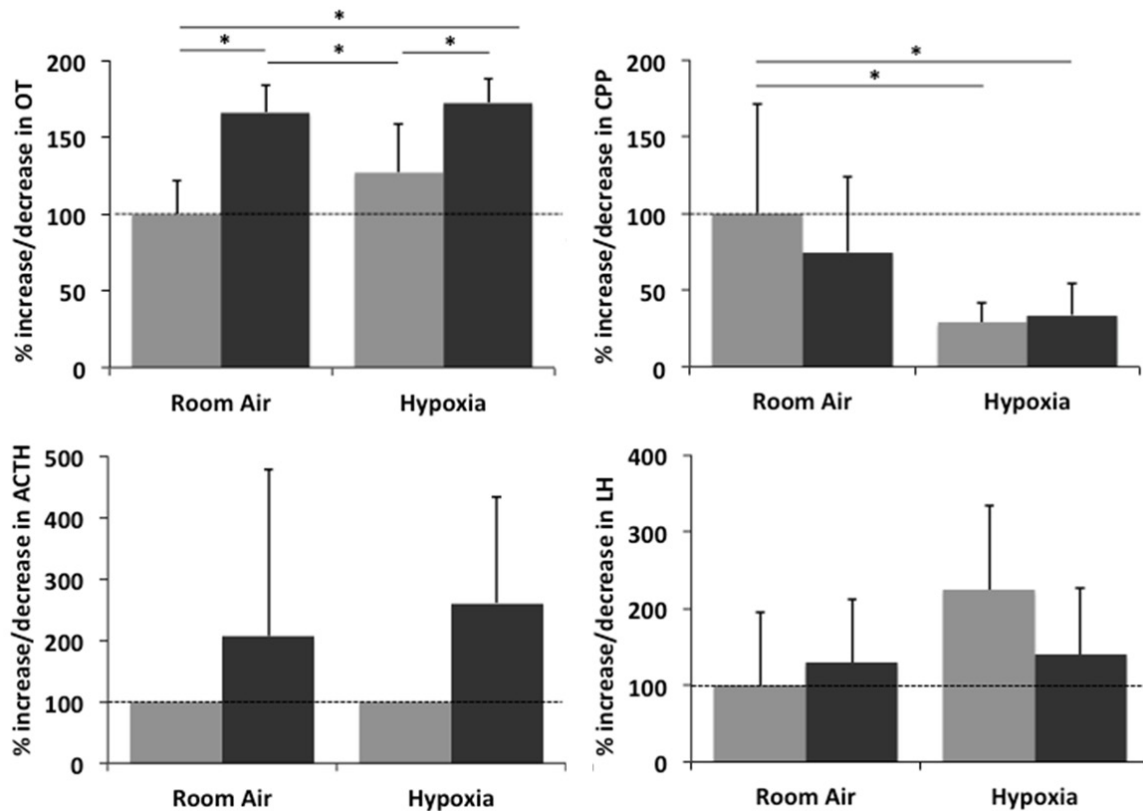


Figure 2. Comparison of serum concentrations of OT (upper left), CPP (upper right), ACTH (lower left) and LH (lower right) between the 4 groups. Dark columns and black columns indicate single and placing with estrous female rats, respectively. Values are means \pm SE, and every value shows the ratio of each hormone to single under room air (in % increase/decrease). *Significantly different between 2 groups ($P < 0.5$).

antibody, Histofine Simple Stain Rat MAX-PO multi (peroxidase-labelled goat-poly, anti-mouse and rabbit primary antibody, 414191F, Nichirei) was allowed to react for 30 min at RT. The Histofine Simple Stain AEC solution (Nichirei) was allowed to react with peroxidase as a substrate for 20 min at RT.

Statistical analysis

All analyses were performed using JMP version 6, Japanese edition (SAS Institute, Cary, NC).

The measurements were expressed as means \pm SE. To determine the independent and combined effects of hypoxia and sexual stimulation, comparisons between groups were performed by repeated two-way analysis of variance, followed by multiple comparison testing using the Tukey-Kramer procedure when at least one was significant in the independent and combined effects. Among all analyses, a *p* value < 0.5 was considered statistically significant.

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Table 2. mRNA expressions of 5 genes in hypothalamus after exposure to hypoxia and/or sexual stimulation

Gene	Means \pm SE				t (p)		
	RS	HS	RX	HX	H	X	H*X
<i>Oxt</i>	1.351 \pm 0.146	1.338 \pm 0.182	1.399 \pm 0.152	1.697 \pm 0.127	0.90 (ns)	1.29 (ns)	1.01 (ns)
<i>Avp</i>	1.487 \pm 0.192	1.881 \pm 0.348	2.089 \pm 0.281	2.463 \pm 0.268	1.40 (ns)	2.16 (0.0389)	-0.04 (ns)
<i>Slc6a4</i>	1.806 \pm 0.355	1.677 \pm 0.232	1.892 \pm 0.191	1.372 \pm 0.246	-1.18 (ns)	-0.35 (ns)	-0.71 (ns)
<i>Hspa4</i>	0.735 \pm 0.065	0.782 \pm 0.062	0.643 \pm 0.053	0.622 \pm 0.025	0.26 (ns)	-2.27 (0.0305)	-0.61 (ns)
<i>Hyou1</i>	0.772 \pm 0.064	0.893 \pm 0.114	0.641 \pm 0.036	0.592 \pm 0.025	0.56 (ns)	-3.08 (0.0045)	-1.23 (ns)

ns, not significant. H, hypoxia; X, sexual stimulation; RS, single under room air; HS, single under hypoxia; RX, sexual stimulation under room air; HX, sexual stimulation under hypoxia. *Oxt*, oxytocin; *Avp*, arginine vasopressin; *Slc6a4*, solute carrier family 6, member 4; *Hspa4*, heat shock protein 4; *Hyou1*, hypoxia up-induced 1.

Results

Serum concentrations of five hormones

Table 1 shows serum concentrations of five hormones in the four groups. Serum concentration of OT was significantly increased by either hypoxia or sexual stimulation, but not in the case of their interaction (p : hypoxia (H): 0.426, sexual stimulation (X): <0.001, interaction of hypoxia and sexual stimulation (H*X): ns). Comparing among the RS, RX, HS and HX groups, the OT concentrations in RX and HX were significantly higher compared with those in RS and HS (**Figure 2**). On the contrary, although serum concentration of CPP was significantly suppressed to less than 50% by hypoxia alone (**Figure 2**), it was not significantly changed by sexual stimulation, and there was no interaction of hypoxia and sexual stimulation (H: 0.04, X: ns, H*X: ns).

ACTH was secreted actively into the blood because of sexual stimulation alone (H: ns, X: 0.234, H*X: ns), whereas LH concentration in serum was significantly elevated under the hypoxic condition only (H: 0.424, X: ns, H*X: ns). However, there were no significant differences in the serum concentrations of ACTH and LH among the groups (**Figure 2**). Serum concentrations of FSH were not significantly changed by either hypoxia or sexual stimulation, or by both.

mRNA expressions of five genes

Table 2 shows mRNA expressions of five genes in the four groups. mRNA expression of *Avp* was significantly increased by sexual stimulation without the interaction of hypoxia and sexual stimulation (H: ns, X: .0389, H*X: ns); however, there was no significant difference in mRNA

expression of *Avp* among the groups (**Figure 3**). mRNA expressions of *Oxt* and *Slc6a4* were not significantly influenced by either hypoxia or sexual stimulation.

Sexual stimulation alone significantly suppressed mRNA expressions of both *Hspa4* (H: ns, X: .0305, H*X: ns) and *Hyou1* (H: ns, X: .045, H*X: ns) without the interaction of hypoxia and sexual stimulation. In particular, the expression of *Hyou1* was significantly suppressed by sexual stimulation under the hypoxic condition, although there were no significant differences in the expression of *Hspa4* among the groups (**Figure 3**).

Immunohistochemical analysis

Neither Hsp70 nor Orp150 was detected in any neurons or glial cells in the hypothalamus in the RS group. Moreover, neither Hsp70 nor Orp150 were expressed in the hypothalamus after sexual stimulation and/or exposure to hypoxia in the RX, HS and HX groups (data not shown).

Discussion

Sexual behavior is organized in two phases: the anticipatory phase, which includes motivation towards and searching for an adequate partner for copulation, and the consummatory phase, in which penile erection, seminal emission, and ejaculation characterise the male sexual response [20]. When sexual (visual, auditory, olfactory, tactile, and imaginative) stimuli come through the central nervous system, neural pathways are activated which convey sexual information from the higher centres of the brain to the genital apparatus to induce penile erection in the male through the spinal cord and the autonomous nervous system, enabling sexual

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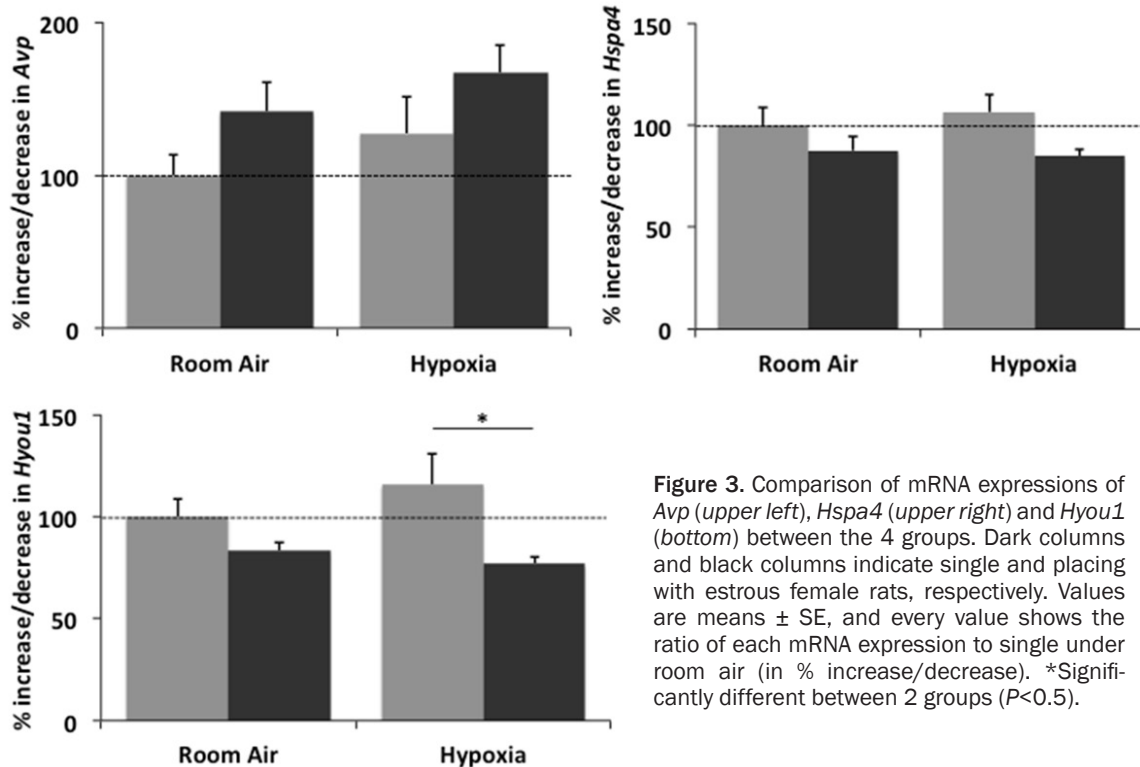


Figure 3. Comparison of mRNA expressions of *Avp* (upper left), *Hspa4* (upper right) and *Hyou1* (bottom) between the 4 groups. Dark columns and black columns indicate single and placing with estrous female rats, respectively. Values are means \pm SE, and every value shows the ratio of each mRNA expression to single under room air (in % increase/decrease). *Significantly different between 2 groups ($P < 0.05$).

intercourse, then culminating with orgasm. In this study, every experimental male rat placed with oestrous female rats would be in the anticipatory phase, which was defined as sexual stimulation.

OT is produced in the hypothalamus and secreted via the posterior pituitary gland [21]. It mediates uterine contractions during labour and milk ejection during lactation in mammals [21, 22]. OT also facilitates erectile function and sexual behaviour, including ejaculation in the male, as well [23, 24]. It has been reported that hypoxia induces elevation of serum OT concentration because of alteration of renal hemodynamics [25, 26]. In this study, even though mRNA expression of *Oxt* was not significantly changed by any stimulations, the serum concentration of OT was significantly increased by sexual stimulation but also by hypoxia. The OT elevation resulting from hypoxia could be caused by the temporal secretion of stored OT without *Oxt* up regulation. Additive/synergistic increase of OT secretion was anticipated by sexual stimulation under a hypoxic condition; however, OT secretion induced by sexual stimulation was suppressed by hypoxia. Thus, from the viewpoint of OT secretion, it is possible that

sexual arousal obtained by sexual stimulation would not be enhanced by hypoxia.

Regarding sexual activity, ACTH acts in the hypothalamus, and then induces penile erection and ejaculation [20]. In humans, however, it is controversial whether ACTH facilitates sexual activity because activation of the hypothalamic-pituitary-adrenal (HPA) axis by stress would inhibit the secretion of sex hormones such as oestrogen and testosterone [20, 27]. In this study, serum ACTH concentration was significantly increased by sexual stimulation, but it was not changed by hypoxia. Thus, the HPA axis activated by sexual stimulation would not be influenced by hypoxia. On the contrary, neither LH nor FSH, which are gonadotropins, was affected by sexual stimulation. Nevertheless, the serum concentration of LH was increased by hypoxia. As with the OT secretion, however, LH secretion induced by sexual stimulation was suppressed by hypoxia. The hypothalamus-pituitary-gonadal (HPG) axis also has crucial roles in reproduction and sex hormones [28, 29], but sexual arousal might not be enhanced by hypoxia at least. Considering the alterations of ACTH and LH, it is possible that reproductive function could be affected by sexual stimulation under hypoxic condition.

AVP is also produced in the hypothalamus, stored in the posterior pituitary gland and secreted in response to both increased plasma osmolality and decreased blood pressure levels [30]. Additionally, AVP might have facilitator effects on male sexual activity [23, 24]. Even though the elevation of CPP concentration resulting from hypoxia was anticipated [26], CPP was significantly decreased by hypoxia. In consideration of the up regulation of *Avp* mRNA expression, hypoxia might promptly induce hypermetabolism of AVP, resulting in the decrease of the absolute amount of AVP. Because AVP, along with corticotropin-releasing hormone, regulates ACTH in the HPA axis [31, 32], it is possible that the regulation of AVP might be influenced by alterations of HPA and HPG axes, as well as alteration of circulation dynamics, such as hypertension induced by hypoxia. At any rate, there was no evidence to suggest that hypoxia could enhance sexual arousal via AVP secretion, but, in reverse, it is possible that hypoxia would undermine male sexual activity by decrease of AVP. The effects of 5-hydroxytryptamine (5-HT)/serotonin are diverse, because stimulation of 5-HT₂ receptor impairs sexual functioning, but stimulation of 5-HT_{1A} receptor facilitates it [33, 34]. In this study, though we did not determine the concentration of 5-HT in the cerebral parenchyma or serum, mRNA expression of *Slc6a4*, which codes serotonin transporter, was not significantly changed by sexual stimulation under a hypoxic condition. Thus, it was still unclear whether hypoxia alters sexual arousal via secretion of 5-HT/serotonin.

Hsp70 and Orp150 are molecular chaperones: Hsp70 is transcriptionally activated for a short time after several stresses, and Orp150 is especially induced by hypoxia/ischaemia. They directly correlate with cytoprotection, reduced tissue damage and accelerated healing [35-40]. In this study, hypoxia changed neither expressions of Hsp70 and Orp150 proteins nor mRNA expression of *Hspa4* and *Hyou1*, which code Hsp70 and Orp150, respectively. Nevertheless, sexual stimulation significantly suppressed mRNA expressions of *Hspa4* and *Hyou1*, which indicates that sexual stimulation might lead to waning with a tolerance to acute hypoxia/ischaemia in brain tissue [36, 40].

The results of this study suggested that hypoxia might reduce sexual arousal induced by sexual

stimulation rather than enhancing it. Nevertheless, some behaviours to obtain sexual arousal, including hanging or smothering, tend to be more frequently and more excessively conducted, because it is possible that these behaviours themselves induce sexual arousal, which exceeds the reduction of sexual arousal by hypoxia, and thus enhancing sexual arousal. Then, when excessive cerebral hypoxia/ischaemia induced by hanging or smothering overwhelms the reduced tolerance to hypoxia/ischaemia, the individual would become unconscious, resulting in AEA/AED. Therefore, we propose that AED/AEA itself would not be a simple accident and would have some pathogenesis.

In conclusion, hypoxia would complicate the response to sexual stimulation. Moreover, AED/AEA could be attributed to a reduced tolerance to hypoxia/ischaemia induced by attempts to obtain sexual arousal under a hypoxic condition. These results imply that AED/AEA would have multiple and complex aetiologies.

Disclosure of conflict of interest

None.

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