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Oxytocin levels in the plasma and cerebrospinal fluid of male rats: effects of circadian phase, light and stress

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Abstract

Oxytocin is involved in the regulation of reproductive and social behaviours, mood and stress responses. Previous work has indicated that oxytocin levels are regulated by circadian phase in brain tissue and plasma of both monkeys and rats, but in the cerebrospinal fluid (CSF) only of monkeys. We examined oxytocin levels in plasma and CSF of rats at two daily phases in darkness (mid subjective day and late subjective night) and after a 30 min exposure to light. We found that an apparent day–night difference in plasma oxytocin levels was eliminated by prior habituation to handling and injections. A previously reported daily oxytocin rhythm in rat plasma may instead reflect a rhythm of responsiveness to stressful experimental procedures. We also report for the first time that oxytocin levels in the CSF of rats are regulated by circadian phase and by prior exposure to light.

Keywords: Enzyme immunoassay (EIA); Sprague–Dawley rat; Neuropeptide; Entrainment; Handling; Injections

The levels of many hormones vary with circadian phase in either or both the general circulation and the cerebrospinal fluid (CSF) [5,19,20]. Rhesus monkeys, for example, show a daily rhythm in levels of oxytocin (OXT) and vasopressin in the CSF [13]. In rats, plasma and brain tissue levels of OXT rise during the light and decrease during the dark phase of a light–dark cycle, but this OXT rhythm has not been detectable in CSF [11,14,20].

We recently observed that OXT immunoreactivity in the tissue lining the third ventricle in the C57 mouse varies with time of day and in response to light exposure (unpublished observations). We interpreted these data to reflect rhythms in central OXT secretion in mice. We therefore attempted to assess whether CSF levels of OXT were affected by time of day and light, but using rats rather than mice because of their larger CSF volumes.

Adult male Sprague–Dawley rats (230–280 g; Charles River, Montréal, Canada) were housed singly under a 12-h light:12-h dark cycle. Phases of a light–dark cycle are defined conventionally as Zeitgeber Time (ZT), with ZT0 indicating time of lights on and ZT12 indicating lights off. The light was ∼300 lx in intensity and light pulses (see below) were 30 min in duration.

In Experiment 1, subjects were entrained to the lighting cycle for a week and were handled daily (at ZT0 or ZT9). The lights were prevented from coming on as usual at ZT0 on the day of the experiment for all animals to assess whether any observed rhythm was a response to light or internally generated. Four rats received a light pulse (LP) during ZT7-7.5 and were killed at ZT8 in darkness (group LP8). Other rats received no light exposure and were killed at ZT8 in darkness (group NL8, $n = 3$). CSF and blood were extracted at ZT8 from rats in both groups without exposing them to additional light. Two groups of rats ($n = 3$ each) were killed at ZT23 in darkness, either after a light pulse (ZT22-22.5; group LP23) or not (group NL23) and CSF and blood were extracted. OXT protein levels in the CSF (single samples) and plasma (in duplicate) were analyzed using an enzyme immunoassay (EIA) kit (Assay Designs Inc., Ann Arbor, MI, USA).

Because 200 μl of CSF was needed to run EIA samples in duplicate and each rat yielded only ∼100 μl of CSF, only a single measurement was available for each rat in Experiment 1. There were no significant differences in

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OXT levels among samples from rats in the same treatment group. Therefore, in Experiment 2 (see below) CSF samples from pairs of animals in the same treatment group were pooled to yield enough CSF to run undiluted duplicate samples for assessment of assay variability. Pooled CSF samples from each pair of rats were treated as a single sample for statistical calculations.

Experiment 2 included the same control and experimental groups as described for Experiment 1, but was run in two successive cohorts (final $n = 8$ per group); CSF samples from pairs of animals in the same groups were pooled. Experiment 2 differed from Experiment 1 in that, during the week of habituation to the lighting cycle and handling, rats were also habituated to injections by daily intraperitoneal (i.p.) saline injections at ZT0 or ZT9.

On the day of the experiment, rats were anesthetised (Ketamine/Xylazine, 80/10 mg/kg, i.p.) in darkness with the aid of an infrared viewer (NVS 300-Powerplus Goggle with Illuminator, Kennesaw, GA, USA). The eyes were protected with Lacrilube (Allergan Inc., Markham, Ont., Canada), then covered with black tape and velvet blindfolds to maintain darkness during CSF collection, for which bright light was required. CSF samples were collected by aspiration from the cisterna magna using a method described in detail by Consiglio and Lucion [2]; samples were stored at −80 °C for subsequent analysis.

The rats received no retinal illumination before receiving an anesthetic overdose (sodium pentobarbital, 70 mg/kg, i.p.) after CSF had been sampled. 0.5 ml of blood was collected from the heart and drawn into chilled Eppendorf tubes containing 0.5 mg EDTA. Blood samples were centrifuged (Brinkmann Eppendorf Centrifuge 5415; Mississauga, Ont., Canada) at 4 °C, 1400 × g for 15 min and the plasma was aliquoted and stored at −80 °C.

Instructions included in the OXT EIA kit were followed without modification, and OXT was not extracted from the plasma. Samples were thawed for assay and no sample was repeatedly frozen and thawed. Data output from a microplate ELISA reader were analyzed using SoftMaxPro (version 2.2.1; Sunnyvale, CA, USA). Data were analyzed with a two-way ANOVA (factors of time and light exposure) and group differences were considered significant when $P < 0.05$.

The acceptable range for the percentage coefficient of variation (CV%, a measure of variability between duplicate samples) varies among experimenters, but 10% is a common cut-off for the OXT EIA (personal communication, Dr. K. Kramer, March 18, 2002). We measured OXT in 45 duplicate samples of plasma; all samples had a CV% below 10% except for 2 in the NL8 group of Experiment 1, which had CV% of 16 and 21%. Among 16 duplicate samples of CSF in Experiment 2, 3 were above 10% (11, 12 and 17%). Including or excluding these data from the analysis did not affect the statistical significance of any tests used. All data collected were, therefore, used to calculate the results reported below.

The plasma OXT concentrations that we report are higher than those found previously using different methods, so we conducted a control study with known concentrations of OXT. The results indicated that the EIA kit had a detection sensitivity of 3.21 pg/ml. The intra-assay CVs for low (3.9 pg/ml), medium (500 pg/ml) and high (1000 pg/ml) concentrations of OXT were 7.54, 6.44 and 6.28%, while the inter-assay CVs were 14.87, 14.07 and 14.73%, respectively. The manufacturer has reported this kit to have very low cross-reactivity for other peptides, such as vasopressin and somatostatin. These results indicate that this method of measuring OXT is sensitive, specific and replicable. Previous studies using radioimmunoassays (RIA) to measure OXT required extraction of the peptide from the sample. Loss of some OXT in the sample during the RIA extraction process and the fact that we used a different antibody may account for the different levels reported.

In Experiment 1, there was a significant main effect of time ($F_{(1,11)} = 26.67$, $P = 0.001$), with OXT plasma levels significantly higher in the subjective-day sample (ZT8) than in the subjective-night sample (ZT23). There was no significant main effect of light exposure on plasma OXT levels and no significant interaction (Fig. 1A). These results are

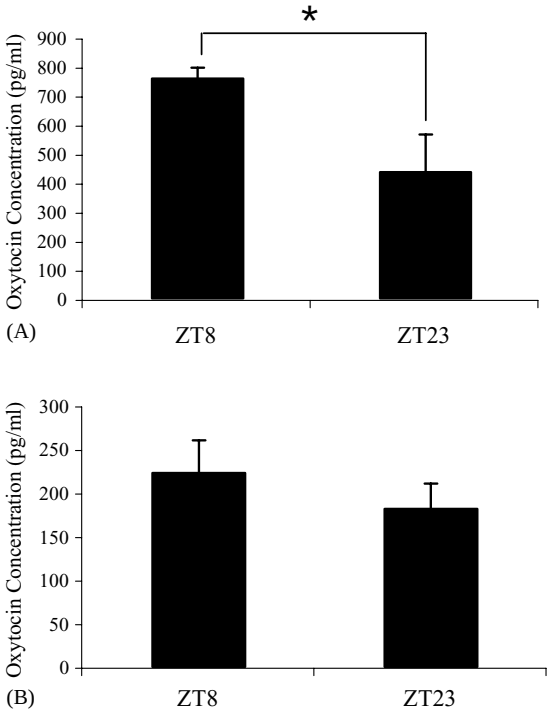

Fig. 1. (A) Plasma oxytocin levels at two circadian phases in rats that were not habituated to injections (Experiment 1). There were no significant effects of light exposure and no interactions, so data were collapsed across light exposure conditions. There was a significant main effect of circadian phase ($F_{(1,11)} = 26.67$; $^*P = 0.001$), with higher plasma oxytocin levels at ZT8 than at ZT23. (B) Plasma oxytocin levels in rats habituated to daily injections for a week before sampling (Experiment 2). There were no significant effects of circadian phase or light exposure and no significant interactions.

consistent with previous findings showing elevated plasma OXT levels during the day in rats maintained in a normal light–dark cycle or housed in constant light [20].

There were no significant differences between the data from the two replicate cohorts in Experiment 2 so these data were combined ($n = 8$ per treatment group). In Experiment 2, unlike the first study, day and night plasma OXT levels sampled in darkness did not differ significantly from each other (Fig. 1B). The only difference between the two studies was that rats in Experiment 2 were injected daily with saline for a week to habituate them to this procedure. This raises the possibility that the data from Experiment 1 reflect a response to the stress of the injections or to the responses of other animals to this procedure.

Windle et al. [20] did not report prior handling or habituation of their animals before decapitation to collect trunk blood for analysis of OXT levels, which suggests that their results, like those in our Experiment 1, might reflect the stress of the experimental procedures used to collect samples, and not simply circadian phase. This interpretation would require a very rapid increase in plasma OXT in response to stress; one study has confirmed rapid, significant increases in plasma OXT in response to restraint stress, within 2.5 min of the onset of stress [6].

Windle et al. [20] did not report whether rats were transported to another room before decapitation, which could itself be a stressor [16], or whether they were killed in the same room in which they were housed. Sharp et al. [17] have reported stress responses in rats that are exposed to the death of other rats, presumably through responses to ultrasounds or olfactory cues. Thus, the daily rhythm in 'baseline' OXT reported in Windle et al. [20] and in our Experiment 1 might actually reflect a rhythm in the amplitude of stress responses. Our findings support this interpretation in that we observed a day–night difference in plasma OXT only if animals were not first habituated to the anesthetic injection procedure.

Physical and pharmacological stress have both been shown to increase OXT levels in the plasma of rats [9,7,22], and OXT increases have been associated with a number of anti-stress effects, such as decreasing heart rate and corticosterone levels [4]. The adrenal cortex is strongly circadian in its physiology, and it is known to vary in its responsiveness to corticotrophin at different times of day in rats [8].

Acute stressors, such as footshocks, increase plasma corticosterone levels in rats when they are applied in the early light phase, but not at night [15]. After repeated exposure to footshocks over a number of days, they fail to elicit an increase in plasma corticosterone at any time point. These results are consistent with our interpretation that habituation to injections blunted a circadian phase-dependent stress response to these treatments on the day on which CSF and plasma OXT were measured (Experiment 2).

These data suggest that rats do not show a spontaneous daily rhythm of OXT in plasma, but rather that circadian phase modulates the extent of stress-induced OXT release into the circulation. Failure to attend to circadian phase-dependence of stress responses may contribute to variability in the results obtained in studies assessing the role of OXT in attenuating stress responses (see [4] for review).

OXT is made in the supraoptic and paraventricular nuclei (PVN) [4]. Blocking active transport with intracerebroventricular injections of colchicine reduces immunohistochemical staining of OXT around the third ventricle while increasing OXT staining in the PVN (unpublished observations). OXT is probably actively released into the CSF from dendrites originating in the PVN that contact the third ventricle [10].

OXT levels in CSF from parallel groups in Experiments 1 and 2 did not differ significantly from each other, so these data were pooled for analysis. We found a significant main effect ($F_{(1,24)} = 7.79$, $P = 0.011$) of circadian time, with higher OXT levels at ZT23 than at ZT8. This difference was consistent across these studies, indicating that it was not affected by habituation to the injection procedure, and did not represent a circadian phase-dependent stress response.

There was also a main effect ($F_{(1,24)} = 7.73$, $P = 0.013$) of light exposure, with light pulses decreasing CSF OXT levels significantly at both time points tested (Fig. 2). These results reflect a dissociation between mechanisms regulating plasma and CSF OXT levels that has been reported

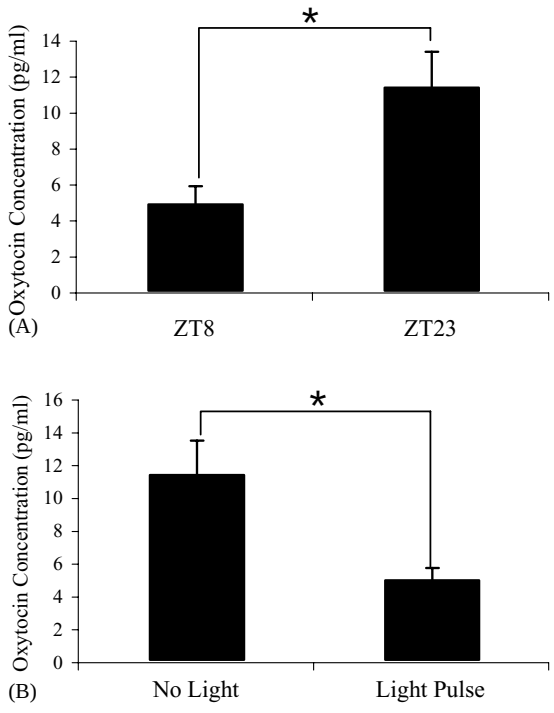

Fig. 2. (A) Oxytocin levels in cerebrospinal fluid at two circadian phases (Experiments 1 and 2). There was a significant effect of circadian time ($F_{(1,24)} = 7.79$; $^*P = 0.011$), with higher oxytocin levels in the late subjective night (ZT23) than during mid subjective day (ZT8). (B) There was also a significant effect of light exposure on oxytocin levels ($F_{(1,24)} = 7.73$; $^*P = 0.013$), with lower levels 30 min after exposure to a 30 min light pulse.

previously [1,4]. No previous study has, however, identified a stressor that releases OXT into the plasma but not the CSF, as we hypothesize occurred in Experiment 1. Injection stress has not been examined in this context before, and it is well known that different stressors cause different patterns of physiological responses [18]. While OXT is released into both the plasma and CSF in response to major stressors, like forced swimming, social defeat and novel environment stress [3,7,22], a milder stressor, such as an injection, may release OXT only into the plasma.

Rats usually avoid bright light [21], so in keeping with a role as a stress-responsive hormone, we would have predicted an increase in OXT in the CSF after light exposure. Instead, the light pulses caused significant decreases in CSF OXT at both phases studied. It is not known whether brief exposure to other mild stressors might have similar effects, or whether this decrease reflects a specific response to retinal illumination, just as melatonin levels decline in response to nocturnal light exposure [12]. A role for CSF OXT in responding to lighting conditions is supported by the observation that CSF OXT levels change when daily lighting cycles are altered [11,20].

OXT has been studied extensively in relation to various physiological and behavioural effects, but its effects on memory consolidation and pain tolerance are particularly striking [4]. Circadian and light-dependent differences in CSF OXT levels could affect the results of studies on pain or memory, and could contribute to discrepancies among studies in which these variables are not controlled. It remains to be determined how its regulation by light and circadian phase affect the many functions served by OXT.

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References

[1] J.A. Amico, S.M. Challinor, J.L. Cameron, Pattern of oxytocin concentrations in the plasma and cerebrospinal fluid of lactating rhesus monkeys (*Macaca mulatta*): evidence for functionally independent oxytocinergic pathways in primates, J. Clin. Endocrinol. Metabol. 71 (1990) 1531–1535.

[2] A.R. Consiglio, A.B. Lucion, Technique for collecting cerebrospinal fluid in the cisterna magna of non-anesthetised rats, Brain Res. Protoc. 5 (2000) 109–114.

[3] M. Engelmann, K. Ebner, R. Landgraf, F. Holsboer, C.T. Wotjakl, Emotional stress triggers hypothalamic but not peripheral release of oxytocin in rats, J. Neuroendocrinol. 11 (1999) 867–872.

[4] G. Gimpl, F. Fahrenholz, The oxytocin receptor system: structure, function and regulation, Physiol. Rev. 81 (2001) 629–683.

[5] G.H. Greeley, M. Morris, J.C. Eldridge, J.S. Kizer, A diurnal plasma vasopressin rhythm in rats, Life Sci. 31 (1982) 2843–2846.

[6] K. Hashimoto, K. Murakami, T. Takao, S. Makino, M. Sugawara, Z. Ota, Effect of acute ether or restraint stress on plasma corticotrophin-releasing hormone, Acta Med. Okayama 43 (1989) 161–167.

[7] T. Iványi, V.M. Wiegant, D. de Weid, Differential effects of emotional and physical stress on the central and peripheral secretion of neurohypophysial hormones in male rats, Life Sci. 48 (1991) 1309–1316.

[8] M. Kaneko, K. Kaneko, J. Shinsako, M.F. Dallman, Adrenal sensitivity to adrenocorticotropin varies diurnally, Endocrinology 109 (1981) 70–75.

[9] R.E. Lang, J.W.E. Heil, D. Ganten, K. Hermann, T. Unger, W. Rascher, Oxytocin unlike vasopressin is a stress hormone in the rat, Neuroendocrinology 37 (1983) 314–316.

[10] M. Ludwig, Q.J. Pittman, Talking back: dendritic neurotransmitter release, TINS 26 (2003) 255–261.

[11] W.B.J. Mens, A.D. Andringa-Bakker, T.B. Van Wimersma Griedanus, Changes in the cerebrospinal fluid levels of the rat during various light–dark regimes, Neurosci. Lett. 34 (1982) 51–56.

[12] R.Y. Moore, D.C. Klein, Visual pathways and the central neural control of a circadian rhythm in pineal serotonin *N*-acetyltransferase activity, Brain Res. 10 (1974) 17–33.

[13] M.J. Perlow, S.M. Reppert, H.A. Artman, D.A. Fisher, S.M. Seif, A.G. Robinson, Oxytocin, vasopressin and oestrogen stimulated neurophysin: daily patterns of concentration in cerebrospinal fluid, Science 216 (1982) 1416–1418.

[14] S.M. Reppert, W.J. Schwartz, H.G. Artman, D.A. Fisher, Comparison of the temporal profiles of vasopressin and oxytocin in the cerebrospinal fluid of the cat, monkey and rat, Brain Res. 261 (1983) 341–345.

[15] S. Retana-Marquez, H. Bonilla-Jaime, G. Vazquez-Palacios, E. Dominguez-Salazar, R. Martinez-Garcia, J. Velazquez-Moctezuma, Body weight gain and diurnal differences of corticosterone changes in response to acute and chronic stress in rats, Psychoneuroendocrinology 28 (2003) 207–227.

[16] I.A. Seggie, G.M. Brown, Stress response patterns of plasma corticosterone, prolactin and growth hormone in the rat following handling or exposure to novel environment, Can. J. Physiol. Pharmacol. 53 (1975) 629–637.

[17] J. Sharp, T. Zammit, T. Azar, D. Lawson, Does witnessing experimental procedures produce stress in male rats? Contemp. Top. Lab Anim. Sci. 41 (2002) 8–12.

[18] A.M.M. Van Erp, M.R. Kurk, W. Meelis, D.C. Willekens-Bramer, Effect of environmental stressors on time course, variability and form of self-grooming in the rat: handling, social contact, defeat, novelty, restraint and fur moistening, Behav. Brain Res. 65 (1994) 47–55.

[19] D.C. Wathes, V.J. Ayad, M. Kumari, E.L. Matthews, C.M. Wathes, A comparison of plasma vasopressin and oxytocin concentrations during the oestrus cycle of the ewe, J. Endocrinol. 134 (1992) 107–113.

[20] R.J. Windle, M.R. Forsling, J.W. Guzek, Daily rhythms in the hormone content of the neurohypophysial system and release of oxytocin and vasopressin in the male rat: effect of constant light, J. Endocrinol. 133 (1992) 283–290.

[21] R. Woodhouse, N. Greenfeld, Responses of albino and hooded rats to various illumination choices in a six-chambered alleyway, Percept. Mot. Skills 61 (1985) 343–354.

[22] C.T. Wotjak, J. Ganster, G. Kohl, F. Holboer, R. Landgraf, M. Engelmann, Dissociated central and peripheral release of vasopressin, but not oxytocin, in response to repeated swim stress: new insights into the secretory capacities of peptidergic neurons, Neuroscience 8 (1998) 1209–1222.