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Hemorrhage- and Restraint-induced Analgesia in Male and Female Conscious Rats

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It is well known that stress induces analgesia. This study was designed to demonstrate the stress-induced analgesia by employing hemorrhage and restraint and to investigate its mechanism and sex difference. The degree of pain was assessed by measuring the magnitude of jaw opening reflex produced by a noxious electrical stimulation in the dental pulp and by measuring the latency to withdraw the tail from a heat ray. Restraint showed an antinociceptive response. A significant increase in pain threshold on bleeding was shown and the increase was larger in male group than in female group. The tail flick latency (TFL) on bleeding after AVP antagonist injection into the ventricle was decreased and the decrease was greater in male rats than in female rats. Castration resulted in a significant reduction of TFL. This effect was reversed by treatment with sex hormones. TFL was decreased during hemorrhage in castrated rats. This response was opposite to that in non-castrated rats. TFL was further decreased during hemorrhage after infusion of AVP antagonist, and there was a significant sex difference. These results suggest that both restraint and hemorrhage produce an antinociception and that, in hemorrhage-induced analgesia, AVP and sex hormones may play an important role and male rats show a greater analgesic response.

Key Words: Hemorrhage, Stress, Vasopressin, Sex difference, Gonadal steroid

INTRODUCTION

Living organisms are exposed to various kind of stress. Experimental designs to induce stress include electric stimulation, forced swimming, restraint, hemorrhage, and so forth. Analgesia is one of the common symptoms observed in various forms of stress. In 1976, three laboratories (Akil et al, 1976; Hayes et al, 1976; Rosecrans & Chance, 1976) simultaneously published on stress-induced analgesia. In addition to stress, analgesia can be induced by many other factors including increased arterial pressure and neuropeptides such as arginine vasopressin (AVP). Stress-induced analgesia can be evaluated with different methods yielding different results.

An interrelationship between blood pressure and pain regulation was suggested by Zamir & Shuber (1980). Both acute and chronic increases in arterial blood pressure have been associated with a decrease in nociception in humans and rats (Randich & Thurston, 1991). Hypertensive humans have greater tolerance to electrical tooth pulp stimulation than normotensive controls (Zamir & Shuber, 1980). Spontaneously or experimentally-induced hypertensive rats have elevated pain thresholds relative to appropriate normotensive controls (Zamir et al, 1980). These studies all suggest a link between the cardiovascular regulation and pain modulation. That is to say, the activation of high pressure baroreceptors by increased arterial blood pressure produced antinociception via activation of a spinopetal noradrenergic system (Thurston & Randich, 1990). The fall of blood pressure caused by hemorrhage may also be closely related to antinociception.

In the meantime, AVP is released into the systemic circulation from the neurohypophysis either in response to decreased blood volume and blood pressure

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sensed by the low pressure volume receptors and the arterial baroreceptors, respectively, or to increased plasma osmolality detected by brain osmoreceptors (Rocha et al, 1969; Andrews & Brenner, 1981). Generally, AVP-induced antidiuresis and vasoconstriction help to maintain normal plasma osmolality, blood volume and blood pressure during bleeding. That is, AVP is a neuropeptide against stress. Brain areas controlling arterial blood pressure contain AVP fibers (Weindl & Sofroniew, 1985). AVP-containing fibers are also found in the brain areas important in pain modulation (Millan et al, 1984), thereby implicating AVP as a possible modulator of nociception. Therefore it is suggested that hemorrhage causes a profuse secretion of AVP which mediates the hemorrhage-induced analgesia. However, few studies were done on the antinociceptive action of lowered arterial pressure.

Meanwhile, it was reported that sex differences exist in nociception and the perception of pain may be affected by the gonadal steroid environment (Beatty & Beatty, 1970). Sex differences in human responses to nociceptive stimuli and painful pathological conditions have generally indicated that women report higher pain levels or exhibit less tolerance than men for given stimulus intensities. Male rats also have been reported to show greater antinociception than female rats following systemic administration of morphine (Craft et al, 1996; Cicero et al, 1996, 1997) or following exposure to stress (Bodnar et al, 1988). Likewise, females receive more medications for pain-management practices (Raftery et al, 1995). However the assumption is still in debate as others (Katz & Criswell, 1996) report no sex difference in antinociception. If there is a gender difference, it may be due to sex hormones. Therefore castration has been commonly employed to investigate gender difference in antinociception.

Collectively, hemorrhage may induce antinociception but it is not clear that there is a gender difference in hemorrhage-induced antinociception although stress-induced antinociception in general is greater in males than in females. Therefore the purpose of the present study was to demonstrate the stress-induced antinociception caused by restraint and hemorrhage, to elucidate its mechanism, and to compare the responses in male and female rats.

METHODS

Animals and drug administration

Age-matched male (375~500 g) and female (240~300 g) Sprague-Dawley rats, which had free access to water and rat chow, were used. The drugs administered were [β-mercapto-β,β-cyclopenta-methylenepropionyl^1, O-Me-Tyr^2, Arg^8]-vasopressin, an AVP V_1-antagonist (5 μg/4 μl aCSF, i.c.v.), and sodium nitroprusside (100 μg/5 min, i.v.) which were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The vehicle for drugs microinjected intracerebroventricularly (i.c.v.) was artificial cerebrospinal fluid (aCSF) which contained (in mM) NaCl 128, KCl 3, $CaCl_2$ 1.2, $MgCl_2$ 0.8, NaH_2PO_4 0.25, $NaHCO_3$ 20, and glucose 3.4, and pH was 7.4.

Surgical procedures of vascular cannulation, access to the lateral ventricle, castration and insertion of EMG electrodes were performed 3 days before the experiment. Stress was induced with restraint according to Carr et al (1990) and with hemorrhage.

Bleeding and determination of the mean arterial pressure (MAP)

PE-50 tubes (Clay Adams, Parsipanny, NJ, USA) filled with heparinized (100 mU/ml) saline were cannulated into the both femoral arteries for measurement of MAP and for bleeding and the vein for nitroprusside administration under anesthesia with an intraperitoneal injection of mixed solution of pentobarbital (25 mg/kg) and urethane (625 mg/kg). The cannulas were sealed, exteriorized, and secured at the back of the neck.

In the morning of the experiment, the rat remained unrestrained in a plastic cage and the arterial line was connected to a pressure transducer (Statham P23ID, Grass Inst., Quincy, MA, USA) coupled to a polygraph (model 79, Grass Inst.). Heart rate was recorded through a tachograph (7P4B, Grass Inst.) triggered from arterial pressure signals. Bleeding was induced to lose approximately 20% of total blood volume for 5 min. Degree of nociception was accessed immediately after bleeding. MAP and heart rate were monitored continuously throughout the experiment.

Operation for the lateral ventricle

To inject the AVP antagonist i.c.v., operation for the lateral ventricle was performed as described by Park et al (1997). Briefly, under anesthesia with an intraperitoneal injection of mixed solution of pentobarbital and urethane, the lateral ventricle was cannulated by placing the rat on a stereotaxic device (model 1404, David Kopf Inst., Tujunga, CA, USA) and fixing the 22-gauge guide cannula (using the coordinates: 1.5 mm lateral, 0.8 mm caudal to bregma and 4 mm deep from the bone) with dental cement. A stainless steel obturator was used to seal the cannula. Penicillin (25 mg/kg, i.m.) was administered following the surgical procedures. After the animals were allowed to recover from surgery for at least 2 days, the experiments were performed under a conscious state.

Testing consisted of microinjecting AVP antagonist into the lateral ventricle and measuring nociception. The obturator was removed from the guide cannula placed into the lateral ventricle and replaced with an inner cannula (27-gauge) filled with the drug to be administered. The tip of the inner cannula extended 1 mm beyond the guide cannula. The other side of inner cannula was attached to a 25 μl Hamilton syringe through polyethylene tube (PE-20). At the end of each experiment, India ink was injected through the indwelling cannula and resultant staining of the walls of the third ventricle was used to confirm correct placement of the cannula.

Castration and replacement of gonadal steroids

Male and female rats were surgically castrated under pentobarbital (25 mg/kg) anesthesia. Animals were allowed to recover for two weeks. After two weeks, twelve castrated animals of each sex were treated with the respective steroid for one week. Testosterone cypionate was administered at a dose of 7.5 mg/kg/day and estradiol benzoate was administered at a dose of 100 μg/rat/day. In pilot studies, administration of a higher dose of the steroid did not influence the overall outcome of the experiments.

Jaw opening reflex (JOR)

To evaluate the degree of nociception, JOR was elicited by a noxious stimulation in the orofacial area and quantified by the integrated electromyogram (iEMG) in digastric or lateral pterygoid muscle. JOR design in this study employed a noxious electrical stimulation in the dental pulp and a subsequent recording of digastric iEMG. A pair of stimulating electrodes (150 μm in diameter) was inserted bilaterally into the lower incisor pulp. The electrodes were secured in place with dental acrylic resin. A pair of recording electrodes was inserted into the rostralmost aspect of anterior belly of the digastric muscle (distance 2~3 mm). Stimulating and recording electrodes were led subcutaneously to a miniature cranial connector sealed on the top of the skull with acrylic dental resin.

Electrical shocks (0.5~1.5 mA intensity, 200 μs duration, 0.5 Hz) were delivered to the dental pulp. Intensity of stimulation was adjusted to 2~3 times threshold for evoked EMG. At this range of stimulation and frequency, no consistent behavioral responses apart from the JOR arose. Electromyographic reflex responses were amplified (DAM80, WPI, Sarasota, FL, USA) and fed to a computerized system for on-line digitization (1401 plus, CED, Cambridge, UK). For each trial, control responses were determined throughout the 3 min preceding the test period (60 stimulations).

Tail flick latency (TFL)

As an alternative method for checking the antinociceptive degree, the latency to withdraw the tail from a heated ray (tail flick unit, UGO Basile, Italy) was measured. Each animal's tail was placed in contact with a radiant heat source that consisted of a 500-W projection bulb. The distal third of the tail rested over a 3-mm round aperture in the surface of the enclosure. Lateral displacement of the tail (approximately 5 mm) activated a photocell and an integrated digital timing circuit, which automatically deactivated the heat source. TFL response after restraint as shown in Fig. 1-A was measured using a uniform intensity of stimulation. In all other experiments, the intensity of heat ray applied to each rat was adjusted to a level that would produce a tail flick response latency of 7~8 sec in resting state. If the animal failed to respond within 20 sec, the trial was automatically terminated to prevent tissue damage, and a latency of 20 sec was recorded for that trial. Before the experiment, the rats were habituated to handling if the procedures for handling and testing influenced the results.

Statistical analysis

All data are presented as mean±S.E. Wilcoxon sign-ranks test was performed to assess the differences between rest and bleeding and Mann-Witney test was used to test sex difference. Significance was defined as a probability of less than 5%.

RESULTS

Restraint increased the TFL (sec) in response to a uniform intensity of stimulation from 7.5±0.2 to 10.9 ±0.3 in male group and from 7.4±0.2 to 10.6±0.3 in female group (Fig. 1-A). It showed stress-induced analgesia but no significant sex difference. TFL measured with different intensities of stimulation to obtain a similar level of tail withdrawal time was increased from 7.3±0.3 to 15.3±1.7 in response to bleeding and returned to 7.3±0.4 after retransfusion in male group, and the figures were 7.8±0.1, 11.7± 0.7, and 8.1±0.3 in female group (Fig. 1-B). Thus bleeding, which caused a drop of arterial pressure from 100.9±1.7 to 52.3±4.6 mmHg affected nociception in conscious rats. The increase in pain threshold on bleeding in male group was larger than in female group. Values of iEMG (mV · sec) were 341±42, 255±38, and 316±38 in male group and 366±39, 283±30, and 351±25 in female group (Fig. 1-C). Analgesia was produced but no difference

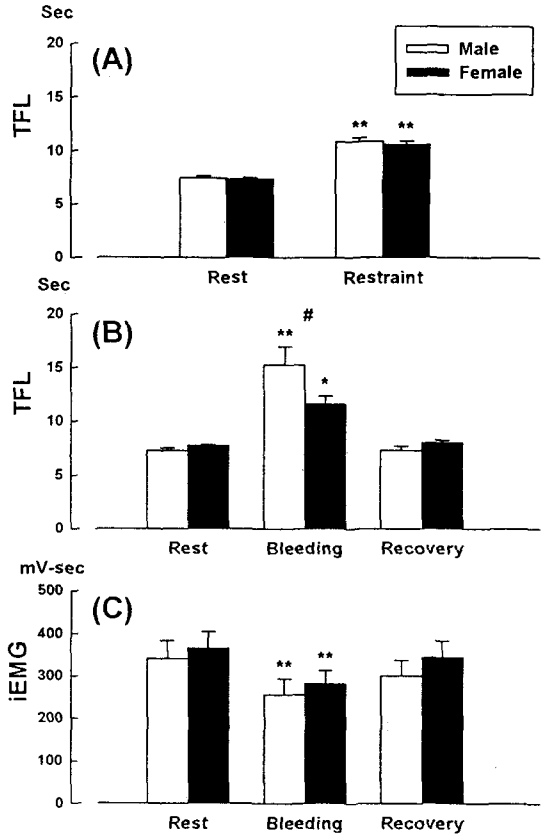

Fig. 1. Analgesic effect of stress (restraint or bleeding) in male and female rats. (A) Tail flick latency (TFL) after restraint. (B) TFL and (C) integrated digastric electromyogram (iEMG) in response to bleeding and retransfusion (recovery). Each bar represents the mean and one standard error of the mean. Number of experiments in A, B and C was 30, 9 and 11, respectively. * $p<0.05$, ** $p<0.01$ vs. Rest, # $p<0.05$ vs. Male.

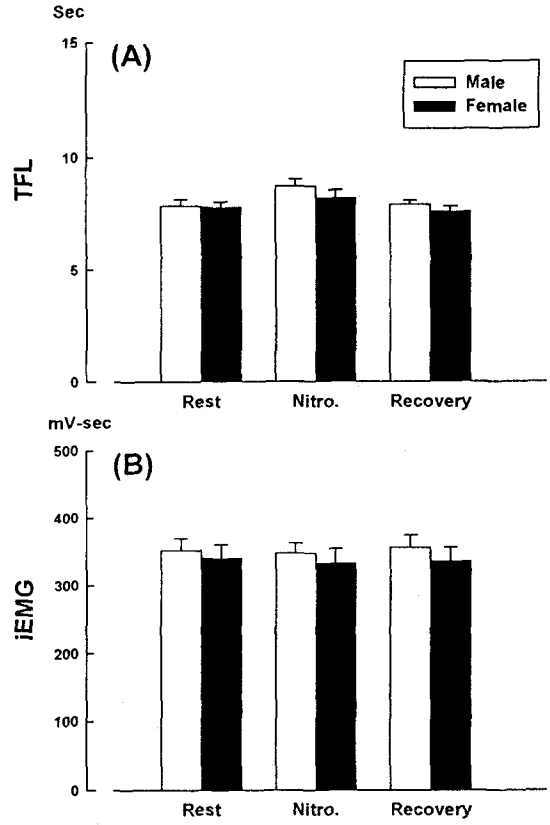

Fig. 2. Tail flick latency (TFL) and integrated digastric electromyogram (iEMG) in response to intravenous administration (100 μg) of nitroprusside (Nitro.) which lowered mean arterial pressure to 50 mmHg. Each bar represents the mean and one standard error of the mean. Number of each experiment was 7.

between male and female rats was evident in the magnitude of JOR during bleeding.

In order to investigate the mechanism of hemorrhage-induced analgesia, nitroprusside was injected intravenously to lower the blood pressure without causing a blood volume change (Fig. 2). The TFL in male group was 7.9±0.3, 8.7±0.3, and 7.9±0.2 and in female group was 7.8±0.3, 8.2±0.4, and 7.6±0.2 in the rest, nitroprusside injection and recovery phases (Fig. 2-A). The iEMG in male rats was 352±18, 347 ±15, and 355±19 and in female rats was 340±21, 332±21, 335±21 (Fig. 2-B). Thus no significant differences existed among the treatments or between two sexes.

To block the effect of increased release of AVP during hemorrhage, AVP V1-antagonist was injected i.c.v. before bleeding in conscious rats (Fig. 3). In male group, basal TFL without infusion of AVP antagonist was 7.8±0.3, and it was decreased to 3.9 ±0.4 on bleeding induced at 2 min after infusion of AVP antagonist, and returned to 7.9±0.4 with retransfusion. TFL in female group was also decreased from 7.8±0.3 to 6.5±0.2 and returned to 7.9 ±0.4 (Fig. 3-A). Bleeding after microinjection of AVP antagonist into the lateral ventricle produced greater nociception and a significant difference in TFL between male and female rats was produced. The

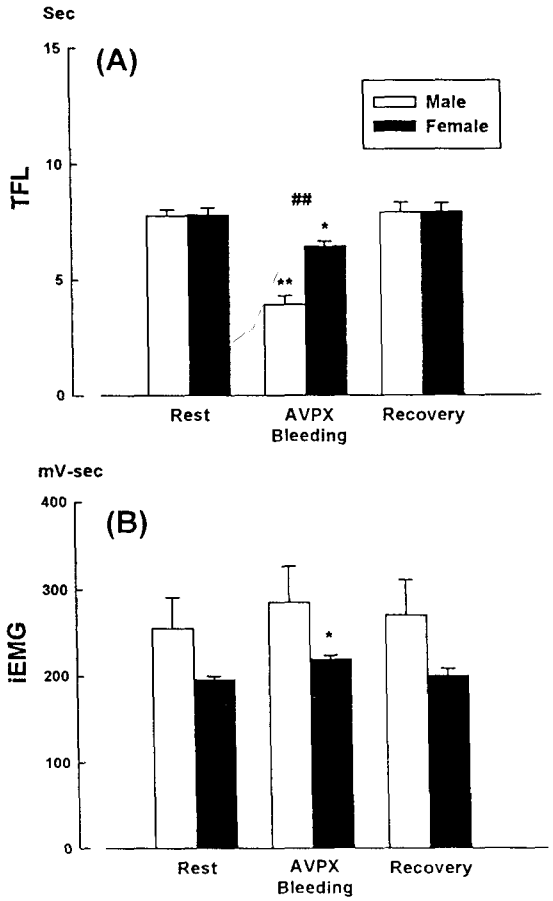

Fig. 3. Tail flick latency (TFL) and integrated digastric electromyogram (iEMG) in response to bleeding after intracerebroventricular administration (5 μg/4 μl) of AVP V1-antagonist (AVPX) and retransfusion (recovery). Each bar represents the mean and one standard error of the mean. Number of experiment was 10 (A) and 11 (B). * $p<0.05$, ** $p<0.01$ vs. Rest, ## $p<0.01$ vs. Male.

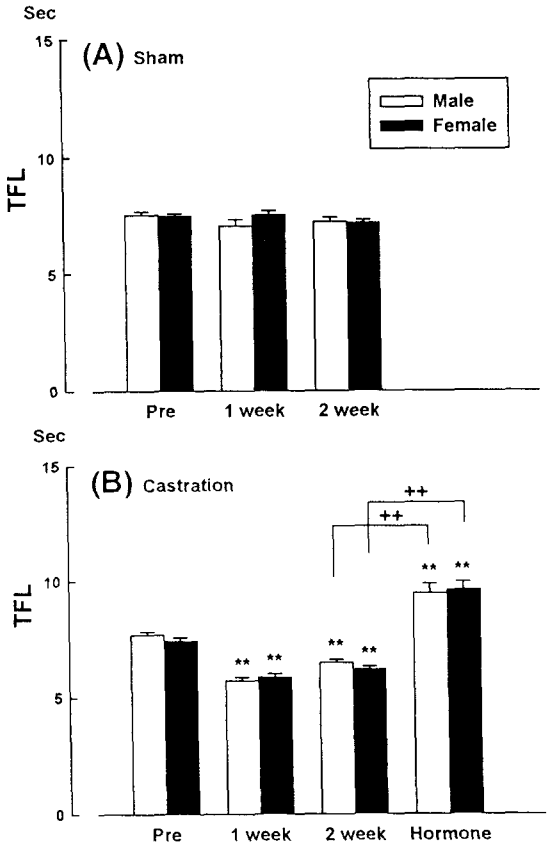

Fig. 4. Tail flick latency (TFL) of sham-operated (A) and castrated (B) rats. Castrated rats were treated with sex hormones for 1 week from 2 weeks after castration. Hormone injected to males and females was testosterone cypionate (7.5 mg/kg/day, s.c.) and estradiol benzoate (100 μg/day, s.c.), respectively. Each bar represents the mean and one standard error of the mean. Number of experiment was 9 (A) and 12 (B). ** $p<0.01$ vs. Pre, ++ $p<0.01$ vs. 2 week. Pre; pre-operation.

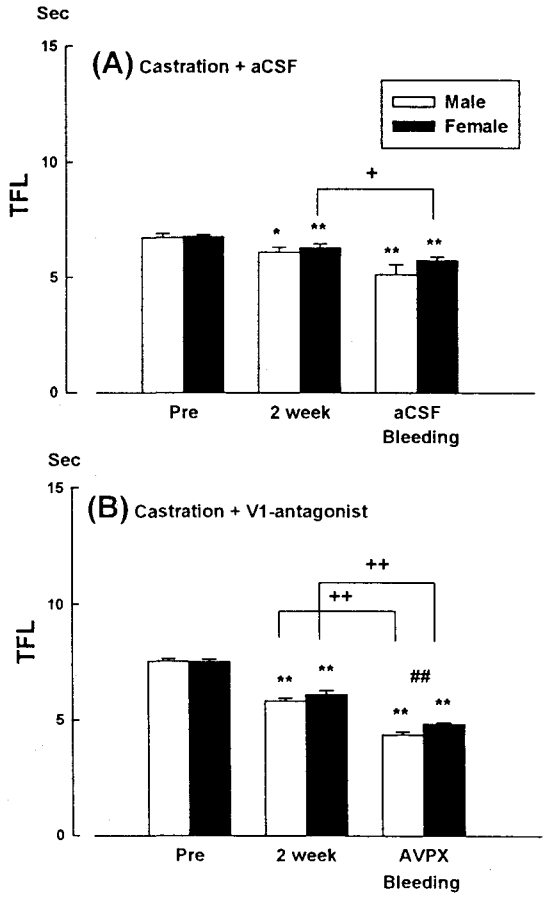

Fig. 5. Tail flick latency (TFL) on bleeding after intracerebroventricular administration of aCSF (4 μl) (A) or AVP V1-antagonist (AVPX, 5 μg/4 μl) (B) in castrated rats. Pre; pre-castration, 2 week; before injection of V1-antagonist at 2 weeks after castration. Each bar represents the mean and one standard error of the mean. Number of experiment was 10 (A) and 8 (B). ** $p<0.01$ vs. Pre, ## $p<0.01$ vs. Male.

iEMG was 255±36, 285±41, and 270±41 in male group, and 196±4, 219±5, and 200±8 in female group (Fig. 3-B). No significant differences between male and female group existed despite of some differences attributable to different intensities of stimulation.

In order to investigate whether sex hormones affected pain threshold, gonads were removed. In sham-operated rats, TFL was 7.5±0.2, 7.1±0.3, and 7.2±0.2 in male group and 7.5±0.1, 7.5±0.2, and 7.2±0.1 in female group before and 1 week and 2 weeks after operation, respectively (Fig. 4-A). There were no significant differences within and between the groups. In the castrated rats, TFL was decreased from 7.7±0.2 before operation to 5.7±0.2 and 6.5±0.2 at 1 and 2 weeks after operation, respectively, in the male group and from 7.4±0.2 to 5.9±0.2 and 6.2±0.1 in the female group (Fig. 4-B). Castration resulted in a greater nociception as the reaction time to hot heat ray diminished significantly during the two weeks following castration. But no sex difference was evident following castration. When sex hormones were injected to the castrated rats, TFL was increased significantly to 9.5±0.4 and 9.6±0.4 in males and females, respectively, without showing a significant sex difference. Thus castration significantly reduced the reaction time to painful stimulation and this effect was reversed by treatment with sex hormone.

TFL changes on bleeding were tested at 2 weeks after castration (Fig. 5). TFL was decreased from the pre-operation value of 6.7±0.2 to 6.1±0.2 in male group and from 6.8±0.1 to 6.3±0.2 in female group at 2 weeks after castration (Fig. 5-A). TFL on bleeding after injection of aCSF was 5.1±0.5 in male group and 5.7±0.2 in female group, which was still significantly lower than the respective pre-operation value (Fig. 5-A). This result, with the disappeared sex difference, presented a contrast to the increased TFL in response to the same amount of hemorrhage without castration. In another group of castrated rats, TFL was decreased from the pre-operation value of 7.6±0.1 to 5.8±0.1 in males and from 7.6±0.1 to 6.1±0.2 in females at 2 weeks after operation. The TFL during bleeding after injection of AVP antagonist was significantly ($P<0.01$) further decreased to 4.4±0.1 in males and 4.8±0.1 in females (Fig. 5-B).

DISCUSSION

Restraint stress experiment showed stress-induced analgesia. This result is consistent with Calcagnetti et al (1990) who observed that restraint stress potentiated the magnitude and duration of analgesia compared to unstressed rats. There was no sex difference in the restraint-induced analgesia in contrast to the clear sex difference shown in the bleeding experiment. This discrepancy might be due to the way in which stress was given (Cannon et al, 1984; Bell et al, 1998).

The TFL on bleeding, a kind of stress, was significantly increased compared to rest in both male and female rats. Despite no sex difference in antino-

ciception was demonstrated by iEMG, a significant difference in TFL was noticed between males and females. This result is consistent with the observation of Romero et al (1988b) who reported sex differences in opioid-modulated stress-induced analgesia. Gender-specific differences have also been reported in the reaction to somatic discomfort induced by footshock (Beatty & Fessler, 1976) and in opioid and non-opioid stress-induced analgesia (Ryan & Maier, 1988), with females being more affected than males. This result suggests that bleeding might partially modulate gender differences in pain sensitivity. The greater antinociception in male compared to female rats might be due, at least in part, to differences in the sex hormone and in the output of the rostral ventral medulla. The periaqueductal gray (PAG) and rostral ventromedial medulla (RVM) are parts of the well characterized system involved in the modulation of nociception (Basbaum & Fields, 1984). RVM neurons have been shown to project to the dorsal horn of the spinal cord (Fields et al, 1995) where the activity of nociceptive neurons are modulated (Rivot et al, 1980). This neural system in male rats might be different from that in females. The greater antinociception following microinjection of morphine into the RVM of male compared to female rats (Boyer et al, 1998) was consistent with the reported sex differences following systemic or i.c.v. administration of morphine (Cicero et al, 1996; Bartok & Craft, 1997). Although the role of difference in neural systems cannot be excluded, the attenuated antinociception following castration as observed in this study suggests an important role of sex hormone.

If gender difference is assumed to be mediated by sex hormone, sexual cycle is expected to be closely related with pain sensation in female rats. Actually pain threshold was reported to vary with menstrual stage: the lowest thresholds occurred in the periovulatory stage, whereas the highest thresholds always occurred in the luteal phase (Giamberardino et al, 1997). Ovariectomy abolished these fluctuations, and administration of estradiol prolonged the response times whereas progesterone had little effect (Martinez-Gomez et al, 1994). In contrast, Gear et al (1996) stated that no significant difference in analgesia was observed among females in different phases of the menstrual cycle. In order to eliminate the effect of sexual cycle, period of ovulation was determined based on vaginal smear and only the female rats within this period were used in this study.

While the iEMG was significantly decreased in male and female rats implying that pain threshold was increased during hemorrhage, there was no sex difference. This result may look inconsistent with the TFL response that was longer in males. The discrepancy might be due, at least in part, to the different integration sites of the two reflexes, TFL relayed within the spinal cord, while iEMG at a supraspinal level.

In order to investigate whether the changes in pain threshold were simply due to change of blood pressure, we tested nitroprusside, a smooth muscle relaxant, by intravenous injection. The result showed that a simple decrease in blood pressure did not affect pain threshold. The role of other factors such as increased vascular tone resulting from hemorrhage remain to be investigated.

Meanwhile, TFL on bleeding after AVP antagonist administration was significantly decreased. This result demonstrates that antinociception during hemorrhage is dependent, at least in part, on AVP. And the greater analgesic response in male rats suggests a greater effect of AVP in males than in females.

Both in castrated male and female rats, the withdrawal of gonadal steroids resulted in a shortened reaction time to heat ray. Moreover administration of gonadal steroids produced a significant increase in the TFL in both sexes. The present results are consistent with the finding of other investigators. For example, stress-induced analgesia was diminished by castration in female rats (Ryan & Maier, 1988) and castration of male rats resulted in an attenuation of stress-induced analgesia (Chatterjee et al, 1982). The reversal effect of sex hormone treatment was also consistent with the observation of Romero et al (1988a). The results obtained in this study may be attributed to the ability of gonadal steroids to modulate the activity of neurotransmitters and other neuropeptides which participate in antinociception (Basbaum & Fields, 1984) as well as to the effects of gonadal steroids on neuromuscular components.

The TFL on bleeding after castration was significantly decreased and showed a sex difference. It means that hyperalgesia was produced by bleeding after castration. This result is opposite to that in non-castrated rats. It remains to be investigated why the hemorrhage-induced analgesia and not the restraint-induced analgesia showed a sex difference and why the effect of AVP was greater in male rats.

In summary, both restraint and hemorrhage induced

an antinociception and AVP and sex hormones might play important roles in hemorrhage-induced analgesia.

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