Hormones and Behavior 60 (2011) 239–243

Contents lists available at ScienceDirect

Hormones and Behavior

journal homepage: www.elsevier.com/locate/yhbeh

Urinary oxytocin as a noninvasive biomarker of positive emotion in dogs

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ARTICLE INFO

Article history:
Received 9 February 2011
Revised 9 May 2011
Accepted 10 May 2011
Available online 14 June 2011

Keywords:
Dog
Urinary oxytocin (OT)
Positive emotion
Radioimmunoassay

ABSTRACT

A reliable assay based on physiological parameters that does not require subjective input from the owners is required to assess positive emotions in dogs. In addition, when viewed from an animal welfare perspective, physiological parameters should be collected in a noninvasive manner. Oxytocin (OT) is a biomarker that may be associated with a calm, relaxed state, and positive emotion. We measured the time-lapse in the concentration of plasma OT relative to urinary OT using a radioimmunoassay with sufficient sensitivity and low variability, and examined the relationship between OT and cortisol. Six dogs were injected with exogenous OT intravenously to increase the blood OT concentration. As a result, the highest concentration of urinary OT occurred 1 h after the injection, although there was little change in urinary cortisol. Moreover, to evaluate the influence of stimuli on urinary OT and cortisol, we provided three stimuli of eating food, exercising and stroking, all of which were assumed to inspire a positive emotion in dogs, and significantly increased urinary OT concentrations. Our findings indicate that urinary OT might be useful as a noninvasive and objective biomarker of positive emotion in dogs.

Introduction

Many studies have examined the relationship between humans and dogs, and in particular the mental and physical health benefits that humans may experience while in these relationships. Although some of these studies have used behavioral characteristics, such as tail wagging and ear position (Abrantes, 1987; Quaranta et al., 2007), to assess a dog's positive emotion, measurement of physiological parameters would likely provide a more reliable assessment of emotion.

Odendaal and Meintjes (2003) measured plasma oxytocin (OT) in dogs as an indicator of their positive emotion. OT, which is composed of nine amino acids, is produced in the paraventricular nucleus and supraoptic nucleus of the hypothalamus and secreted from the posterior pituitary gland into the circulating blood (Buijs et al., 1983). OT is well known for its physiological role in smooth muscle contractions associated with parturition and the lactation of mammals (Ludwig, 1998; Russell, 1998). OT may also inhibit the hypothalamus-pituitary-adrenal (HPA) axis (DeVries et al., 2003; Neumann, 2000). Exogenous OT induces a decrease in basal plasma cortisol and ACTH in men (Legros et al., 1987, 1988). Previous studies have reported that various stimuli, such as sight (Strathearn et al., 2009), touch (Bello et al., 2008; Grewen et al., 2005; Matthiesen et al., 2001), smell (Hernandez et al., 2002), vocalization (Seltzer et al., 2010), food intake (Verbalis et al., 1986), and exercise (Kasting, 1988; Landgraf et al., 1982; Michelini, 2001), elicit the release of OT. Miller et al. (2009) suggested that the increases in OT would be related to a calm and relaxed state. Some researches (Fonberg et al., 1981; Kostarczyk and Fonberg, 1982; Okamoto et al., 2009) indicated that stroking could cause the relaxation with heart rate deceleration in dogs. Therefore, eating food, exercising and stroking might be good stimuli to inspire a positive emotion.

OT also has been a hormone that affiliated with social behavior. Plasma OT concentrations have been shown to increase in affiliative bonding between prairie voles (Gingrich et al., 1997), and the OT concentration in cerebrospinal fluid is positively correlated with social behaviour in rats and monkeys (Haller et al., 2003; Winslow et al., 2003). Moreover, OT plays an important role in stress relief (Bello et al., 2008), trust (Kosfeld et al., 2005; Witt et al., 1992), social attachment (Uvnäs-Moberg, 1997), social affiliation (Pedersen et al., 1988) and bonding (Carter, 2003; Lim et al., 2004; Panksepp, 1992). While examining the relationship between dogs and their owners, Nagasawa et al. (2009) demonstrated that there was a strong correlation between the frequency of interactions initiated by the dog's gaze and an increase in urinary OT concentration of the owners. These increases in OT concentration were associated with a high degree of satisfaction and communication with their dogs and an extended gaze from the dog (Nagasawa et al., 2009). Odendaal and Meintjes (2003) detected increased plasma OT concentrations in humans and dogs after positive interactions. These results indicate that OT contributes to social behavior, such as social contact or affiliative behaviors, even between different species.

Most of these studies measured the concentration of OT in samples of blood or cerebrospinal fluid, although the radiolabelled studies of

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0018-506X/$ – see front matter
doi:10.1016/j.yhbeh.2011.05.012

blood to urine excretion in human (Amico et al., 1987) and common marmoset monkeys (Seltzer and Ziegler, 2007) were reported. Noninvasive methods for measuring OT that are associated with naturally occurring behavioral events would be of benefit for further studies on the relationship between oxytocin and social behavior.

The aim of this study is to elaborate the time frame that excretes plasma OT into the urine and establish a connection between OT and cortisol in dogs. In this study two experiments were performed. The first experiment was designed to measure the time-lapse in the concentration of plasma OT relative to urinary OT by injecting OT intravenously and measuring the urinary concentration of OT using radioimmunoassay. The second experiment examined the relationship between OT and cortisol by the stimulus. Our goal was to assess the influence of positive emotion on urinary OT, and to establish the suitability of urinary OT as a noninvasive biomarker.

Materials and methods

Experiment 1

Animals

Six healthy Labrador Retrievers (males, 5.29 ± 0.95 years old, Table 1) that were not taking any prescription medication were used for this study. Five dogs were from a canine training center (Murase Dog Training Center, Kanagawa, Japan) and the other was a housedog. Intact male dogs were chosen to facilitate frequent urine sampling. All dogs could urinate at the direction of human.

Experimental procedure

Dogs were injected intravenously with exogenous OT through a lateral saphenous venous catheter four times every 5 min. Blood was also collected from the saphenous venous catheter. OT (Fuji Phama Co., Japan) concentration (500 pg/ml) was quantified followed the method of Amico et al. (1987). Although Amico et al. infused OT continuously using catheter for 1 h, it was difficult for dogs to move. If the exogenous OT was administered once and metabolized rapidly, we might have been not able to find the increase of plasma OT concentration. Therefore we injected 0.25 ml of OT 4 times every 5 min (0.25 ml × 4, total: 500 pg/ml), as outlined in Fig. 1. Dogs were demanded to keep quiet in their own cages after injection. Blood and urine samples were collected 30 min before injection (Resting: Time −30), immediately prior to injection (Time 0), immediately after the fourth injection (Time 15), 15 min after the fourth injection (Time 30) and every 30 min thereafter (Time 60, 90, 120, 150, 180, 210) using an indwelling needle. Immediately after collection, blood samples were transferred into vacuum blood collection tubes with EDTA and were centrifuged at 1600 × *g* for 15 min at 4 °C. Plasma and urine were frozen at −80 °C until analyzed.

Measurement of oxytocin

Plasma and urinary OT concentrations were measured by radioimmunoassay (Higuchi et al., 1985). Urinary samples were extracted using SEP-Columns containing C18 (WAT020515, Waters Corporation, USA). SEP-Columns were equilibrated by washing two times with 5 ml MeOH followed by four washes with 5 ml distilled water. Urinary samples (400 μl) and an equal volume of 0.1 N HCl were loaded into the equilibrated columns with a 5 ml syringe (SS-05SZ, Terumo, Japan). Columns were washed two times with 5 ml of 4% CH_3COOH and residual moisture was eliminated with a plunger. The peptide was eluted with 1.5 ml MeOH and the elutant was evaporated to dryness under a flow of nitrogen gas.

Phosphate buffer (100 μl, 0.01 M, pH7.4) containing 1% bovine serum albumin (A-8022, Sigma-Aldrich, USA), 50 μl standard solution of OT (4084-v, Peptide Institute, Japan) or sample, 50 μl anti-OT serum diluted with phosphate-buffered saline (pH7.4) containing 0.5% normal rabbit serum, and 50 μl ^{125}I-labelled oxytocin solution (approx. 10,000 c.p.m., NEX187, PerkinElmer, USA) were mixed in glass tubes (10 × 75 mm) and incubated at 4 °C for 72 h. Diluted sheep anti-rabbit IgG secondary antibody (50 μl, diluted 20-fold in 0.01 M phosphate buffer containing 1% bovine serum albumin, PA1-85601, Thermo Fisher Scientific, USA) was then added and the mixture was incubated at 4 °C for 72 h. The separation of free OT from OT bound to antibody was carried out by centrifugation at 1600 × *g* for 30 min at 4 °C. Radioactivity in the precipitate was counted with a gamma scintillation spectrophotometer (ARC-370, ALOKA, Japan) after the supernatant fluid was aspirated. Urinary OT levels are expressed as the OT to creatinine ratio.

Measurement of urinary cortisol

Urinary cortisol concentration was determined by enzyme immunoassay (EIA) as modified by a previous study (Mogi et al., 2008). The assay employed Cortisol-3-CMO-HRP (FKA 403, Cosmo Bio, Japan), Cortisol-3-CMO-BSA IgG (FKA 404-E, Cosmo Bio, Japan) and commercial cortisol standards (086-08241, Wako Pure Chemical, Japan). The EIA was completed in 96-well microtiter plates (3881-096, Asahi Glass Co., Japan) layered 24 h beforehand with goat anti-rabbit IgG (100 μl/well; diluted 400-fold in coating buffer; 0.05 M Na2CO3/NaHCO3, pH 9.6, 111-005-045, Jackson ImmunoResearch Laboratories, USA) and incubated overnight at room temperature. Non-bound antibody was removed from the wells of the plates with wash buffer (0.05% Tween 80, 169-24081, Wako Pure Chemical, Japan). The sample was diluted three times and 15 μl were used in the assay. The plate was read at an optical density of 405 nm using a microplate reader (Model 680, Bio-Rad Laboratories, USA). Urinary cortisol concentration was standardized using the concentration of creatinine.

Measurement of urinary creatinine

Creatinine concentrations were measured by the Jaffe reaction using 96-well microplates (3881-096, Asahi Glass Co., Japan). The plate was read at an optical density of 450 nm using a microplate reader.

Experiment 2

Animals

Nine dogs were used (Flat-coated Retriever, German shepherd dog, Golden Retriever, Jack Russell Terrier, Labrador Retriever, Miniature Schnauzer, Shiba, and 2 Standard Poodles, 3.17 ± 2.12 years old, Table 2) for this experiment; five were housedogs, and four were from Azabu University (Kanagawa, Japan). The dogs were healthy and took no prescription medication. All dogs could urinate at the direction of human.

Experimental procedure

To evaluate the influence of stimuli on urinary OT, the dogs were exposed to three stimuli of eating food, exercising and stroking. These are widely used as reinforcers that could cause pleasant sensations for dog training and inspire a positive emotion or feeling of safety. Drinking water was providing as a control condition. During the feeding, experimenters (a 30 year old male and two 21 year old females) offered dogs dry food that they consumed each day. The experimenters did not rush the dogs, they ate at their own pace until finished eating. For the exercising, dogs

Table 1
Profiles of the dogs at Experiment 1.

Individual	Breed	Sex	Age
A	Labrador retrievers	Male	6.8
B	Labrador retrievers	Male	4.7
C	Labrador retrievers	Male	4.8
D	Labrador retrievers	Male	4.1
E	Labrador retrievers	Male	5.3
F	Labrador retrievers	Male	6