FULL PAPER *Ethology*

Intracerebroventricularly Administered Oxytocin Attenuated Cortisol Secretion, but not Behavioral Responses, during Isolation in Holstein Steers

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ABSTRACT. In rodents, intracerebroventricular oxytocin administration attenuated hypothalamo-pituitary-adrenal (HPA) responses and anxiety behavior during stress. We examined the effects of intracerebroventricular injection of oxytocin on isolation-induced stress responses in cattle. In a methodological test, we determined the dosage of oxytocin applied in a main test which did not induce an increase in plasma cortisol concentration or stereotyped behaviors. In a main test, 5 steers aged from 199 to 250 days were assigned to the following three treatments randomly: T1, no isolation after injection of 200 μl of artificial cerebrospinal fluid (aCSF); T2, isolation after aCSF injection; and T3, isolation after 0.5 μg of oxytocin in 200 μl aCSF injection. The isolation was conducted by leaving the experimental steer alone in its stall for one hour while its peers were taken outside. In T2, the isolation induced a rapid increase in plasma cortisol concentration. The maximum %-changes from the pre-isolation value were significantly attenuated by oxytocin injection (T2 vs. T3, $p<0.05$). The isolation also induced an increase in the frequency (number of occurrences/1 hr isolation) of vocalizations and body orientation changes, and a decrease in the percentage of time spent lying and ruminating. The effect of oxytocin on these behavioral responses to isolation was not apparent. These results indicate that intracerebroventricularly injected oxytocin at low dose attenuated the cortisol response to isolation in steers while the effect on behavior was very small in this experimental condition.
KEY WORDS: behavior, cortisol, isolation, oxytocin, steer.

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In response to various stressors, oxytocin is released not only peripherally, but also intracerebrally to regulate pituitary-adrenocortical and behavioral stress responses [11]. Recent studies in rodents have shown that oxytocin suppresses the hypothalamo-pituitary-adrenal (HPA) response to stressors [11, 12, 18] and has anxiolytic properties [9, 19]. There are, however, few studies about the anti-stress effect of central oxytocin in animals other than rodents. In sheep, oxytocin infusions directly into the posterior pituitary, but not into paraventricular nucleus of the hypothalamus, suppressed cortisol responsiveness to an acute psychological stressor, a barking dog [4]. They, however, did not elucidate the anxiolytic properties of oxytocin because of their experimental design.

Recently, Ludwig *et al.* reviewed evidence that neuropeptides, such as oxytocin and vasopressin, are released from dendrites and diffuse to distant targets to exert long-lasting changes on behavior [8]. We tried to mimic this phenomenon by intracerebroventricular administration of oxytocin.

We used steers in the present study. The anti-stress effect of central oxytocin has not been elucidated in cattle. In our previous study in steers, intracerebroventricularly infused corticotropin-releasing hormone (CRH) or arginine vasopressin (AVP) activated HPA axis activity and induced stereotyped behaviors [22]. We suspected that the role of CRH or AVP in regulating stress responses may be different from that in sheep [21, 23]. Because oxytocin might suppress the activity of these neuropeptides that induce stress responses, the central role of oxytocin could be different between sheep and cattle.

In the present study, we examined the effects of intracerebroventricularly infused oxytocin in steers on adrenocorticotropic and behavioral responses to mild psychological stressor, isolation from peers, to elucidate its central roles in regulating stress responses.

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MATERIALS AND METHODS

Experimental procedures and care of the animals were approved by the Institute Committee for Animal Use and Care at the National Institute of Agrobiological Sciences.

Animals and surgery: Six steers (199 to 250 days old, 248–261 kg) were used in the methodological test and 5 of them were used in the main test. They were castrated at least 2 months before the experiment. After stereotaxic surgery, animals were individually reared in a stanchion stall in an experimental room. The animals were fed 2 kg of concentrate feed and chopped timothy hay twice a day at 09.00 and 16.00 hr. The amount of hay was changed according to the body weight of the steers to maintain 0.9 kg daily body weight gain. Water was available *ad libitum*.

More than one month before the experiment, an 18 gauge

stainless cannula (Eicom, Kyoto, Japan) was stereotaxically implanted into the third cerebral ventricle of each steer under isoflurane anesthesia according to a procedure reported earlier [7]. Briefly, the head of each steer was placed in a stereotaxic apparatus for calves [14]. The skin was excised and a small hole was drilled through the skull to the dura mater. An 18 gauge spinal needle attached to a micromanipulator was carefully lowered until the tip reached the lateral ventricle. One m*l* of radio-opaque material (Iopamiron 300, Nihon-Schering, Osaka, Japan) was infused slowly into the third ventricle via the lateral ventricle and a lateral X-ray was taken. According to the X-ray, the tip of the cannula was placed into the third ventricle through a midline puncture. Efflux of cerebrospinal fluid from the cannula was confirmed. The cannula was secured to the skull with acrylic dental resin and screws, and protected by a stainless cap, also fixed to the skull.

During the pre-experimental period, steers were continually tamed and allowed to adapt to their environment in order to minimize the impact of the stress of handling associated with intracerebroventricular infusions and blood sampling. On the day prior to the first experiment, each animal was fitted with an indwelling jugular catheter (Terufusion IVH catheter kit, Terumo, Tokyo, Japan) to collect blood samples.

Treatment solutions: The infusion vehicle was an artificial cerebrospinal fluid (aCSF: NaCl, 125 mM; KCl, 2.5 mM; NaH_2PO_4, 0.5 mM; Na_2HPO_4, 1.2 mM; $CaCl_2$, 1.2 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 27 mM) [6]. The pH of the medium was adjusted to 7.4 with 4–6 m*l* of 0.5 N HCl. For methodological tests, synthetic oxytocin (Peptide Institute, INC., Osaka, Japan) were dissolved in the aCSF solution at dose of 2.5, 25, and 250 μg/m*l* and divided into 0.5 m*l* aliquots, which were stored at –20°C before use. For the main test, 2.5 μg/m*l* was used.

Experimental procedure; *Methodological test*: Six animals were assigned to the following 4 treatments randomly at intervals of at least 2 days. Experiments were performed between 13.30 and 15.30 hr. At 14.00 hr (0 point of the experiment), the animal was lightly restrained with a rope and 0.5, 5, and 50 μg of oxytocin in 200 μl aCSF or 200 μl of aCSF was injected into the third ventricle via the implanted cannula at a rate of 200 μl/20 sec. Serial blood samples for measurements of plasma cortisol (CORT) concentrations were collected via indwelling jugular catheters at –30, 0, 10, 20, 30, 40, 50, 60, and 90 min.

Experimental procedure; Main test: Five animals were assigned to the following three treatments randomly at intervals of at least 2 days: T1, no isolation after injection of aCSF; T2, isolation after aCSF injection; and T3, isolation after 0.5 μg of oxytocin in 200 μl aCSF injection. The isolation was conducted by leaving the experimental steer alone in its stall for one hour from 30 to 90 min while its peers were taken outside instead of taking the experimental steer to another environment alone, which would have induced excessive reactions to human handling and a novel environment [3].

Experiments were performed between 13.30 and 16.00 hr. At 14.00 hr (0 point of the experiment), the animal was lightly restrained with a rope and 0.5 μg of oxytocin in 200 μl aCSF or 200 μl of aCSF was injected into the third ventricle via the implanted cannula at a rate of 200 μl/20 sec. Serial blood samples for measurements of plasma CORT concentrations were collected via indwelling jugular catheters at –30, 0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 120 min in T2 and T3.

Data analysis: Blood samples were taken into prechilled tubes containing EDTA sodium and stored on ice until the end of the experiment. After the experiment, the tubes were centrifuged and plasma samples were stored at –20°C until assay. The plasma CORT concentrations were obtained by enzyme immunoassay [15] using peroxidase-labeled cortisol (FKA403, 1:40,000 final dilution; Cosmo Bio Co., Ltd., Tokyo, Japan) and anti-cortisol serum (FKA404E, 1:70,000 final dilution; Cosmo Bio Co., Ltd., Tokyo, Japan). The standard curve ranged from 0.16 to 160 *n*g/m*l*, and the ED_{50} of the assay was 3.2 *n*g/m*l*. The intra assay coefficient of variation (CV) was 3.5 % at 24 *n*g/m*l*. The inter assay CV was 8.6 % at 24 *n*g/m*l*. The areas under the CORT concentration curve between 0 and 90 min after the injection were calculated for the methodological tests. In the main test, raw data for CORT was converted to percent-changes from the pre-infusion value (mean of -30 and 0 min).

Continuous behavior sampling was performed for 90 min after injection in the methodological test and for the 60 min isolation period in the main test using the videotaped record. In the methodological test, we observed maintenance behavior such as self-grooming, grooming and water access, and abnormal behavior such as rubbing, head shaking, abnormal licking, tongue playing and head-up posture. The percentages of time spent lying or ruminating for 90 min after the injection were calculated. In the main test, we observed vocalizations, body orientation change, head shaking and grooming, and categorized these behaviors as conflict behaviors. The total number of these behaviors and the percentage of time spent lying or ruminating for the 60 min isolation period were analyzed.

Statistical analysis: In the methodological test, the effects of time and treatments on CORT were analyzed using repeated statement of the SAS GLM procedure with animals and treatments as the sole source of variation in the whole plot, and time as the source of variation in the subplot. The differences in variation with time depend on whether the time-treatments interaction was significant or not. Moreover, after transposing the dataset with respect to each animal, the variations with time within each treatment were analyzed as a randomized blocks design of the SAS GLM procedure with animals and time as the main effect in the model. If there was a significant main effect of time, the statistical differences from the pre-injection value (–30 and 0 min) within each treatment were analyzed using the contrast statement of the SAS GLM procedure followed by calculations of the Scheffe F-value. Additionally, the areas under the CORT curve (AUC) were calculated from 0 to 120 min

with basal concentrations subtracted during each treatment. The AUCs were analyzed as a randomized blocks design using the GLM procedure of SAS with animals as the block, and treatments as the main effect in the model. If there was a significant main effect of treatments, the statistical differences among the treatments were analyzed using the contrast statement of the SAS GLM procedure followed by calculations of the Scheffe F-value.

In the main test, percent changes from the pre-injection value (means of –30 and 0 min) of CORT were calculated; these were statistically assessed by Friedman's test followed by a Nemenyi multiple comparison if there was a significant effect of time. The maximum %-changes in CORT during isolation period were also statistically compared between T2 and T3 by Friedman's test followed by a Nemenyi multiple comparison if there was a significant effect of treatment. In both tests, behavioral data were statistically assessed by Friedman's test followed by a Nemenyi multiple comparison if there was a significant effect of treatment.

RESULTS

Methodological test: Only 50 μg of oxytocin injection evoked an increase in CORT (Fig. 1A). There was a significant time-treatment interaction in CORT ($P<0.05$). Significant time effects ($P<0.05$) were seen with 50 μg oxytocin treatment. There was a significant main effect of treatment ($P<0.02$) on the AUC. The AUC response to 50 μg of oxytocin was significantly higher than those in response to aCSF ($P<0.05$) and 5 μg ($P<0.02$) of oxytocin. The AUC response to 50 μg of oxytocin tended to be higher than that in response to 0.5 μg of oxytocin ($P<0.06$) (Fig. 1B).

Table 1 summarizes data for maintenance and abnormal behaviors exhibited for 90 min after injection. There was a significant difference among the four treatments in the percentage of time spent ruminating (Friedman's test, $\chi^2=9.40$, p=0.022). The percentage of time spent ruminating after 50 μg of oxytocin injection was significantly less than that after aCSF injection (Nemenyi multiple comparison, p<0.02). There tended to be a difference among the four treatments in the total number of rubbings (Friedman's test, $\chi^2=7.14$, p=0.050). The total number of rubbings after 50 μg of oxytocin injection tended to be higher than that after aCSF injection (Nemenyi multiple comparison, p<0.1).

Main test: The isolation induced a rapid increase in the %-change of CORT (Fig. 2A). Significant differences in %-change of CORT between time were observed both in T2 and T3 (T2: Friedman's test, $\chi^2=25.27$, p=0.005, T3: $\chi^2=21.56$, p=0.018). In T2, the %-change of CORT at 40 min was significantly higher than those at 10, 20 and 30 min (Nemenyi multiple comparison, p<0.05). The maximum %-changes from the pre-isolation value in T2 (631.3 ± 387.1 %) was significantly attenuated by oxytocin injection (T3: 386.0 ± 273.0%) (Friedman's test, $\chi^2=5.00$, p=0.025; Nemenyi multiple comparison, p<0.05) (Fig. 2B).

Table 2 summarizes data for conflict behaviors and maintenance behavior exhibited for the 60 min isolation period.

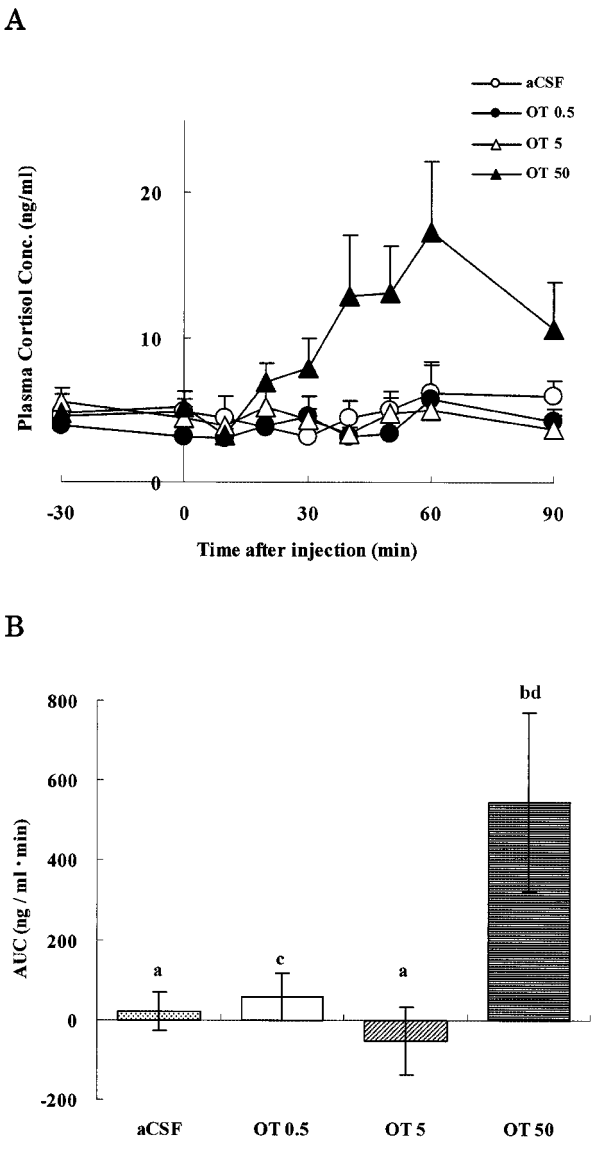

Fig. 1. The effects of intracerebroventricular injections of 200 μl of aCSF and 0.5, 5 and 50 μg of oxytocin in 200 μl aCSF on temporal changes in mean (+ SEM) plasma cortisol concentrations in steers (A) and the mean (± SD) area under the plasma cortisol concentration curve from 0 to 90 min after the injection (B). Different superscript letters indicate statistical differences (p<0.05: between a and b, p<0.06: between c and d).

There was a significant difference among the three treatments in the total number of vocalizations (Friedman's test, $\chi^2=7.60$, p=0.04), in the total number of body orientation changes (Friedman's test, $\chi^2=7.60$, p=0.04), and in the percentage of time spent lying (Friedman's test, $\chi^2=8.40$, p=0.024). The total number of vocalizations significantly increased by isolation (T1 vs. T2: 0 vs. 62.8 ± 30.7, Nemenyi multiple comparison; p<0.05). The total number of body orientation changes tended to increase (T1 vs. T2: 4.2

Table 1. Percentage of time spent lying or ruminating and occurrence of maintenance and abnormal behavior for 90 min after injection

		aCSF	Oxytocin 0.5 μg	Oxytocin 5 μg	Oxytocin 50 μg	χ^2	p
Lying (%)	Mean	56.2	49.6	56.8	32.5	3.80	0.338
	SD	13	19.9	25.5	19.1		
Ruminating (%)	Mean	42.5	34.4	32.2	4.6 *	9.40	0.022
	SD	13.9	13.9	23.4	10.5		
Self-grooming (No.)	Mean	6.8	17.3	13.0	13.0	5.48	0.132
	SD	7.2	6.2	16.1	3.9		
	Number of Animals exhibiting the behavior	(4/6)	(6/6)	(5/6)	(6/6)		
Grooming (No.)	Mean	2.2	2.0	1.2	1.7	0.84	0.864
	SD	4.0	3.2	2.0	3.2		
	Number of Animals exhibiting the behavior	(2/6)	(2/6)	(2/6)	(2/6)		
Water Access (No.)	Mean	1.8	1.3	5.7	6.2	5.32	0.144
	SD	1.7	2.4	6.8	5.7		
	Number of Animals exhibiting the behavior	(5/6)	(2/6)	(5/6)	(5/6)		
Rubbing (No.)	Mean	3.5	7.7	6.7	11.7†	7.14	0.050
	SD	3.8	6.7	5.2	6.4		
	Number of Animals exhibiting the behavior	(4/6)	(5/6)	(5/6)	(6/6)		
Head Shaking (No.)	Mean	3.0	0.7	2.0	2.5	0.33	0.870
	SD	2.5	0.8	2.7	2.9		
	Number of Animals exhibiting the behavior	(4/6)	(3/6)	(4/6)	(5/6)		
Abnormal Licking (No.)	Mean	10.8	11.0	9.2	14.5	0.60	0.913
	SD	6.9	10.1	9.3	10.9		
	Number of Animals exhibiting the behavior	(6/6)	(5/6)	(5/6)	(6/6)		
Tongue Playing (No.)	Mean	0.8	2.3	1.3	0.3	6.60	0.070
	SD	2.0	4.3	3.3	0.5		
	Number of Animals exhibiting the behavior	(1/6)	(3/6)	(1/6)	(2/6)		
Head-up Posture (No.)	Mean	15.3	6.5	3.3	4.5	3.36	0.362
	SD	14.2	7.3	4.5	4.0		
	Number of Animals exhibiting the behavior	(6/6)	(5/6)	(5/6)	(5/6)		

p: P-value obtained by Friendman's test.
*: Significantly different from aCSF (Nemenyi multiple comparison: $p<0.05$).
†: Tended to differ from aCSF (Nemenyi multiple comparison: $p<0.1$).

$\pm$ 1.6 vs. 25.0 $\pm$ 11.4, Nemenyi multiple comparison, $p<0.1$). No effect of oxytocin on the total number of vocalizations (T3: 53.0 $\pm$ 41.1) and body orientation changes (T3: 31.2 $\pm$ 9.8) was apparent. The percentage of time spent lying significantly decreased with isolation (T1 vs. T2: 59.5 $\pm$ 20.9% vs. 11.6 $\pm$ 10.8%, Nemenyi multiple comparison, $p<0.05$). There was no effect of oxytocin on the percentage of time spent lying (T3: 21.8 $\pm$ 21.4%). The percentage of time spent ruminating tended to decrease with isolation, but the differences among treatments were not significant (T1: 41.4 $\pm$ 19.6%, T2: 11.5 $\pm$ 25.7%, and T3: 10.3 $\pm$ 18.0%).

DISCUSSION

In the present study, intracerebroventricularly administered oxytocin at low doses of 0.5 and 5 μg/ 200 μl had no effect on the basal cortisol level, but 0.5 μg/ 200 μl of oxytocin significantly attenuated isolation-induced cortisol increase in steers. On the other hand, low doses of oxytocin did not affect either normal behavioral patterns, nor isolation-induced behavioral responses, i.e. excessive vocalizations, frequent changes of body orientation, and a relative decrease in lying and ruminating.

Intracerebroventricularly administered oxytocin might diffuse and stimulate oxytocin receptors widely distributed within the brain [2] because oxytocin has a longer half life in the brain (< 20 min) than in the blood (2 min) [10]. Recent studies have shown that oxytocin released from dendrite diffuses and acts at distant targets which in some cases are rich in oxytocin receptors but are innervated by few oxytocin neuron projections [8]. In the methodological study, 50 μg of oxytocin injection induced an increase in the plasma cortisol concentration, a reduction in the percentage of time spent ruminating, and increase in stereotyped rub-

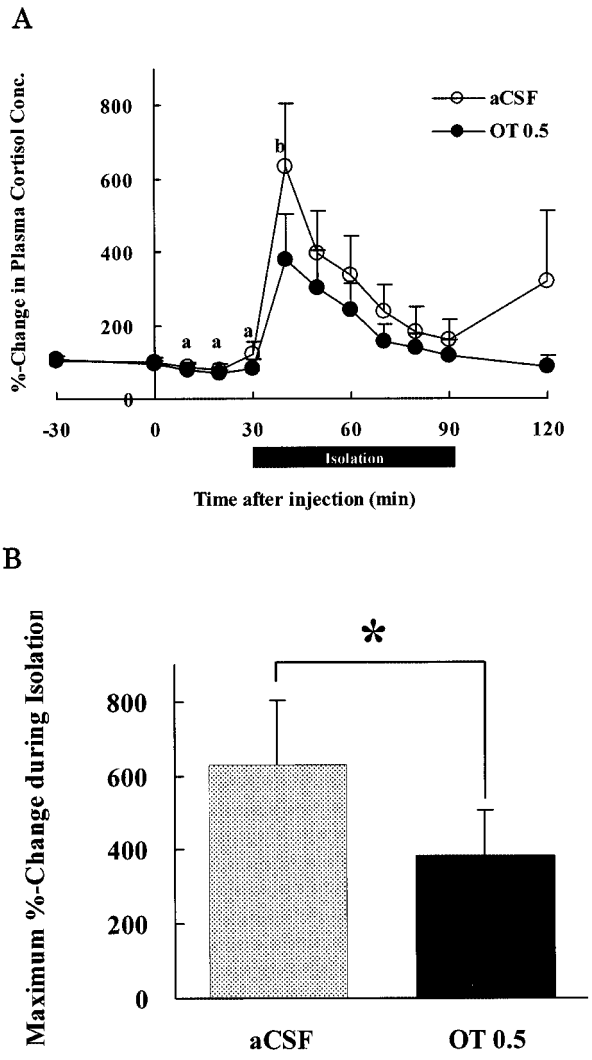

Fig. 2. The effects of intracerebroventricular injections of 200 μl of aCSF and 0.5 μg of oxytocin in 200 μl aCSF on isolation-induced temporal changes in mean (+ SEM) percent-changes of plasma cortisol concentrations from pre-injection values in steers (A) and the maximum percent change from pre-injection values during isolation (B). Different superscript letters a and b indicate statistical differences among times ($p<0.05$) and * indicates statistical difference between the treatments ($p<0.05$).

bing. Schlosser *et al.* [16] reported that the oxytocin-elicited increase in ACTH release is mediated via AVP V1b receptors in rats, whereas oxytocin affinity for the V1b receptor is one order of magnitude less than AVP. Moreover, the V1b receptor has been thought to be involved in the modulatory action of AVP on emotional processes [5]. We recently found that intracerebroventricular infusion of 20 nmol of AVP for 30 min induced increase in plasma concentrations of ACTH and cortisol and stereotyped behaviors including stereotyped head rubbing in steers [22]. Taking these findings into consideration, an excessive amount of oxytocin injected intracerebroventricularly might produce AVP-like effects through V1b receptors. Therefore, in the main test, we used a low dosage of oxytocin (0.5 μg of oxytocin in 200 μl aCSF) which did not induce an increase in CORT or stereotyped behaviors.

The effect of ICV oxytocin on attenuating the plasma cortisol increase in response to isolation was partial in the present study. Cook [4] reported that the plasma cortisol increase in response to a psychological stressor, a barking dog, attenuated to almost the basal level with one or 10 mg of oxytocin injection to the anterior pituitary, but not by direct infusion into the paraventricular nucleus of the hypothalamus (PVN) in lactating and non-lactating ewes. The quantity of oxytocin used in the present study was 0.5 μg, which was far less than in their study. In Holstein dry cows, we measured oxytocin concentrations in the third ventricle (our unpublished observations). Since the diurnal variation of the concentration was < 100 pg/m*l*, the dosage used in this study was closer to the physiological level than in their study.

The partial inhibition of the plasma cortisol response to isolation by oxytocin in this study may have been due to the lower estrogen influence in steers castrated before sexual maturation than that in females. In ovariectomized 17β estradiol benzoate-treated rats, low doses of oxytocin infusion (1 and 10 *n*g/hr) attenuated ACTH and corticosterone responses to 30 min restraint. Increased expression of CRH mRNA within PVN was also attenuated by oxytocin, which could be linked to the inhibition of c-fos expression in PVN, ventrolateral septum and the dorsal hippocampus [18]. More recently, Ochedalski *et al.* reported that the ability of central oxytocin to inhibit HPA axis activity depends on the levels of circulating oestradiol [13]. On the other hand, Neumann *et al.* reported gender-independent inhibitory effects of brain oxytocin on basal release of ACTH and stress-induced secretion of ACTH [12]. The effect of gonadal steroid on brain oxytocin function should be examined in future studies.

There may also be a sex difference in the anxiolytic effect of oxytocin. Reports on the anxiolytic effect of oxytocin were mainly studies in female rats [19] and mice [9]. The anti-anxiolytic effect of oxytocin knockout has not been confirmed in males [1, 20]. In addition, central oxytocin activity to attenuate anxiety response to a mild stressor might be under the strong regulation of gonadal steroid in mice and rats [17], suggesting a strong relation between oxytocin and gonadal steroids. No inhibition of behavioral response to isolation by oxytocin in this study also may have been due to lower estrogen influence in steers castrated before sexual maturation than that in females.

In the present study, intracerebroventricularly injected oxytocin attenuated cortisol response to isolation in steers, while the effect on behavior was very small in this experimental condition. These results suggest that the oxytocin system within the brain regulates HPA axis inhibition during stress, but that the system might not work to regulate behavioral responses to stress in this experimental condi-

Table 2. Percentage of time spent lying or ruminating and occurrence of conflict behaviors exhibited for the 60 min isolation period

		aCSF no isolation	aCSF isolation	Oxytocin 0.5 μg Isolation	χ^2	p
Lying (%)	Mean	59.5	11.6*	21.8	8.40	0.024
	SD	20.9	10.8	21.4		
Ruminating (%)	Mean	41.4	11.5	10.3	3.18	0.204
	SD	19.6	25.7	18.0		
Vocalization (No.)	Mean	0	62.8*	53†	7.60	0.040
	SD	0	30.7	41.1		
	Number of Animals exhibiting the behavior	(0/5)	(5/5)	(5/5)		
Body Orientation Change (No.)	Mean	4.2	25†	31.2*	7.60	0.040
	SD	1.6	11.4	9.8		
	Number of Animals exhibiting the behavior	(5/5)	(5/5)	(5/5)		
Head Shaking (No.)	Mean	1.6	1.8	4.2†	4.53	0.090
	SD	3.6	1.3	5.0		
	Number of Animals exhibiting the behavior	(1/5)	(4/5)	(5/5)		
Self-Grooming (No.)	Mean	8.0	3.4	4.8	0.33	0.870
	SD	8.3	2.5	4.1		
	Number of Animals exhibiting the behavior	(5/5)	(5/5)	(5/5)		

p: P-value obtained by Friendman's test.
*: Significantly different from aCSF no isolation (Nemenyi multiple comparison: $p<0.05$).
†: Tended to differ from aCSF no isolation (Nemenyi multiple comparison: $p<0.1$).

tion. The effect of gonadal steroid on these functions of oxytocin is still to be elucidated.

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