OXYTOCIN AND SOCIAL BEHAVIOUR IN DOGS AND OTHER (SELF-)DOMESTICATED SPECIES METHODOLOGICAL CAVEATS AND PROMISING PERSPECTIVES

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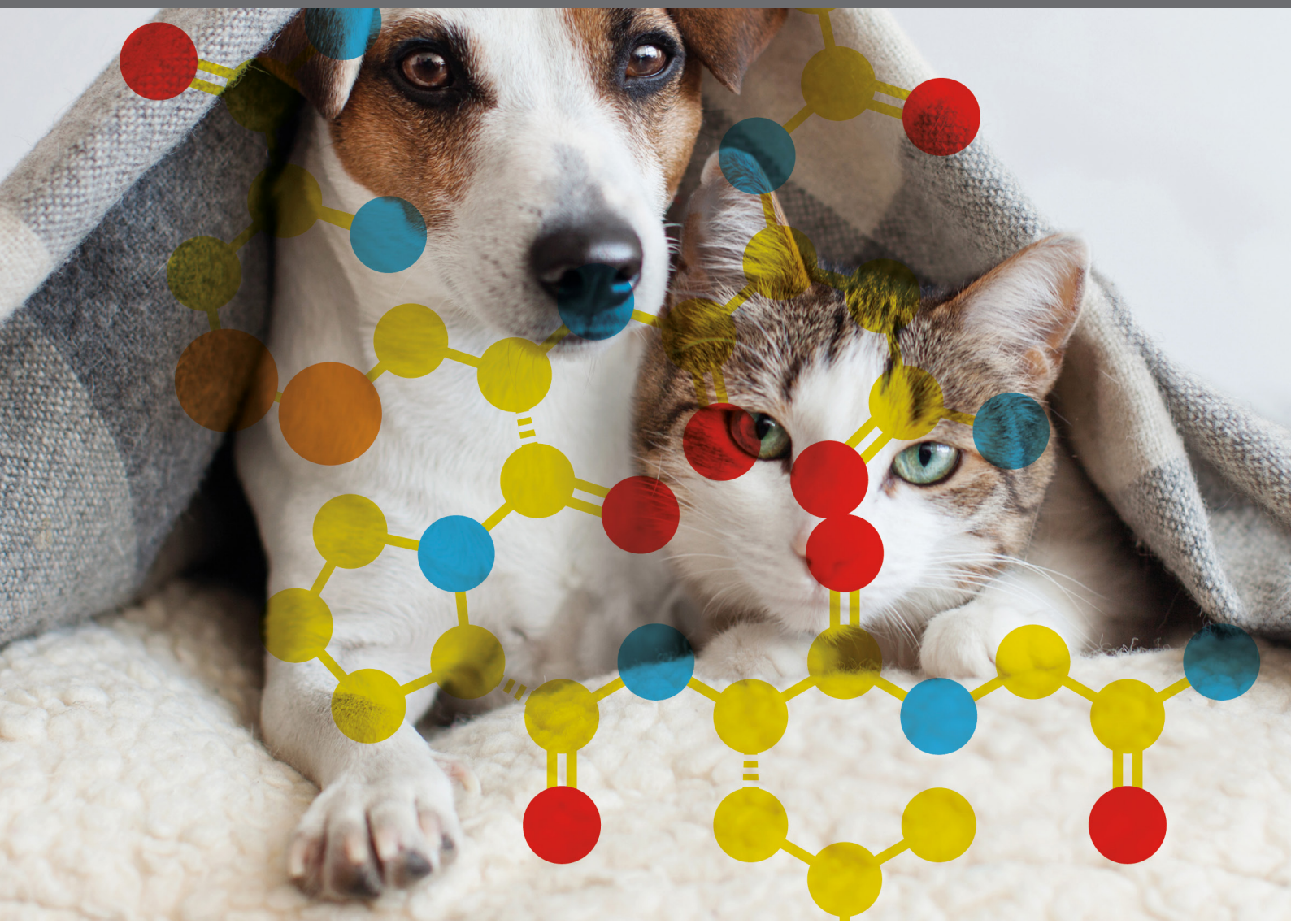

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OXYTOCIN AND SOCIAL BEHAVIOUR IN DOGS AND OTHER (SELF-)DOMESTICATED SPECIES METHODOLOGICAL CAVEATS AND PROMISING PERSPECTIVES

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Studying the relationship between different aspects of social behaviour and the oxytocin system in nonhuman animal species is a promising research area which may also have translational relevance for understanding the neuro-hormonal bases of human social cognitive abilities. In order to advance our understanding of social-behavioural effects of oxytocin, this Research Topic eBook collects together contributions from researchers in social cognition and related fields, whose work addresses cutting-edge questions and important gaps in our knowledge of the behavioural effects of oxytocin in dogs and other domestic species.

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Table of Contents

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EDITORIAL
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Editorial: Oxytocin and Social Behaviour in Dogs and Other (Self-)Domesticated Species: Methodological Caveats and Promising Perspectives

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Keywords: domestic species, oxytocin, social behavior, dog, intranasal administration, gene-behavior associations

Editorial on the Research Topic

Oxytocin and Social Behaviour in Dogs and Other (Self-)Domesticated Species: Methodological Caveats and Promising Perspectives

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Over the past decade the oxytocin system has become a focus of attention for researchers from various fields studying mechanisms underlying different forms of social behavior. Some have even suggested that it is the neurohormone, oxytocin, that has had the most permissive role in the evolution of the human nervous system (Carter, 2014), implying that *Homo sapiens* could not have evolved without it, as the success of this species highly depends on social behavior and cognition. Not surprisingly research into model systems of human social behavior has followed this trend including several discoveries on the relatedness of numerous forms of domestic species' social behavior and their respective oxytocin systems. This is particularly interesting as domestic species are known to have adapted to the human social environment in evolutionary terms, however the proximal and distal mechanisms underlying behavioral parallels between humans and domestic animals still remain largely unexplored.

Among domestic species, dogs are the most studied model of human behavior, and their human-analog socio-cognitive skills have been well-established both at the behavioral (Miklósi and Topál, 2013) and at the neural (Bunford et al., 2017) level. This bias in favor of dogs is also present in the number of research papers published about oxytocin and social behavior, as considerable amount of information has already accumulated about this species over the past few years (reviewed in Kis et al., 2017). The aim of this special issue was to fill in gaps not only for canine oxytocin research, but also for research on other domestic species. An important critical review article by Rault et al. presents literature on dogs, pigs, cattle, and sheep focusing on welfare aspects and outlines both problems and possible solutions for oxytocin research. The other 16 articles in the special issue present original research that keep up with the high methodological standards and present valuable data that the field had thus far been missing.

These include, first of all, research on non-canine domestic species. Bienboire-Frosini et al. present a reliable immunoassay measure of peripheral (plasma) oxytocin in cat, dog, horse, cow, pig, sheep, and goat. Arahori et al. focus on domestic cats and describe microsatellite polymorphisms adjacent to the oxytocin receptor gene revealing moderate associations with owner-rated personality traits.

The remaining 14 original research papers present data on domestic dogs' oxytocin system and social behavior. These include research using various methodological approaches: gene × behavior associations, intranasal oxytocin administration, and peripheral oxytocin measurements. Among the genetic studies is one (Cimarelli et al.) that presents a considerable methodological advance in the field, introducing an epigenetic study and presenting, for the first time, evidence that oxytocin receptor gene (OXTR) methylation is associated with social behaviors of pet dogs. A similarly important conceptual novelty is the introduction of quantitatively measured environmental factors (OXTR polymorphisms in the owners' gene; Kovács et al.) as well as contextual and individual characteristics (Turcsán et al.) into canine OXTR research. Both of these studies highlight significant additional factors to genetic research into dogs' oxytocin system, although at present have only tested these on one specific breed, the Border collie. The importance of breed differences, on the other hand, is highlighted by another paper (Kubinyi et al.) that presents incremental research about the relationship between OXTR polymorphisms and greeting behavior. Building on their previous research on Border collies and German shepherds the authors show a similar relationship in Siberian huskies. Direct comparison with human subjects (infants) is carried out by Oláh et al. investigating gaze-following as a function of OXTR polymorphisms.

The special issue also includes significant new research using intranasal administration of oxytocin (IN-OT) for dogs. While the literature of the field seems to be biased toward reporting of positive results, an important negative finding is highlighted here by Thielke et al., in a study investigating the effects of oxytocin administration on dogs' attachment behavior toward their owners measured via the strange situation test. Results show that contrary to expectations, intranasal administration of oxytocin fails to increase owner-directed proximity and contact seeking, rather it decreases such behaviors (in the baseline phase). A very interesting pair of papers by Somppi et al. and Kis et al. used eye-tracking technology to assess how oxytocin administration modulates dogs' viewing of human faces with different emotional expressions. The two research groups independently carried out studies using the same set of stimuli, and while the general conclusion from both is that there is an effect of oxytocin on the outcome measure, the specifics of the results differ by several points. The special issue also includes a paper presenting important incremental research using IN-OT methodology (Nagasawa et al.), which shows that previous results of the same research group about enhanced gazing behavior following oxytocin treatment can be conceptually replicated in ancient Japanese breeds (Shiba, Kai, and Shikoku).

Studies about canine peripheral oxytocin levels in this special issue include methodological improvements as well as the assessment of different forms of dog-human interaction. Temesi et al. describe the time-course of intranasally and intravenously administered oxytocin on serum and urine oxytocin concentrations by directly comparing these measures in Beagle dogs. MacLean et al. validate their salivary oxytocin measure by comparing it to plasma measures of the same Labrador retriever and Labrador retriever × Golden retriever dogs before and after a free-form social interaction with a human versus control treatment (resting). Two of the papers (Petersson et al.; Rossi et al.) measure both oxytocin and cortisol levels from dogs' blood samples and find that the two hormones are related to behavior during their respective tests. Another paper by MacLean et al. connects to the applied value of oxytocin research focusing on dogs' aggressive behavior: while dogs with and without reported history of aggression only differ in plasma vasopressin levels (and not oxytocin), an interesting difference is found between pet dogs and assistance dogs (that have been bred for affiliative and non-aggressive temperaments), with the latter group having higher oxytocin levels.

In our view the papers of this special issue present a considerable advancement in the field of oxytocin research in domestic species. While the independent research papers collected here use varying methodology and address independent scientific questions, they all nicely tie to the conceptual and methodological gaps that have been highlighted. Studies including non-canine domestic species, as well as different breeds of dogs inform us about both the specificity and the generalizability of oxytocin effects. Conceptual replications and incremental research presented here ensure the robustness of the findings. The novel methodological approaches as well as conceptual innovations described in this issue broaden the scope of the field. Furthermore, the open reporting of negative and controversial findings guarantees transparency of research. The papers of this issue are all good examples for this, and thus together strengthen the view that domesticated animals serve as valuable models for investigating the interrelatedness of social behavior and the oxytocin system.

AUTHOR CONTRIBUTIONS

AK drafted the first version of the manuscript. All authors read and commented the manuscript as well as contributed to its content and approved of the final text.

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MINI REVIEW
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Oxytocin as an Indicator of Psychological and Social Well-Being in Domesticated Animals: A Critical Review

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Oxytocin is often portrayed as a hormone specific to social behavior, reflective of positive welfare states, and linked to mental states. Research on oxytocin in domesticated animal species has been few to date but is rapidly increasing (in dog, pig, cattle, sheep), with direct implications for animal welfare. This review evaluates the evidence for the specificity of oxytocin as an indicator of: 1. Social, 2. Positive, and 3. Psychological well-being. Oxytocin has most often been studied in socially relevant paradigms, with a lack of non-social control paradigms. Oxytocin research appears biased toward investigating positive valence, with a lack of control in valence or arousal. Oxytocin actions are modulated by the environmental and social contexts, which are important factors to consider. Limited evidence supports that oxytocin's actions are linked to psychological states; nevertheless whether this is a direct effect of oxytocin *per se* remains to be demonstrated. Overall, it is premature to judge oxytocin's potential as an animal welfare indicator given the few and discrepant findings and a lack of standardization in methodology. We cover potential causes for discrepancies and suggest solutions through appropriate methodological design, oxytocin sampling or delivery, analysis and reporting. Of particular interest, the oxytocinergic system as a whole remains poorly understood. Appreciation for the differences that social contact and group living pose in domesticated species and the way they interact with humans should be key considerations in using oxytocin as a psychosocial indicator of well-being.

Keywords: affiliation, animal welfare, emotion, human-animal interaction, intranasal administration, oxytocin, positive, social behavior

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INTRODUCTION

Being able to display social behavior is key to the welfare of domesticated animals, who are all social species. Oxytocin (OT) has received increased attention for its involvement in the proximate mechanisms of social behavior, offering opportunities to elucidate the perception or processing of social stimuli.

This review aims to critically evaluate the validity and robustness of OT as an indicator of animal welfare related to the social environment. We focus on the primary literature on OT

in domesticated animal species, because although OT has been well researched in human, non-human primates and rodents (Winslow et al., 2003; Neumann, 2009; Cavanaugh et al., 2016; Freeman and Young, 2016), an emerging theory is that domestication may have influenced the oxytocinergic system (Nagasawa et al., 2015), similarly to the effects of domestication on quantitative behavioral changes (Price, 2002). We discuss findings on the impact of experimental manipulations on endogenous OT concentration, differences in OT receptor gene polymorphisms and OT administration on behavior and human-animal interaction. We excluded the special case of maternal behavior, covered by previous reviews (Neumann, 2009; Kim and Strathearn, 2016), and studies of human-animal interaction focused on the human experience. This review does not intend to exhaustively cover the literature on OT in domesticated species but rather focuses on aspects relevant to behavior and welfare, highlighting findings and gaps in research. Approaches to study animal welfare and animal welfare assessment are covered elsewhere (Fraser, 2008; EFSA, 2012).

REFLECTIONS ON RESEARCH TO DATE

We found 32 relevant studies on OT in domesticated species, with 78% of them published in the last 5 years (**Table 1**). Studies used different methods: measures of central OT (in cerebrospinal fluid 3%, in brain sections 3%) or peripheral OT (in blood plasma or serum 31%, in urine 13%), administration of exogenous OT to study the animal's response (47%), study on brain OT gene expression (3%), and more recently OT receptor gene polymorphisms (13%), with 9% of studies using more than 1 approach. For studies that measured endogenous OT, 47% took a pre- and post-stimulus sample, 40% took repeated samples during the presentation of the stimulus, and 13% only took a sample at 1 time point (comparing between individuals). Only 22% of studies had a control group, whereas the rest used within-subject designs.

Average sample size was 48 subjects, ranging from 5 to 207 subjects. Most studies used dogs (53%: 44% mixed breeds and 9% single breed, and 6% with wolf as a comparison), then pig (22%), cattle (13%), sheep (9%), and cat (3%). Studies on dogs involved a variety of adult age dogs (over 12 months) whereas studies on farm animals involved mostly young, pre-pubertal weaned subjects. As mentioned earlier, we excluded studies of maternal behavior. Studies involved mixed sexes (56%, intact or castrated), females only (34%), males only (6%), or unreported (3%). The more pronounced effects of OT administration in females than males is well-recognized (Rault et al., 2013a; Nagasawa et al., 2015; Kovács et al., 2016; Oliva et al., 2016a), but whether sexes differ in endogenous OT remains unclear as the few studies that included both sexes did not report testing for a sex effect or individual OT profiles or had insufficient sample size.

The majority of OT studies were on human-animal interaction (51%), demonstrating that OT's function cross species boundaries. The rest was composed of intra-species interaction (22%) and social isolation (27%), with 24% of studies using more than 1 paradigm. Furthermore, a variety of experimental testing conditions have been used (**Table 1**).

The following sections explore the premise of OT as an indicator of social, positive and psychological well-being in domesticated animals based on the research to date and the main factors identified for the scope of this review. We discuss research from different animal species when available, but do not assume results should be similar across species given different ethological or evolutionary importance, which we refer to as species-specific social behavior.

ARE OXYTOCIN'S FUNCTIONS SPECIFICALLY SOCIAL?

A large number of studies investigated the impact of social stimuli on endogenous OT concentration. A range of social settings trigger an OT response; the presence and magnitude of the response depending on a range of experimental factors such as familiarity of setting and partner, voluntary contact, and form of contact.

Can Only Social Variables Influence OT Release?

Studies generally showed OT increases in response to social interactions, but unfortunately few studies contained a non-social control situation. This is important because OT increases following stroking but also after exercising and eating in dogs (Mitsui et al., 2011) and OT increase in response to social and non-social stressors in rodents and humans (Nishioka et al., 1998; Olff et al., 2013). Conversely, OT did not differ between sheep kept in their groups *vs.* isolated in an unfamiliar environment (Parrott and Thornton, 1989). Furthermore, basal plasma OT relates to broader behaviors such as negative correlation with curiosity and general activity and positive correlation with fearfulness in dairy cows (Yayou et al., 2010, 2014).

Impact of Different Types of Social Interaction

Visual contact with humans is sufficient to increase OT compared to isolation [dog: (Rehn et al., 2014), artifically-reared sheep: (Guesdon et al., 2016)], and there is a positive feedback loop between OT and gazing (i.e., visual contact) in dogs interacting with humans (Nagasawa et al., 2015). Nevertheless, additional physical contact increases OT for a longer duration (Rehn et al., 2014), and more frequent interactions initiated toward humans correlate with higher OT increase in CSF (Rault, 2016).

We propose that OT is released upon voluntary interaction by the animal rather than contact imposed on the animal, hence depending on the animal's control of the interaction. Indeed, OT was higher when interactions were reciprocated (Romero et al., 2014), whereas time spent near an owner asked to ignore the dog yielded inconsistent results, either correlating with (Pekkin et al., 2016) or with no effect on urine OT (Romero et al., 2014; Nagasawa et al., 2015). Furthermore, stroking imposed on the animal did not activate more OT neurons than human presence in hand-reared lambs (Guesdon et al., 2016), and did not increase plasma OT (Coulon et al., 2013).

TABLE 1 | Summary of studies on OT and social behavior in domesticated species to date.

			Design				Measures	
Ref#	**N & species (gender)**	**OT dose**	**Test design**	**Test category**	**Familiarity + with whom**	**Control condition**	**OT matrix measurement**	**Analysis method**
ENDOGENOUS OXYTOCIN								
Nagasawa et al., 2015	30 dogs; 11 wolves (m/f)	NA	OT pre and post: gazing, verbal, touch	Social (interspecies)	Familiar and unfamiliar person; voluntary contacts	Within	Urine (extracted)	RIA
Mitsui et al., 2011	9 dogs (m/f)	NA	food, water, exercise and touch	Social (interspecies)	Familiar human; imposed contacts	Within	Urine (extracted)	RIA
Rehn et al., 2014	12 dogs (f)	NA	OT during: physical or verbal contact, ignoring	Social (interspecies)/Isolation	Familiar human; imposed contacts	Within	Blood (non-extracted)	EIA
Pekkin et al., 2016	28 dogs (m/f)	NA	OT pre and post: pressure vest on effect noise stress	Social (interspecies)/Stress	Familiar human; voluntary contacts	Within	Urine (extracted)	ELISA
Odendaal and Meintjes, 2003	18 dogs (m/f)	NA	OT pre and post: touch, verbal, low-key play	Social (interspecies)	Familiar and unfamiliar person; imposed contacts	Within	Blood (non-extracted)	HPLC
Handlin et al., 2011	10 dogs (m)	NA	OT pre and post: touch verbal, ignore with female owners	Social (interspecies)	Familiar human; imposed contacts	Within	Blood (non-extracted)	EIA
Rault, 2016	5 pigs (f)	NA	OT pre and post: touch, verbal, positive and negative interaction with person	Social (interspecies)	Familiar human; voluntary contacts	Within	CSF (non-extracted)	ELISA
Bruckmaier et al., 1993	8 cows (f)	NA	OT pre and post: milking in different environments	Stress	Familiar vs. unfamiliar environment	Within	Blood (extracted)	RIA
Yayou et al., 2010, 2014, 2015	20 calves (f)	NA	OT repeatedly: sniffing, touching, mixing with unfamiliar conspecifics; during development	Stress/Novel environment/Social (intra-species)	Familiar and unfamiliar environment; familiar and unfamiliar conspecifics	Within	Blood (extracted)	EIA
Parrott and Thornton, 1989	10 sheep (m/f)	NA	OT pre and post: during isolation and in social environment; effect of opioid agonist and antagonist	Social (intra-species)/Stress	Familiar conspecifics and unfamiliar environment	Within	Blood	RIA
Coulon et al., 2013	16 lambs (f)	NA	OT during: touch, isolation, reunion	Social (interspecies)	Familiar human, voluntary or imposed contacts	Within	Blood	EIA
OXYTOCIN ADMINISTRATION								
Romero et al., 2014	16 dogs (m/f)	40 IU IN	OT pre and post: affiliation, proximity	Social (interspecies)/Social (intra-species)	Familiar human or dog	Within	Urine (extracted)	RIA
Oliva et al., 2016a	75 dogs (m/f)	24 IU IN	Ability to use experimenters' visual cues to find food; questionnaires	Social (interspecies)	Rating by familiar human, task with unfamiliar human	Within	NA	NA
Kovács et al., 2016	39 dogs (m/f)	12 IU IN	Spontaneous preference for biological motion versus non-biological control stimuli	Social (movement)	NA	Within	NA	NA
Hernádi et al., 2015	36 dogs (m/f)	12 IU IN	Response to threatening behavior owner or experimenter	Social (interspecies)/Stress	Familiar and unfamiliar human	Within	NA	NA
Oliva et al., 2015	62 dogs (m/f)	24 IU IN	Use of pointing and gazing cues by experimenter in object choice task	Social (interspecies)	Unfamiliar human	Within	NA	NA
MacChitella et al., 2017	17 dogs (m/f)	2 IU/kg IN	Use of pointing and gazing cues by experimenter in object choice task	Social (interspecies)	Unfamiliar human	Within	NA	NA
Kis et al., 2015	64 dogs (m/f)	12 IU IN	Pointing to find food in cognitive bias test	Social (interspecies)	Unfamiliar human	Between and Within	NA	NA
Romero et al., 2015	16 dogs (m/f)	40 IU IN	Social play between adult dogs	Social (intra-species)	Familiar conspecifics	Within	NA	NA

(Continued)

TABLE 1 | Continued

Ref#	N & species (gender)	OT dose	Design				Measures	
			Test design	Test category	Familiarity + with whom	Control condition	OT matrix measurement	Analysis method
Rault et al., 2013a	24 piglets (m/f)	24 IU IN	Observe distress-related behavior during mixing with unfamiliar conspecifics	Social (intra-species)	Unfamiliar conspecifics	Between and Within	NA	NA
Camerlink et al., 2016	96 pigs (f)	24 IU IN	Observe social contact on return to pen after positive/negative/neural experience	Social (intra-species)	Familiar conspecifics	Between and Within	NA	NA
Rault et al., 2015	144 piglets (m/f)	24 IU IN/80 IU SC	Observe social behavior and food/water intake post-weaning;	Social (intra-species)/Stress	Familiar and unfamiliar conspecifics	Between	NA	NA
Reimert et al., 2015	96 pigs (f)	24 IU IN	Emotional contagion for positive and negative events: observing interaction	Social (intra-species)	Familiar conspecifics	Between and Within	NA	NA
Rault et al., 2013b	24 piglets (f)	24 IU IN	Behavior during isolation after prenatal stress or control	Stress	NA	Between	NA	NA
Rault, 2016	3 pigs (f)	36-60 IU IN	Endogenous OT collection overtime	Home pen normal environment	NA	Within	CSF (non-extracted)	ELISA
Mitsui et al., 2011	6 dogs (m)	24 $\times 10^{-5}$ IU IV in 4 bolus each 5 min	OT pre and post IV OT injection	Unfamiliar cages, non-social	NA	Within	Blood (non-extracted); urine (extracted)	RIA
Nagasawa et al., 2015	27 dogs (m/f)	40 IU IN	OT pre and post, dog behavior: gazing, touch, proximity	Social (interspecies)	Familiar and unfamiliar person	Within	Urine (extracted)	RIA
OT RECEPTORS AND NEURONS								
Oliva et al., 2016b	169 dogs and 12 wolves (m/f)	24 IU IN	Pointing and indicating by experimenter	Social (interspecies)	Unfamiliar human	NA	OTR	PCR
Kis et al., 2014	207 dogs (m/f)	NA	Greeting, threatening, separation with stranger and familiar person	Social (interspecies)	Familiar and unfamiliar humans	NA	OTR	PCR
Ottenheimer-Carrier et al., 2017	97 dogs (un)	NA	Personality questionnaire	Personality	NA	NA	OTR	PCR
Arahori et al., 2016	94 cats (m/f)	NA	Personality questionnaire	Personality	NA	NA	OTR	PCR
Guesdon et al., 2016	24 sheep (f)	NA	Isolation, presence, touch human	Social (interspecies)/Stress	Familiar human	Between	Neuronal activation PVN	Immunohistochemistry
Vellucci and Parrott, 1997	10 young pigs (m)	NA	Restraint	Stress	NA	Between	OT gene forebrain	Autoradiography

m, male; f, female; un, unknown; NA, not applicable; IN, intranasal administration; SC, subcutaneous administration; Between, between-subject control; Within, within-subject control; OT, oxytocin; OTR, oxytocin receptor; Extracted, samples extracted prior to assaying; Non extracted, No mention made of extraction of sample prior to assaying; RIA, radioimmunoassay; EIA, enzyme immunoassay; HPLC, high-performance liquid chromatography.

In summary, social presence can trigger OT release, and physical contact intensify it, but further research is warranted to investigate whether OT release relates to species-specific social behavior and reciprocal interactions rather than contacts imposed on the subject.

Impact of Partner Familiarity

Most human-animal interaction studies used familiar humans. The few studies that included familiar and unfamiliar humans suggest that OT's release is stimulated by familiar partners (Rehn et al., 2014; Hernádi et al., 2015; Nagasawa et al., 2015). Unexpectedly, OT administration reduced dog's friendliness toward their owner whereas it did not affect their response toward a stranger (Hernádi et al., 2015), but exogenous OT administration at supraphysiological levels causes OT to bind to vasopressin receptors, possibly resulting in confounded effects (Manning et al., 2012). Furthermore, in this last study, a stranger was standing behind them in the first situation versus their owner in the second situation. This may have influenced the dog's response because the stranger in their back may have provided a potential threat whereas their owner in the back social support, as dogs looked back more at their owner than the stranger (Hernádi et al., 2015). Overall, findings support that OT is involved with familiar rather than unfamiliar individuals (Bielsky and Young, 2004).

Conversely, in studies that used unfamiliar conspecifics, OT administration often increases negative social behavior and reduces positive social behavior (see Section Is Oxytocin an Indicator of Positive Valence? below). Social cognition is important in situations where animals need to determine whether the social partner is familiar or unfamiliar; an ability linked to oxytocin and vasopressin (Bielsky and Young, 2004).

Summary on Oxytocin and Sociality

Oxytocin has most often been studied in socially relevant paradigms, but with a lack of non-social control paradigms to establish the specificity of OT to social contexts. It is difficult to disentangle it from a general stress coping mechanism in social species (Cavanaugh et al., 2016), in which OT may have evolved as the social arm of homeostatic processes (Buisman-Pijlman et al., 2014). Comparative studies using various species could help assess the relationship between OT and sociality. The presence of a partner increases OT release compared to social isolation, with a possible additional advantage of reciprocated contact, which requires further research with consideration of species-specific social behavior.

IS OXYTOCIN AN INDICATOR OF POSITIVE VALENCE?

In the quest for indicators of positive welfare states, OT is often proposed to reflect situations of positive valence. However, few studies have compared positive to negative or neutral situations. For instance, that urinary OT increases in three positive situations does not prove OT as a "biomarker of positive emotions" (Mitsui et al., 2011) unless a non-positive situation would have been included, although cortisol was included as a measurement of arousal.

Environmental context can modulate OT's actions. For instance, OT administration promoted positive social behaviors of dogs toward both their owners and familiar dogs (Romero et al., 2014), but reduced friendliness toward the owner in the presence of an approaching stranger, as discussed earlier (Hernádi et al., 2015). Opposite findings were found in pigs, in which OT administration in familiar groups reduced social contact in neutral or positive situations but increased it in negative situations (Camerlink et al., 2016). Conversely, CSF (endogenous) OT increased in pigs following positive human interaction, but not negative human interaction (Rault, 2016), although valence and familiarity of the partner were confounded.

The social context (e.g., partner familiarity) may also modulate OT's actions. Calves with high basal plasma OT postnatally showed higher social engagement, both affiliative and agonistic behaviors, in later life (Yayou et al., 2015), and exogenous studies showed that OT administration can increase aggression in pigs (Rault et al., 2013a, 2015). However, these studies involved animals mixed with unfamiliar conspecifics and in unfamiliar environments, i.e., stressful situations. Altogether, these findings are consistent with the in-group vs. out of group OT theory in humans (De Dreu, 2012), with OT's positive actions toward existing social partners and negative actions toward unfamiliar partners.

In summary, OT does not necessarily correlate with positive situations or outcomes. The OT literature appears biased toward investigating positive valence, with a lack of controlled paradigms for valence and arousal. There is evidence that negative situations also mobilize OT. We propose that OT may be evolutionarily linked to social coping strategies (Buisman-Pijlman et al., 2014; Cavanaugh et al., 2016), as the social arm of homeostatic processes, and as such neither positive nor negative but simply adaptive. The valence of OT's actions are modulated by the environmental and social contexts, and OT's theoretical function of preserving existing social bonds (Tops et al., 2014). Environmental and social factors are therefore important to consider in study design and interpretation (Olff et al., 2013).

ARE OXYTOCIN'S ACTIONS LINKED TO SPECIFIC PSYCHOLOGICAL PROCESSES?

Oxytocin is often referred to as the "feel-good" hormone, or as an indicator of positive emotions (Mitsui et al., 2011). Rodent and human data highlight the effect of exogenous OT in increasing trust and reading of social cues, reducing anxiety and other psychological processes (Lee et al., 2009). There is no direct neurobiological evidence yet in domesticated species to support the role of OT in psychological, and particularly emotional, processes. Studies extrapolate their findings to psychological implications based on analogy with human studies (Mitsui et al., 2011). However, OT's role in human psychological processes is still debated (Nave et al., 2015). Particularly, whether the affective "feel good" effect is a direct or indirect effect of OT is unclear,

given that OT antagonists do not block these effects (Uvnas-Moberg, 1998) and that the oxytocinergic system interact with other reward systems, notably opioidergic and dopaminergic systems that also increase in response to social interactions (Odendaal and Meintjes, 2003; Buisman-Pijlman et al., 2014; Tops et al., 2014) and impact on the HPA axis (Buisman-Pijlman et al., 2014; Tops et al., 2014).

Most of the knowledge in psychology is about the effect of intranasal OT administration, rather than correlative studies between endogenous OT and psychological states. Interestingly, OT administration induces a positive cognitive bias in dogs to ambivalent food cues (Kis et al., 2015).

The stage at which OT affects socio-cognitive processes currently debated in humans (perception vs. processing of social cues) has been followed up in dogs, with OT administration posited to reduce the attentional bias to social cues (Kovács et al., 2016), whereas others argue that OT does not alter perceptual salience of social cues or social anxiety but rather motivates social engagement (Romero et al., 2014).

The social motivation vs. social reward hypothetical functions, which appears in the human literature, is also relevant to domesticated animals. The hypothesis that OT increases social motivation is supported by exogenous OT studies, with dogs administered OT initiating more contact toward a familiar dog and owner (Romero et al., 2015), even when owners were instructed to ignore or only briefly reciprocate (Romero et al., 2014; Nagasawa et al., 2015). The hypothesis that OT conditions the rewarding value of social cues is supported by endogenous OT studies, where the failure from humans to reciprocate contact results in lower plasma OT concentration over time compared to the initial reunion (Rehn et al., 2014), but no change in urine OT (Nagasawa et al., 2015). More frequent measurements of OT over time could allow discerning appetitive from consummatory motivations.

In summary, there is currently limited evidence that OT's actions are linked to psychological states. Nevertheless, it remains to be demonstrated that it is a direct effect of OT *per se*. This is a worthwhile area of research given the increasing interest in affective states (feelings, emotion, and cognition) in psychology and animal welfare science.

POTENTIAL AND CURRENT LIMITATIONS OF OXYTOCIN AS AN ANIMAL WELFARE INDICATOR

While findings are coming at a quick pace, the few and discrepant findings make it premature to conclusively decide on OT's potential as an animal welfare indicator.

Oxytocin's Potential as an Animal Welfare Indicator

An animal-based indicator of welfare should be valid and robust (EFSA, 2012). The interpretation of OT as an animal welfare measure requires precise and consistent results. Unfortunately, we highlighted above substantial inconsistencies in findings to use OT as a welfare indicator, possibly due to the exploratory stage of the research. Possible causes of discrepancy are highlighted in **Table 2**, along with potential solutions. Full reporting of the factors listed in **Table 2** would enhance rigor in OT research while abiding by good scientific practices. Standardization of the experimental testing procedures may also help to compare findings, as is commonly done for research on primates and rodents.

Briefly, OT is a peptide hormone, which makes it especially sensitive to sampling collection procedures and analytic methods compared to steroid hormones like cortisol. Given OT's variability between individuals and contexts (Olff et al., 2013), within-subject experimental designs (see Kekecs et al., 2016) and counterbalanced designs should be favored to tackle contextual modulation. Inter-individual variation is a well-known phenomenon in OT research, and worthy data to report (individual data profile can be shared through Supplementary Material, see for instance (Nagasawa et al., 2015)), to help further studies and meta-studies progress our understanding of the OT system's response and actions. The reproducibility crisis of science does not spare OT research (Nave et al., 2015), and we found only one study replication (MacChitella et al., 2017).

Overlooked Areas of Oxytocin Research

The biological significance of OT measured in different matrices (e.g., centrally but also blood, urine, saliva, and milk) remains to be elucidated. The function of the oxytocinergic system as a whole is poorly understood, and most studies focused solely on its circulating hormone (through measurement or administration), rather than OT-secreting neurons or the OT receptor (Freeman and Young, 2016). Oxytocin receptor gene polymorphisms have provided insights into variation in human-animal interaction. Nevertheless, the role of genetic (breed) and epigenetic (rearing) factors remain to be clarified, as the OT receptor gene differs between wolf and dogs (Oliva et al., 2016b) but differences between animals that vary in their sociality returned positive [dogs: (Kis et al., 2014); cats: (Arahori et al., 2016)] or null findings [dogs: (Oliva et al., 2016b; Ottenheimer-Carrier et al., 2017)].

The drawbacks of sampling endogenous OT explain the attractiveness of intranasal OT administration, boosted by pioneering studies in humans (Born et al., 2002; Kosfeld et al., 2005). However, OT dose-response studies are lacking, species-specific metabolic differences in absorption or clearance rate are unknown, and the use of selective OT antagonists would strengthen the evidence for OT-mediated pathways (Guastella et al., 2013; Cavanaugh et al., 2016). For instance, most studies test animals 45 min post-OT administration following human studies, but effects may vary between sampling matrices or species (Mitsui et al., 2011; Nagasawa et al., 2015; Rault, 2016). The biological relevance of commonly administered OT doses is also questionable, as intranasal administration of 36–60 IU increased endogenous CSF OT 20- to 60-fold in pigs (Rault, 2016), well-beyond normal physiological concentrations, although plasma OT increases appear to be only three-fold higher than baseline in dogs after delivery of 40 IU, and to a lower extent but inconsistently in urine (Romero et al., 2014). This also raises the likelihood of activating the

TABLE 2 | Summary of common research design and methodological pitfalls, and potential solutions to enhance validity and comparison in OT research.

Factor	Problems	Potential solutions
Sample size	Low number of subjects	Use power analysis to calculate sample size[a]
	Heterogenous sample: e.g., breed, age, previous experience, sex, hormonal status	Minimize the number of variables between subjects and situations
	High inter-individual variability	Adopt a within-subject design
Testing paradigm	Sole testing paradigm	Use more than 1 paradigm, adapted to the hypothesis (e.g., social vs. non-social; positive vs. negative valence) to determine the specificity of the findings
	No control treatment	Include control group (between-subject design)
	Unknown contextual effects	Adopt a counterbalanced design
	Lack of standardization or measure of (social) stimulus	Standardize the stimulus, or measure covariates to take into account at the data analysis stage
	Too few methodological details	List individual (current characteristics and past experiences) and context description in the methodology to improve content validity of findings. Choose behavioral test and conditions that are species-appropriate; choose settings to fit aim: either familiar or unfamiliar environment/person/animals and control for it
OT sample collection[b]	Different sampling matrices (e.g., plasma, urine, CSF)	Study the correlation between OT in different matrices and biological actions/targets
	Inappropriate time-point for sample collection	Timepoint appropriate to OT release and half-life in the matrix; prefer multiple time-points if possible to assess OT dynamics overtime
	Varying collection procedures (OT is a peptide hormone sensitive to degradation, especially by freeze-thaw cycles)	Uniformization of collection procedures within study, researchers blind to experimental treatments
OT sample analysis: bioanalytic validity and reliability[2]	Sensitivity	Demonstrate that concentration falls within the assay detection limit
	Precision and reliability	Determine intra- and inter-assay CVs in your lab
	Accuracy	Demonstrate quality control steps: e.g., spiking, linear dilution; correlation between analysis technique used and other validated techniques, or cite peer-reviewed published validation
	Specificity	Compare extracted vs. unextracted samples; report cross-reactivity or cite published validation
OT administration	Route of administration	Consider the mode of delivery: subject position, subject habituation and administrator training, product additives, concentration/volume, absorption and clearance rate[c]
	Dose	Assess dose-dependent response through a pilot trial or within the main experiment; aim for minimal dose; administer OT and a selective antagonist
	Timeline for testing post-administration	Use multiple sampling timepoints if possible; time of day
Study replication	Lack of study replication	Use multiple replicates within a study; replicate studies from other researchers
Results analysis	Failure to report initial OT concentration data ("absolute" OT concentrations) or reporting solely correlation	Report absolute concentrations, supplementary file to share large dataset, especially interesting for individual data profile and variation
	Use of incorrect statistical analysis	Correct for multiple comparisons, baseline data, etc
	Omitting or discarding data	Identify causes for outliers, justify the treatment of outliers
Publication of findings	Large bias toward positive over null findings[d]	Lay out the soundness of the experimental design and proper analysis of the findings[e]

[a] *See http://www.3rs-reduction.co.uk/html/6__power_and_sample_size.html*
[b] *For instance (Robinson et al., 2014).*
[c] *For instance (Guastella et al., 2013).*
[d] *For instance (Lane et al., 2016).*
[e] *For instance (Kilkenny et al., 2010).*

vasopressinergic system by OT administration, resulting in potential confounding behavioral effects (Manning et al., 2012). Interestingly, dogs with lower endogenous OT concentrations were more responsive to exogenous OT administration than dogs with higher endogenous OT concentrations (Romero et al., 2014).

The responsiveness of the OT system (synthesis, pulsatile release, receptor numbers, and binding) to stimuli remains poorly understood, especially as most studies only sampled at a couple of timepoints. Studying OT's role along with complementary physiological systems (vasopressinergic, opioidergic, dopaminergic, and the HPA axis) is also crucial to comprehend OT's function.

The potential modulation of the OT system through development and experience (Buisman-Pijlman et al., 2014), and particularly its epigenetic bases, warrant further research. For instance, basal plasma OT related to behavioral traits in the neonatal calves (Yayou et al., 2010) but not with their behavior in later life (Yayou et al., 2014) or only in specific conditions (Yayou et al., 2015). There is a crucial lack of knowledge of the ontogeny of the oxytocinergic system in domesticated species.

Oxytocin and Social Communication

There is an increasing body of evidence that OT mediates social communication and social cognition, particularly using human-dog interaction as a model (Nagasawa et al., 2015; Kovács et al., 2016). Oxytocin administration enhances dogs' performance using human momentary distal pointing cues (Oliva et al., 2015; MacChitella et al., 2017), increases gaze to owner (Nagasawa et al., 2015), decreases aversion to unfamiliar human gaze (Oliva et al., 2015), but also block the ability of owner to predict the performance of their dog (Oliva et al., 2016a). The stage at which OT intervenes in socio-cognitive processes remains unclear (see Section Are Oxytocin's Actions Linked to Specific Psychological Processes?).

Intriguing evidence suggests that OT administration may not only influence the treated animal, but also non-treated conspecifics in the same environment. For instance, OT administration to a pig altered the behavior of a conspecific unable to see the OT-administered pig, reducing defecation during the negative situation and reducing low tail during the positive situation (Reimert et al., 2015). Similarly, OT administration affected cage mates through olfactorily-mediated stress inhibiting effects in rats (Agren and Lundeberg, 2002) and in humans (Weisman et al., 2012).

The involvement of OT in social communication promises to be a fascinating area of research, while emphasizing the need to monitor complementary measures such as behavior and vocalization.

IMPLICATIONS: CAN OXYTOCIN BE TRUSTED AS AN ANIMAL WELFARE INDICATOR?

Focusing on the biological significance of OT in the regulation of psychological and behavioral states may help reconcile findings. A greater understanding of the effects of genetic, epigenetic and ontogeny on the oxytocinergic system is highly relevant to domesticated animals. Accumulating evidence in other species also shows that OT's actions are moderated by context and inter-individual differences. This is determinant to the use of OT as an animal welfare indicator sensitive to the state of interest and robust to extraneous factors. Furthermore, classic parameters for animal welfare measures such as sensitivity, specificity, and repeatability remain to be tested. Indeed, research on OT in domesticated species brings the advantage of potentially well-controlled experiments. It also has direct implications for animal welfare given the importance of social factors and the ability for human management practices to include situations conducive to OT system's development and stimulation.

AUTHOR CONTRIBUTIONS

Mv screened the existing literature and drafted **Table 1**. JR and FB analyzed and interpreted the literature database and wrote the draft of the manuscript. JR, Mv, and FB reviewed and approved the final manuscript.

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Microsatellite Polymorphisms Adjacent to the Oxytocin Receptor Gene in Domestic Cats: Association with Personality?

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A growing number of studies have explored the oxytocin system in humans and non-human animals, and some have found important genetic polymorphisms in the oxytocin receptor gene (*OXTR*) associated with the bonding system, social behaviors, and personality in several species. Although single nucleotide polymorphisms in *OXTR* have been well-examined in various species, microsatellites (or short tandem repeats) adjacent to *OXTR* have rarely been studied, despite some suggestions that microsatellite polymorphisms near genes might play a role in genetic transcription and translation. In this study, we surveyed microsatellites in the upstream, intron, and downstream regions of *OXTR* in domestic cats (*Felis catus*). We succeeded in amplifying 5 out of 10 regions, and recognized these five regions as polymorphic. We compared allele frequencies in these five regions between mongrel cats in Japan (n = 100) and cats of 10 pure breeds (n = 40). There were significant differences in allele frequencies between the two populations in all microsatellite regions. Additionally, the owners of mongrel cats answered a comprehensive personality questionnaire, and factor analysis extracted four factors (Openness, Friendliness, Roughness, and Neuroticism). We examined the association between the microsatellite genotypes, age, sex, neutering status, and personality scores. Compared to their counterparts, younger cats tended to score higher on Openness, male cats scored higher on Friendliness, and female and neutered cats scored higher on Roughness. When we divided the sample into three groups depending on the length of alleles, we found a marginally significant association between Friendliness and MS3. Additionally, we found a sex-mediated effect of genotypes in MS4 on Friendliness, resulting in different effects on females and males. Our findings that mongrel cats had longer alleles in MS3 and MS4 than purebred cats, and that those cats tended to score higher on Friendliness, supported the previous findings. However, future studies such as comparison between purebred cats with apparently different origin or personality are required to determine the association of genetic variants in the *OXTR* with personality.

Keywords: domestic cat, microsatellite polymorphism, mongrel cat, oxytocin receptor gene, personality, purebred cat

INTRODUCTION

Many studies focusing on the oxytocin system in humans have revealed that some genetic polymorphisms are associated with a large variety of individual differences in, for example, empathy (e.g., Rodrigues et al., 2009; Wu et al., 2012; Laursen et al., 2014), attachment anxiety (e.g., Chen and Johnson, 2012), prosociality (e.g., Shang et al., 2017), and pair-bonding behavior (e.g., Walum et al., 2012). In parallel, studies on non-human animals have explored genetic polymorphisms in the oxytocin receptor gene (*OXTR*) as a candidate gene related to the bonding system, social behaviors, and personality traits (e.g., Aspé-Sánchez et al., 2016). For *OXTR* in non-human primates, Staes et al. (2014) tried to find genetic differences between chimpanzees and bonobos at the locus of rs53576 in the intron 3 region of *OXTR*, which had been shown to be involved in many associations in humans (e.g., Rodrigues et al., 2009; Laursen et al., 2014); they expected this locus would contribute to species differences in empathy between chimpanzees and bonobos. Although they did not find polymorphism at this locus, they found novel polymorphisms near rs53576 in these two species. To date, these polymorphisms have not been shown to be associated with behavioral traits within species (chimpanzee; Staes et al., 2015); other studies have suggested the impact of the oxytocin system on primates' bonding systems (Lee et al., 2011; Vargas-Pinilla et al., 2015). In fact, New World monkeys showed a new type of the oxytocin gene (*OXT*) (one amino acid was changed compared to the wild type) with co-evolved *OXTR* with positive selection, indicating an association with different systems in animals' mating (e.g., monogamy). Concerning companion animals, Kis et al. (2014) reported that three single nucleotide polymorphisms (SNPs) in domestic dogs were associated with dogs' responses to unfamiliar humans; however, Ottenheimer-Carrier et al.'s (2017) questionnaire study failed to provide further support to this finding in various breed groups, suggesting the possibility that gene-personality associations may reflect multiple factors, including breed, early experiences, and experimental contexts. Our previous study on domestic cats (*Felis catus*) (Arahori et al., 2016) reported an association between one SNP and a personality trait (Roughness). However, the molecular and functional mechanisms are still unclear.

Most studies referring to gene-behavior associations have focused on SNPs. However, in addition to SNPs, microsatellites (or short tandem repeats) adjacent to genes have been suggested to play a role in genetic transcription and translation (Sawaya et al., 2013). In particular, microsatellites near to or on the promoter region, such as TATA boxes (transcription start sites), GC-rich regions, and CAAT boxes within upstream regions could affect gene expression. In non-human animals, Oliva et al. (2016) compared microsatellites between wolves and dogs in the region close to *OXTR*, and found differences in the frequencies, but no differences in performance (scores) in an object choice task depending on length of alleles. Lonn et al. (2017) revealed that microsatellites in the 5′ regulatory region of *OXTR* and arginine vasopressin receptor gene 1a (*AVPR1A*) were associated with reproductive success demonstrated in field experiments and gene expression in the brains of bank voles. However, little research on *OXTR* has been conducted on animals, including domestic cats.

Domestic cats, which are common companion animals, are thought to have been domesticated almost 10,000 years ago in the Near East (Driscoll et al., 2007). Their gene-personality associations have been under-researched—our previous research is an exception (Arahori et al., 2016). These associations are providing an important clue to understand how African wild cats (the ancestors of domestic cats) adapted to humans and human societies by comparing personality-related genes found in domestic cats from an evolutionary developmental biology point of view, and infer their pathway from wildcats to pets. The association would also be important for cats' welfare because personality-related genes could suggest matching between potential owners and cats.

In addition to the early phase of cat domestication, many cat breeds have more recently been created through various degrees of artificial selective pressure, according to humans' preferences (mainly in relation to appearance, although sometimes also temperament—e.g., the Ragdoll with its high placidity; Bradshaw et al., 1999). Some previous studies have described breed-specific personality traits as reported by veterinarians and owners in response to questionnaires. Takeuchi and Mori (2009) reported that Japanese domestic cats (mongrel cats) and American Shorthair cats ranked higher on friendliness, playfulness, demand for affection, and novelty seeking than 10 other pure breeds examined in the study, whereas Chinchilla cats were ranked highest on aggression to humans and cats, timidity, and nervousness. Wilhelmy et al. (2016) examined the links between appearance (e.g., coat color) and personality, but concluded that most individual differences stemmed from breed differences. In sum, the artificial selection of cat breeds may have influenced breed-specific personality, suggesting that some heritable genes would be linked to personality. Therefore, examining differences in terms of *OXTR* between mongrel cats and purebred cats might be one of the keys to reveal the effects of *OXTR* on the personality of cats.

In this study, we explored the microsatellites adjacent to *OXTR* in cats. First, we compared allele frequencies in microsatellites between mongrel cats and purebred cats because we expected that *OXTR* in purebred cats under human selective pressures would be different genetically from that in mongrel cats. Possibly, purebred cats must have been selectively bred for appearance by humans, although it is unclear that such selection could also influence behavior and personality. On the other hand, mongrel cats are not generally selected by humans. In fact, in 2016 in Japan, 57.1% of cat owners reported that they adopted free-roaming cats as their pet(s), either by themselves or via organizations (e.g., animal shelters) (Japan Pet Food Association, 2016), suggesting free-roaming cats' histories were much different from purebred cats in Japan. Therefore, we expected that sequences in *OXTR*, the candidate gene related to social behavior, would differ between mongrel cats (free-roaming for several generations) and purebred cats (having undergone artificial selection). Second, we examined the association with personality scores in mongrel cats to ascertain the effects of *OXTR* polymorphisms on personality. Only mongrel cats were examined due to the difficulty of finding

single breed subjects with little or no kinship. We considered not only *OXTR* genotypes and the effects of age, sex, and neutering on cat personality, but also sex-mediated effects connected with *OXTR*, as suggested in human studies (e.g., Stankova et al., 2012).

MICROSATELLITES IN *OXTR* AND THE DIFFERENCES BETWEEN MONGREL CATS AND PUREBRED CATS

Materials and Methods

Subjects

We included 100 cats (54 males, 46 females; mean age = 60.95 months; standard deviation = 52.05 months) in Japan. All cats were mongrels born in Japan, suggesting that their ancestors could not be identified as purebred according to the owners. According to the owners, 35 cats were free-roaming cats that had been taken home by the owners themselves, 23 were adopted by the owners via their acquaintances, 17 were adopted by owners via animal shelters or volunteers, 3 comprised siblings of cats that owners already kept, and we were unable to obtain answers from 22 cat owners. A total of 88 cats were neutered (48 males, 40 females), while 12 cats were not (6 males, 6 females); 85 were household pets (48 males, 37 females) and the remaining 15 were residents in "cat cafés" (6 males, 9 females), where guests interact with them, resulting in extensive contact with people almost every day. All cats had one or two owners (caretakers). Based on owners' reports, none of the cats in the sample were genetically related. We included more than one cat belonging to the same owner if these cats showed different haplotypes in examined microsatellites.

To compare allele frequencies between mongrel and purebred cats, we selected an additional 40 genetically unrelated cats of 10 different typical cat breeds (4 cats each: 2 males, 2 females; Abyssinian, American Curl, American Shorthair, Scottish Fold, Somali, Persian, Himalayan, Chinchilla Persian, Maine Coon, and Russian Blue). These cats were used in a previous study (Kato et al., 2007; in Japanese), and they were offered to us via veterinary hospitals from cat owners (Veterinarians confirmed their breeds, and the owners offered information about their kinships).

Microsatellite Searching

From the cat genome sequence (GenBank assembly accession: GCA_000181335.3) we searched for microsatellites in the upstream region (8 kb) of exon 1, the intron region between exons 1 and 2, and the downstream region (3 kb) of exon 2 in *OXTR* using WebSat software (Martins et al., 2009). We found 10 regions including microsatellites (4 in the upstream region, 5 in the intron region, and 1 in the downstream region; **Figure 1**); however, we analyzed only 5 (MS1, 2, 3, 4, and 5) of these 10 regions. We could not amplify those in the intron region when considering polymerase chain reaction (PCR) conditions because there were probably numerous mutations in this region.

Sample Collection and Genotyping

For mongrel cats, we collected buccal cell samples after obtaining permission from their owners. Buccal cell samples were collected using cotton swabs (JCB INDUSTRY LIMITED, Ginza, Chuou-ku, Japan) in 2 ml of 0.9% saline solution, then 9 ml of 99.5% ethanol was added for preservation, and stored at 4°C until DNA extraction.

DNA was extracted from the buccal cells of cats using a QIAamp blood and tissue kit (QIAGEN, Valencia, CA, United States). Five microsatellite regions were amplified by PCR with a 10 μl mixture for each sample, containing 5 μl of Multiplex PCR kit (QIAGEN, Valencia, CA, United States), 1 μl template DNA, 3.5 μl H_2O, and 0.05 μl for each forward and reverse primer (see **Table 1** for primer information). The PCR conditions consisted of 95°C preheating for 15 min, 35 cycling at 94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 60°C for 30 min as the last extension. Subsequently, we sequenced PCR products using the Applied Biosystems *3130xl* Genetic Analyzer (Applied Biosystems, Foster City, CA, United States). The sizes of PCR products were genotyped using the GENESCAN software package (PerkinElmer, Foster City, CA, United States). We checked peaks (the sizes of PCR products), and repeated the analysis if the peaks were unclear to determine.

Statistics

We assessed linkage disequilibrium among all microsatellites. We used Fisher's exact test to assess the differences in allele frequencies between mongrel and purebred cats. We used R (v. 3.4.2) for all statistical analyses (R Core Team, 2017) using the package: *genepop* (Rousset, 2008).

Results

Linkage Disequilibrium among Five Microsatellite Loci

All five regions were polymorphic in both (mongrel and purebred) cat groups. For mongrel cats, the test for genotypic linkage disequilibrium (LD) revealed non-random associations among microsatellite loci ($p < 0.01$) other than MS4 and MS5 ($p = 0.694$).

Allele Frequency Differences between Mongrel and Purebred Cats

Overall, alleles of mongrel cats in MS1, MS2, MS3, and MS4 were longer than in purebred cats, but the difference in MS5 was reversed (**Table 2**). In MS2 and MS4, mongrels had more alleles than purebred cats did. Fisher's exact test revealed significant differences between mongrels and purebred cats in all alleles (MS1: $p < 0.0001$; MS2: $p < 0.01$; MS3: $p < 0.0001$; MS4: $p < 0.0001$; and MS5: $p < 0.001$).

GENE-PERSONALITY ASSOCIATIONS IN MONGREL CATS

Materials and Methods

Subjects and DNA Collection

We used the same mongrel cat subjects ($n = 100$) as those used to assess differences between mongrel cats and purebred cats in microsatellites adjacent to *OXTR* (see section "Microsatellites in

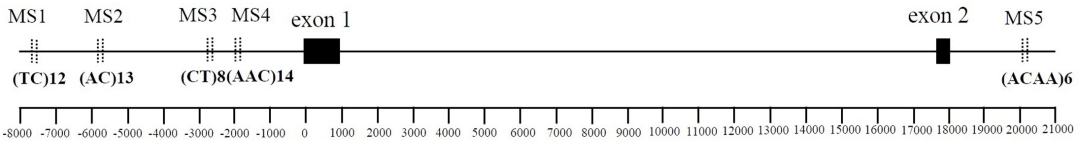

FIGURE 1 | Conceptual diagram of *OXTR* and the locus of microsatellites in cats.

TABLE 1 | Primer sequence used for this study.

MS	Primer (5′–3′)	Fluorescent dyes	Repeat unit	Size (bps)	No. of alleles
1	CTCCTGAATGTGGGTGGGAC	NED	(TC)12	217	4
	TCAGAGCGCCTGTGAATGAG				
2	AAAGGTGAAGCAGAAAGTGGAG	FAM	(AC)13	396	7
	ACCTCCAGTGAAAAGTGACAGA				
3	GGTGGCTCAGTCAGTGACTC	HEX	(CT)8	235	2
	GGTGATGTGGGGCTTAGCAT				
4	TGGTTTTCCCTGTCTTCATTCT	HEX	(AAC)14	365	9
	TTTGTTCCTATTCCCATTCCTG				
5	CCTGCATTTGGGGTAGAGATTA	FAM	(ACAA)6	308	2
	CACCAGCAACGTATGAGAGTTC				

TABLE 2 | Allele frequencies observed in Japanese mongrel cats and purebred cats.

Locus	*He*	Allele frequencies								
MS1		211	215	217	219					
Mongrel	0.61	0.14	0.195	0.57	0.095					
Pure breed	0.74	0.138	0.288	0.3	0.275					
MS2		392	396	406	408	410	412	414		
Mongrel	0.81	0.08	0.015	0.08	0.13	0.2	0.2	0.295		
Pure breed	0.81	0.288	0	0.063	0.125	0.125	0.213	0.188		
MS3		235	237							
Mongrel	0.44	0.33	0.67							
Pure breed	0.49	0.6	0.4							
MS4		356	359	362	365	368	371	374	377	380
Mongrel	0.78	0	0.005	0.235	0.325	0.185	0.15	0.08	0.005	0.015
Pure breed	0.58	0.013	0.125	0.15	0.613	0.1	0	0	0	0
MS5		304	308							
Mongrel	0.28	0.83	0.17							
Pure breed	0.47	0.625	0.375							

He represents expected heterozygosity in each population. The numbers next to each population mean allele frequencies in each allele. Underlined numbers indicate the alleles divided into L.

OXTR and the Differences between Mongrel Cats and Purebred Cats").

Rating of Cat Personality

We used a personality questionnaire developed for Japanese Akita dogs (Konno et al., 2011), which was used in our previous study with cats (Arahori et al., 2016). It consists of 30 questions measured on a six-point scale. The caretaker of each mongrel cat (including "cat café" owners) completed the questionnaire. Additionally, we included data from the cat sample (only mongrel cats) in Arahori et al. (2016) (32 males, 25 females; mean age = 60.0 months; standard deviation = 50.55 months), in which test–retest reliability was satisfactory.

Statistics

First, we inspected the scree plot by running a parallel analysis to determine the number of factors. Next, we performed a factor analysis with maximum likelihood estimation and promax rotation to reveal the cats' personality structure, and extracted items with factor loadings greater than $|0.5|$. We calculated factor scores using the regression method.

For gene-personality association, we divided samples into three groups by genotype (*L/L*, *S/L*, *S/S*; see **Table 2** for grouping) to make the sample size and number of alleles in *S* and *L* of mongrel cats as equal as possible. After dividing, pairwise LD measurements (D′) were calculated to assess the linkage of each microsatellite. We used a generalized linear model

(GLM) to examine the association between factor scores as the response variable and independent variables; age (months), neutering status, sex, target genotype (*L/L*, *S/L*, *S/S*) and interaction of sex and genotypes were included as fixed effects. We visually checked the residual plots and normal Q–Q plots to confirm our assumption (normal distribution and homogeneity of variance) for each model. We used R (v. 3.4.2) for all statistical analyses (R Core Team, 2017), as well as the packages: *psych* (Revelle, 2015), *genetics* (Warnes and Warnes, 2007), *LDheatmap* (Shin et al., 2006), and *car* (Fox and Weisberg, 2011).

Results

Factor Analysis

We excluded questionnaire data (gene-behavior association test) of two owners due to missing data. Both the scree plot and a parallel analysis indicated four factors, and a factor analysis revealed the cats' personality structure based on the questionnaire. The items in each factor were almost identical to those in Arahori et al. (2016). Therefore, we named these four factors Openness, Friendliness, Roughness, and Neuroticism (**Table 3**). The 23 items had loadings over |0.5| on each factor except for the item "adaptable," which had a negative loading on Neuroticism but a positive loading on Friendliness (**Table 3**; underlined values). The Cronbach's alpha for each factor was acceptable (**Table 3**).

Linkage of Genotypes (after Dividing into *L* and *S*)

We plotted the correlation of genotypes on the heatmap using pairwise LD measurements (D′; **Figure 2A**). When dividing each allele into two groups (*S* and *L*) based on the sample size and number of alleles, the test showed that seven combinations out of 10 were in significant linkage disequilibrium (For MS2–MS4: $p < 0.01$; For MS1–MS2, MS1–MS4, MS2–MS3, MS2–MS5, MS3–MS4, and MS3–MS5: $p < 0.001$). There was no pair showing a complete linkage.

TABLE 3 | Factor loadings for the questionnaire.

	Openness	Friendliness	Roughness	Neuroticism
Playful	**0.923**	−0.04	−0.273	−0.088
Active	**0.807**	−0.139	−0.208	−0.244
Curious	**0.722**	0.231	0.131	−0.072
Inquisitive	**0.692**	0.096	0.106	−0.013
Inventive	**0.633**	0.037	0.012	0.051
Focused	**0.629**	0.223	−0.128	0.147
Mischievous	**0.523**	−0.106	0.168	−0.023
Vigilant	−0.119	−0.015	0.06	**0.755**
Fearful	−0.018	−0.065	0.113	**0.75**
Attentive	−0.137	0.054	0.008	**0.743**
Nervous	−0.116	0.125	0.167	**0.709**
Timid	0.046	−0.24	−0.068	**0.683**
Anxious	0.06	−0.18	0.117	**0.554**
Irritable	−0.131	−0.007	**0.889**	0.007
Moody	−0.15	0.022	**0.807**	0.148
Defiant	0.014	0.068	**0.797**	0.099
Dominant	−0.018	0.013	**0.782**	0.004
Aggressive	−0.167	−0.086	**0.576**	−0.066
Gentle	0.012	**0.745**	−0.184	0.088
Sociable	0.018	**0.725**	0.091	−0.235
Calm	−0.016	**0.671**	−0.033	−0.32
Friendly	0.056	**0.656**	0.074	−0.469
Adaptable	0.02	**0.632**	0.143	**−0.555**
Distractible	−0.136	−0.142	0.373	−0.295
Impulsive	0.295	−0.14	0.381	−0.058
Excitable	0.382	−0.134	0.44	0.062
Restless	0.304	−0.12	0.156	0.098
Quitting	−0.182	0.053	0.316	−0.196
Affectionate	0.143	0.363	0.053	0.059
Cautious	−0.005	0.382	−0.02	0.296
Variance explained (%)	13.9	10.8	13	14.3
Cronbach's alpha	0.85	0.86	0.88	0.87

The leftmost row represents all items used in this study. Cronbach's alpha is shown in the bottom. The underlined values mean the item which showed both positive and negative loadings. The bold numbers indicate factor loadings of items included in each factor.

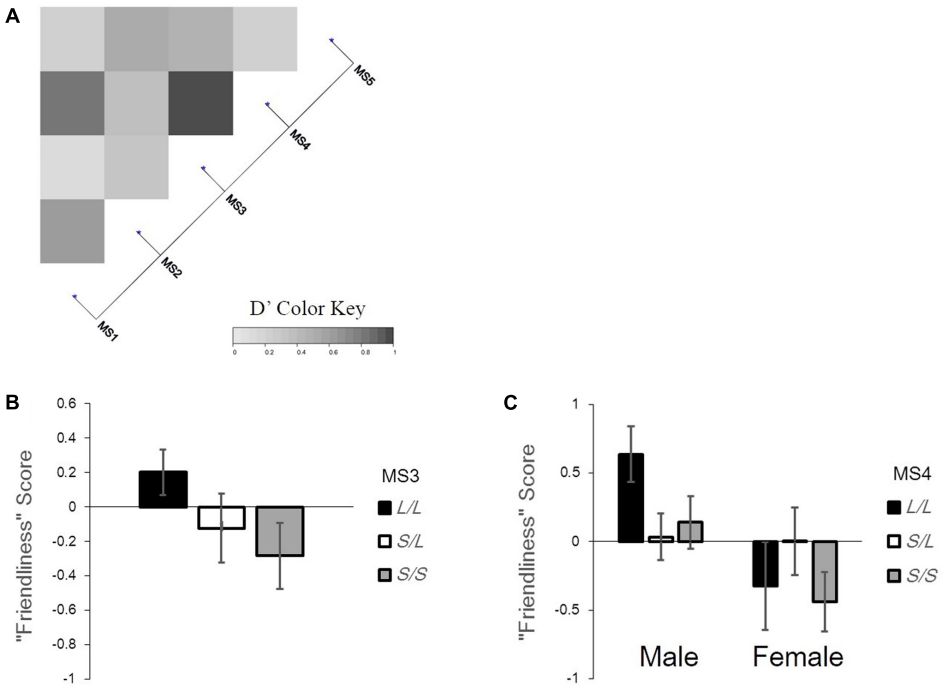

FIGURE 2 | The linkage of microsatellites grouped for gene-personality analysis and the gene-personality association found in this study. **(A)** Pairwise LD measurements and the heatmap plot of five microsatellite grouping. **(B)** The association between the genotypes of MS3 and "Friendliness" scores. **(C)** The association between sex, the genotypes of MS4 and "Friendliness" scores. The error bars represent standard errors.

Association between Independent Variables and Personality Scores

We checked our assumptions to see the residual plots and normal Q–Q plots (**Supplementary Figures S1**, **S2**) as satisfactory. When grouping into three groups (*L/L*, *S/L*, *S/S*), a GLM analysis (see **Supplementary Table S1** for all results) revealed a significant difference in Openness according to age, with older cats scoring lower than younger cats ($\beta = -0.008$, standard error (SE) = 0.002, 95% Wald confidence interval (CI) = [−0.012, −0.003], $\chi^2(1) = 12.558$, $p < 0.001$). The effect of sex was significant on Friendliness, with females being less friendly than males ($\beta = -2.251$, $SE = 1.333$, 95% CI = [−4.863, 0.361], $\chi^2(1) = 5.365$, $p = 0.021$). MS3's effect on Friendliness was marginally significant; cats with *S/S* alleles tended to score lower than cats with *L/L* and *S/L* alleles (**Figure 2B**; $\beta = -1.227$, $SE = 0.595$, 95% CI = [−2.393, −0.062], $\chi^2(2) = 4.963$, $p = 0.084$). Additionally, the interaction effect of sex × genotype in MS4 was significant (female × *S/L*: $\beta = 1.784$, $SE = 0.696$, 95% CI = [0.419, 3.148], $\chi^2(2) = 8.308$, $p = 0.016$; **Figure 2C**). Among males, the Friendliness score was higher in *L/L* groups than in other groups. On the other hand, among females, the Friendliness score was higher in *S/L* groups than in other groups. Sex and neutering effects were significant on Roughness, with females and neutered cats scoring higher than males and intact (not neutered) cats (sex: $\beta = 2.266$, $SE = 1.344$, 95% CI = [−0.368, 4.899], $\chi^2(1) = 6.885$, $p = 0.009$; neutering: $\beta = 0.798$, $SE = 0.34$, 95% CI = [0.131, 1.465], $\chi^2(1) = 5.498$, $p = 0.019$). We could not find any significant effect on the Neuroticism score.

DISCUSSION

This is the first study to survey microsatellites in the adjacent region of *OXTR* in cats. Microsatellites in the adjacent region of *OXTR* have been identified as important candidate genes related to social behavior in various species. We compared (1) allele frequencies between mongrel cats and purebred cats, and (2) examined the association between genotype and personality scores in mongrel cats.

First, the allele frequencies in five microsatellites were markedly different between mongrel and purebred cats. However, the test revealed significant linkage disequilibrium among almost all of the microsatellite loci, perhaps because they were located in the same gene.

Second, we analyzed the association between personality scores and the length of microsatellites in mongrel cats, and one significant and one marginal significant association were found with genotypes; the length of MS3 alleles and interaction of MS4 alleles and sex were associated with Friendliness. Taken together with the first finding, mongrel cats tended to have longer alleles in MS3 and MS4 than purebred cats, and those with longer alleles scored higher on Friendliness (**Figures 2B,C**). Takeuchi and Mori (2009) reported that Friendliness in Japanese domestic cats (mongrel cats) scored higher than in any other purebred cats examined. Our reports were consistent with their study; however, the functional reasons for the association for MS3 and MS4 were beyond the scope of our study. Because MS3 and MS4 were highly genetically correlated when dividing alleles into *L* and *S*

(**Figure 2A**) and the locations on *OXTR* were near (**Figure 1**), they might have shown similar effects. We also found that male cats scored higher on Friendliness than did female cats, and their scores changed depending on the length of MS4 alleles. In humans, some studies have reported sex-mediated effects of genotypes in terms of *OXTR* (e.g., Stankova et al., 2012), and our previous study also found sex × neutering × *OXTR* genotype effects on Roughness scores in cats (Arahori et al., 2016).

Our study had several limitations. First, our purebred samples were mainly from eastern populations, and Persian, Chinchilla, and Himalayan have a similar origin (Alhaddad et al., 2013; The Cat Fanciers' Association, 2017). Moreover, our sample size was too small for comparison among purebred cats. For future approaches, it would be recommended to carefully select 2 or 3 cat breeds using large sample size of each, with unique and easily comparable origins and selective pressures to reveal genetic differences related to their personality. As potential candidates one could think of using the Bengal breed (Gershony et al., 2014) for example, which is a hybrid of wildcats and domestic cats, or other carefully selected breeds that have already been revealed to differ in personality from mongrel cats. Lastly, we must note that GLM analysis was conducted after recognizing the high correlation (genetic linkage) among alleles/genotypes with possibilities of multicollinearity because they were positioned within the same gene.

Future studies have the potential to study other personality-related genes in cats and other felid species in terms of microsatellites. For example, *AVPR1A* is an important candidate gene related to social behavior in animals. In primates and prairie voles, microsatellites near (in the regulatory region of) *AVPR1A* are known to be related to mating systems, social organization, and sexual preferences (e.g., primates: Rosso et al., 2008; prairie voles: Castelli et al., 2011), as a result of comparing closely related species with different social systems. Only lions, cheetahs, and domestic cats are considered "social" felid species (Bradshaw, 2013), and this candidate-gene approach could reveal the *AVPR1A* effect.

CONCLUSION

Our study has shown polymorphisms in microsatellites in domestic cats. Associations in mongrel cats and differences in allele frequencies with purebred cats showed consistency with previous findings (Takeuchi and Mori, 2009). However, the role of microsatellites in these non-coding regions is still unclear and further research is therefore necessary, using different carefully selected breeds of cats.

ETHICS STATEMENT

This study adhered to the ethical guidelines of Kyoto University, and was approved by the Animal Experiments Committee of the Graduate School of Letters of Kyoto University (Approval reference number: 17-11).

AUTHOR CONTRIBUTIONS

MA designed this study, collected DNA sample and questionnaires, conducted genotyping, analyzed data, and drafted the manuscript. HA designed primers for genotyping. HC, ST, and BB contributed to data collection. MI-M and KF provided critical discussion regarding the analyses and the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2017.02165/full#supplementary-material

FIGURE S1 | Residual plots were shown for checking visually the assumption for normality of residuals. **(a)** Openness, **(b)** Friendliness, **(c)** Roughness, and **(d)** Neuroticism.

FIGURE S2 | Normal Q–Q plots were shown for checking visually homogeneity of variance. **(a)** Openness, **(b)** Friendliness, **(c)** Roughness, and **(d)** Neuroticism.

TABLE S1 | In each sheet, the GLM results for Openness, Friendliness, Roughness and Neuroticism were shown.

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frontiers in Neuroscience

ORIGINAL RESEARCH
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Validation of a Commercially Available Enzyme ImmunoAssay for the Determination of Oxytocin in Plasma Samples from Seven Domestic Animal Species

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The neurohormone oxytocin (OT) has a broad range of behavioral effects in mammals. It modulates a multitude of social behaviors, e.g., affiliative and sexual interactions. Consequently, the OT role in various animal species is increasingly explored. However, several issues have been raised regarding the peripheral OT measurement. Indeed, various methods have been described, leading to assay discrepancies and inconsistent results. This highlights the need for a recognized and reliable method to measure peripheral OT. Our aim was to validate a method combining a pre-extraction step, previously demonstrated as essential by several authors, and a commercially available enzyme immunoassay (EIA) for OT measurement, using plasma from seven domestic species (cat, dog, horse, cow, pig, sheep, and goat). The Oxytocin EIA kit (EnzoLifeSciences) was used to assay the solid-phase extracted samples following the manufacturer's instructions with slight modifications. For all species except dogs and cats, concentration factors were applied to work above the kit's sensitivity (15 pg/ml). To validate the method, the following performance characteristics were evaluated using Validation Samples (VS) at various concentrations in each species: extraction efficiency via spiking tests and intra- and inter-assay precision, allowing for the calculation of total errors. Parallelism studies to assess matrix effects could not be performed because of too low basal concentrations. Quantification ranges and associated precision profiles were established to account for the various OT plasma concentrations in each species. According to guidelines for bioanalytical validation of immunoassays, the measurements were sufficiently precise and accurate in each species to achieve a total error $\leq$30% in each VS sample. In each species, the inter-assay precision after 3 runs was acceptable, except in low concentration samples. The linearity under dilution of dogs and cats' samples was verified. Although matrix effects assessments are lacking, our results indicate that OT plasma levels can reliably be measured in several domestic animal species by the method described here. Studies involving samples with low OT plasma concentrations should pay attention to reproducibility issues. This work opens new perspectives to reliably study peripheral OT in a substantial number of domestic animal species in various behavioral contexts.

Keywords: oxytocin, measurement, enzyme immunoassay, analytical validation, extraction, pets, farm animals, mammals

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INTRODUCTION

Oxytocin (OT) is a 9-amino-acid neurohormone that is universally present in mammals and plays a key role in regulating a broad variety of social behaviors ranging from social memory and affiliation (sexual and parental behaviors, attachment, pair bonding) to aggression ("mate-guarding," maternal aggression) (for reviews, see Lim and Young, 2006; Lee et al., 2009; Ziegler and Crockford, 2017). Emotional behaviors, such as anxiety-related behaviors and stress coping are also modulated by OT (Uvnäs-Moberg, 1998; Amico et al., 2008; Neumann, 2008). In particular, OT has been described as being involved in positive social interactions in intra-species interactions (Grewen et al., 2005; Baumgartner et al., 2008; Ditzen et al., 2009; Dunbar, 2010; Smith and Wang, 2012; Romero et al., 2015; Numan and Young, 2016) and inter-species interactions, such as human-animal interactions (Odendaal and Meintjes, 2003; Beetz et al., 2012; Rault, 2012, 2016; Romero et al., 2014). Some authors even suggest a role of OT in enhancing well-being (Uvnäs-Moberg and Petersson, 2005; Ishak et al., 2011).

Consequently, there is a growing interest in investigating and explaining the role of OT in behavioral neuroscience, which has led to an increasing number of studies on the topic. Some of these studies have focused on behavioral effects in different contexts of OT administration. Routes for OT administration are variable: of note, the intranasal administration of OT has recently solicited considerable interest because of its non-invasive nature, its ease of use, and its direct access to the brain, circumventing the blood-brain barrier and the rapid degradation of OT in blood (Veening and Olivier, 2013; for reviews, see Olff et al., 2013). Importantly, Lee et al. (2017) developed a method based on spectrometry to distinguish endogenous and exogenous OT that showed that intravenously or intranasally administrated OT were able to reach the brain in a similar way. Another approach investigating the behavioral/emotional effects of OT is based on correlational studies measuring OT levels in biological samples, such as excretory fluids (urine, saliva), blood or cerebrospinal fluid (Alves et al., 2015). However, the assessment and interpretation of urine and saliva measurements provide inconsistent findings and require further investigation, notably using efficient assay methods (Horvat-Gordon et al., 2005; Anderson, 2006; Carter et al., 2007; Young and Anderson, 2010). Conversely, plasma and CSF samples, despite being more invasive (though to a lesser extent for plasma), displayed interesting results with established correlations between OT concentrations in CSF and plasma samples in several species (Neumann et al., 2013; Carson et al., 2014; Dal Monte et al., 2014). Of note, Dal Monte et al. (2014) and Freeman et al. (2016) obtained contradictory results regarding the correlation of OT concentration in CSF and plasma in the same species *Macaca mulatta*, which can be explained by looking more closely at the measurement methods used: Dal Monte et al. (2014) used extracted plasma to assay OT and found correlation between OT levels in plasma and CSF while (Freeman et al., 2016) used unextracted plasma and did not find any correlation.

Indeed, there are several methodological issues regarding OT measurement reliability since the methods used to analyze plasma OT greatly vary in published reports that investigate the role of OT in social behavior, leading to some inconsistent results. The assay methods described in these studies vary widely and may or may not apply plasma extraction (with different types of extraction protocols), as previously highlighted (Christensen et al., 2014; Robinson et al., 2014). Historically, radioimmunoassay (RIA) procedures were used, usually involving prior extraction procedures (Kendrick et al., 1991; Gilbert et al., 1996; Wotjak et al., 1998; Cool and Debrosse, 2003; Handler et al., 2003). Little by little, enzyme immunoassays became more prevalent than RIA due to their safety and ease of use, and for unknown reasons, extraction steps were also gradually abandoned by some researchers as highlighted by Dal Monte et al. (2014), McCullough et al. (2013), and Nave et al. (2015). But, since the beginning of enzyme immunoassay (EIA) use to assay OT in plasma, some authors have added methodological caveats after having firmly demonstrated that extraction was crucial in order to remove interfering immunoreactive molecules and obtain consistent OT concentrations with the first studies involving RIA (Szeto et al., 2011; Robinson et al., 2014). Notably, in an elegant study, Szeto et al. (2011) proved the necessity of performing extraction both in RIA and EIA to eliminate compounds that artificially increase the apparent plasma OT levels. Indeed, they demonstrated that the linearity and the accuracy of measurements was only verified in extracted samples and that there was no correlation between OT values in extracted samples and OT values in unextracted samples. Additionally, using HPLC characterization of immunoreactive fractions, they showed that some non-OT immunoreactive products (with molecular masses larger than that of OT) persist even in the extracted samples. The authors suggest that these products could come from OT degradation. The critical importance of extracting plasma to assay OT in order to obtain reliable results was again underlined in various reviews (McCullough et al., 2013; Leng and Ludwig, 2016), and even by the EIA manufacturer (ENZO Life Sciences, 2015).

Furthermore, immunoassays require validation as highlighted and defined by Andreasson et al. (2015), DeSilva et al. (2012), and Kelley and DeSilva (2007). Specifically, the European Medicines Agency (EMA) set up guidelines for bioanalytical validation in 2011 (European Medicines Agency, 2011) to facilitate the adoption of these good analytical practices. In human pharmaceutical research, this approach is now standard. However, this is not necessarily the case when it comes to animal science. In addition, some immunoassay kits designed for use with human samples are directly used with animal samples without prior validation (Young and Anderson, 2010; McCullough et al., 2013). This can be problematic since basal levels of analyte may differ between species and may be subject to matrix effects due to the variable presence of interfering substances in biological fluids. It is then important to validate methods of measurement, even pre-existing or commercial methods, when changing the sample's origin and/or nature to obtain reliable results (Kelley and DeSilva, 2007; European Medicines Agency, 2011). The procedure for validating the method can be adapted according to the application and the intended use of the measured analyte (Lee et al., 2006; Valentin et al., 2011).

The goal of this study is to provide a detailed analytical validation of a reliable and universal method for assaying OT in plasma samples from various domestic animal species. Indeed, in some domestic species, such as pets and farm animals, plasma is the biological fluid most readily available, unlike CSF (which is assessed more easily in studies involving lab animals like mice or rats). The panel of domestic species was chosen to meet the needs that may exist in behavioral/emotional studies involving OT, notably on human-animal interactions (Coulon et al., 2012; Rault et al., 2013; Kis et al., 2015; Oliva et al., 2015; Thielke and Udell, 2017), maternal behavior (Castrén et al., 1993; Hernandez et al., 2002; Poindron et al., 2007; Ishii et al., 2008), or stress coping (Yayou et al., 2010; Sutherland and Tops, 2014), where OT can be assessed as a biomarker for a particular behavioral or emotional state. For the reasons stated earlier, we decided to perform an analytical validation based on the EMA guidelines for OT measurement using extracted plasma samples followed by EIA.

MATERIELS AND METHODS

Study Animals

Seven adult mammal species of both sexes were involved in this study. Dogs (*Canis familiaris*), cats (*Felis domesticus*), horses (*Equus caballus*), pigs (*Sus scrofa*), goats (*Capra hircus*), and sheep (*Ovis aries*) were from IRSEA breeding (Apt, France, approval n° A 84-400-1). Cows (*Bos taurus*) were from the experimental farm of the Institut Agricole Régional (I.A.R., Reg. La Rochère 1/A, 11100 Aoste, Italy). The housing, husbandry and the use of animals in the procedures described in this article were carried out following the French and European legislation and in compliance with the principles of replacement, reduction and refinement. All procedures were performed with approval from the Ethics Committee C2EA125, in harmony with French and European legislation.

Blood Sampling

During the morning (7:00–13:00), blood was collected by a veterinarian from the jugular vein of animals involved in this study in pre-chilled EDTA-Aprotinin tubes (BD® tubes, Elvetec, Pusignan, France) and centrifuged at 4°C at 1,200 g to recover plasma. The volume of blood drawn varied according to species. In cases of delay (max. 3 h) between the sampling and centrifugation, tubes were kept at 4°C in order to avoid OT degradation, which was also enhanced by the use of aprotinin as a protease inhibitor. Recovered plasma was aliquoted in several tubes to avoid repeated freeze/thaw cycles, which could degrade OT, and stored at −20°C until further use. To limit OT degradation during the storage, all samples were assayed within 2 months (Marnet et al., 1994).

Oxytocin Enzyme ImmunoAssay

Plasma OT was measured using the EIA kit initially developed by Assay Designs Inc. and currently provided by Enzolifesciences (Villeurbanne, France, catalog No. 900-153A), following the manufacturer's instructions. This kit has been increasingly used in studies assaying OT in various animal species in the last decade (Robinson et al., 2014; Leng and Ludwig, 2016). Notably, it was previously assessed by Szeto et al. (2011) in a thorough work studying the effect of OT extraction from plasma prior to RIA and EIA. According to the manufacturer, the kit's sensitivity is 15.0 pg/ml; intra-assay precision at low, medium and high concentrations are 12.6, 10.2, and 13.3% respectively; inter-assay precision at low, medium and high concentrations are 20.9, 16.5, and 11.8% respectively.

Plasma Oxytocin Extraction and Concentration

Plasma OT was measured after a solid-phase extraction (SPE) using C18 columns following the manufacturer's instructions with the following modifications. The C18 columns used were 1 g HyperSep (Thermofischer, Illkirch, France) to ensure their capacity to deal with large volumes of plasma and so 2 ml of acetonitrile was used to equilibrate them. The elution buffer was 60% acetonitrile/40% TFA 0.1% since during prior observations on several animals' plasmas, this elution buffer displayed better recovery than the elution buffer recommended by the provider (95% acetonitrile/5% TFA 0.1%). The samples were dried overnight at room temperature in a centrifugal concentrator under vacuum. A volume of 600 µl of Assay buffer was used to reconstitute the samples in order to run the assay in triplicates and to avoid pipetting insoluble material which may be observed in some samples (here, it was particularly the case for dog samples). Before carefully pipetting the reconstituted samples into the EIA plate wells, tubes were also quickly spun to move the insoluble material downward. SPE is strongly recommended by the kit provider (ENZO Life Sciences, 2015). The extraction helps eliminate potential interfering molecules and diminishes the matrix effect. In addition, during the extraction step, OT molecules can be concentrated from the sample prior to EIA for aid in measurement. In this study, sample concentration was necessary for cow (20-times), horse (15-times), pig (10-times), goat (10-times), and sheep (10-times) samples to reach concentration values within the kit's standard range. Dog and cat samples were not concentrated. Consequently, the volume of extracted plasma from each species was adjusted from the beginning of the procedure so that the volume of assay buffer could be held constant at 600 µl and the concentration factors specified above could be applied (for instance, the volume of treated pig plasma was 6 ml in these conditions).

Assessment of Validation Criteria

According to the EMA Guideline on bioanalytical method validation (2011), commercial kits need to be revalidated to ensure that the sample analysis is performed accurately and precisely. Additionally, a change of biological matrix or species is a reason to perform a partial validation, which "can range from the determination of the within-run precision and accuracy to an almost full validation." The precision of an analytical procedure is defined as "the closeness of agreement between a series of measurements obtained under the prescribed conditions" and is expressed as the ratio of standard deviation/mean (%), also known as coefficient of variation (CV%). Two types of

precision can be evaluated: the intra-assay precision or within-run precision and the inter-assay precision or between-run precision. The accuracy of an analytical procedure is defined as "the closeness of the determined value to the value which is accepted either as a conventional true value or an accepted true value." In accordance with EMA recommendations and the principle of the Fit-for-Purpose method validation for biomarker measurements (Lee et al., 2006; Valentin et al., 2011), the authors have chosen to perform a partial validation of the Enzolifesciences Oxytocin EIA kit, including assessment of within-run and between-run precision and accuracy. Of note, parallelism studies to detect possible matrix effects or differing antibodies' affinities between the endogenous analyte and the standard, that assess serially diluted study samples along the standard curve, are also a crucial step of the validation procedure (European Medicines Agency, 2011; Valentin et al., 2011; DeSilva et al., 2012; Andreasson et al., 2015). Regrettably, in view of the low basal levels of plasma OT eventually found in each species, it was not possible to conduct this test since, as soon as the actual samples were diluted, the authors faced the issue of an OT level decrease below the kit's sensitivity. For the assessment of precision and accuracy, three validation samples (VS), which are spiked plasma samples at three levels of concentration, were prepared by spiking study samples with 50 pg of OT standard before the SPE. Thanks to the high concentration of the OT standard (10,000 pg/ml), we were able to ensure the spiking solution represented <5% of the final volume, as recommended by Valentin et al. (2011). They were stored and treated according to the same procedures used for the analysis of study samples, including the extraction and concentration steps, so that the global procedure could be evaluated and validated. It is precisely because this is the global procedure (including possible concentration, extraction and assay steps) which is assessed through the VS spiked at the beginning of the process that the term "accuracy" is now replaced by "extraction efficiency" when specifically relating to the study outcomes, in order to take into account the combination of the SPE efficiency and the proper accuracy of the EIA, which cannot be distinguished in this case. Study samples being run in triplicate, the within-run precision was determined using 3 replicates of measures. Three independent runs were performed on different days, using two different lots of EIA kits from Enzolifesciences, to establish the between-run precision (the kit lot used in run 3 was different from the kit lot used in run 1 and 2). The VS levels of concentration were chosen according to the range of quantification preliminarily established for each species, including at least one level close to the Lower Limit of Quantification (LLOQ) observed within the study samples. The LLOQ is defined as the lowest concentration in a sample which can be quantitatively determined with acceptable levels of precision and accuracy and is different from the Limit of Detection (LOD), or sensitivity, which is defined by the manufacturer in a sample-independent manner. To preliminarily determine the range of quantification for each species, numerous study samples were assayed in triplicate during multiple runs and a precision profile was established, from which the LLOQ and ULOQ (Upper Limit of Quantification) were determined. In this context and in order to stay in close touch with the real samples, all values found along the standard range described by the manufacturer were taken into account, including the ones found above the 80% intercept. Acceptance criteria were as defined by the EMA, as follows: regarding extraction efficiency, the absolute mean bias % relative error (RE) should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ) and the precision should not exceed 20% of CV (25% at the interval between 1x and 3xLLOQ). Furthermore, the total error is a concept that expresses the closeness of agreement between a measured test result and its theoretical true value (DeSilva et al., 2012). The term total error describes the combination of systematic error (mean bias) and random error (precision): it is calculated by summing the absolute value of the % RE and the % CV and should not exceed 30% (40% at the interval between 1x and 3xLLOQ). At least 67% of VS samples should reach these acceptance criteria. In addition, the linearity under dilution was assessed in samples where no concentration was required (i.e., dogs and cats' samples) by spiking two pools of plasma samples with two different high amounts of OT standards (800 and 500 pg) to ensure reaching high concentrations within the kit's dynamic range and supporting five points of 1:2 serial dilutions.

RESULTS

Precision Profiles

The precision profiles obtained for each of the seven studied species are presented in **Figure 1** and contain values from neat and spiked samples tested throughout the study. Spiked samples served to extend the range of quantification with higher OT concentrations than neat samples. The range of quantification in this study was delimited by the LLOQ determined for each species and shown in boxes inside the charts. Importantly, precision was satisfactory in all the samples tested for each species throughout the quantification range with CV <20%, or <25% for concentrations comprised in an interval of 1xLLOQ-3xLLOQ. Dog and cat samples were not concentrated and consequently displayed narrower ranges of quantification (0–150 pg/ml) than other animal samples whose concentrations were multiplied by at least a factor of 10 during the extraction step, leading to a range of quantification extending to a maximum of 350 pg/ml in sheep. When the specified concentration factors are applied, mean plasma OT concentrations can be calculated from neat samples in each species population: 26.6 ± 10.5 pg/ml in dogs (n = 9); 10.9 ± 5.0 pg/ml in cats (n = 10); 4.6 ± 3.9 pg/ml in horses (n = 14); 2.9 ± 1.5 pg/ml in cows (n = 30); 11.3 ± 9.0 pg/ml in sheep (n = 10); 9.1 ± 6.7 pg/ml in goats (n = 12); and 6.7 ± 4.1 pg/ml in pigs (n = 11).

Within-Run Precision, Extraction Efficiency and Total Error Assessment

According to the precision profile of each species, VS with OT concentrations falling within the range of quantification determined here were tested to assess within-run precision and extraction efficiency (**Tables 1**–**7**) and calculate the total errors. All three dog, cat, cow, sheep, goat and pig VS displayed

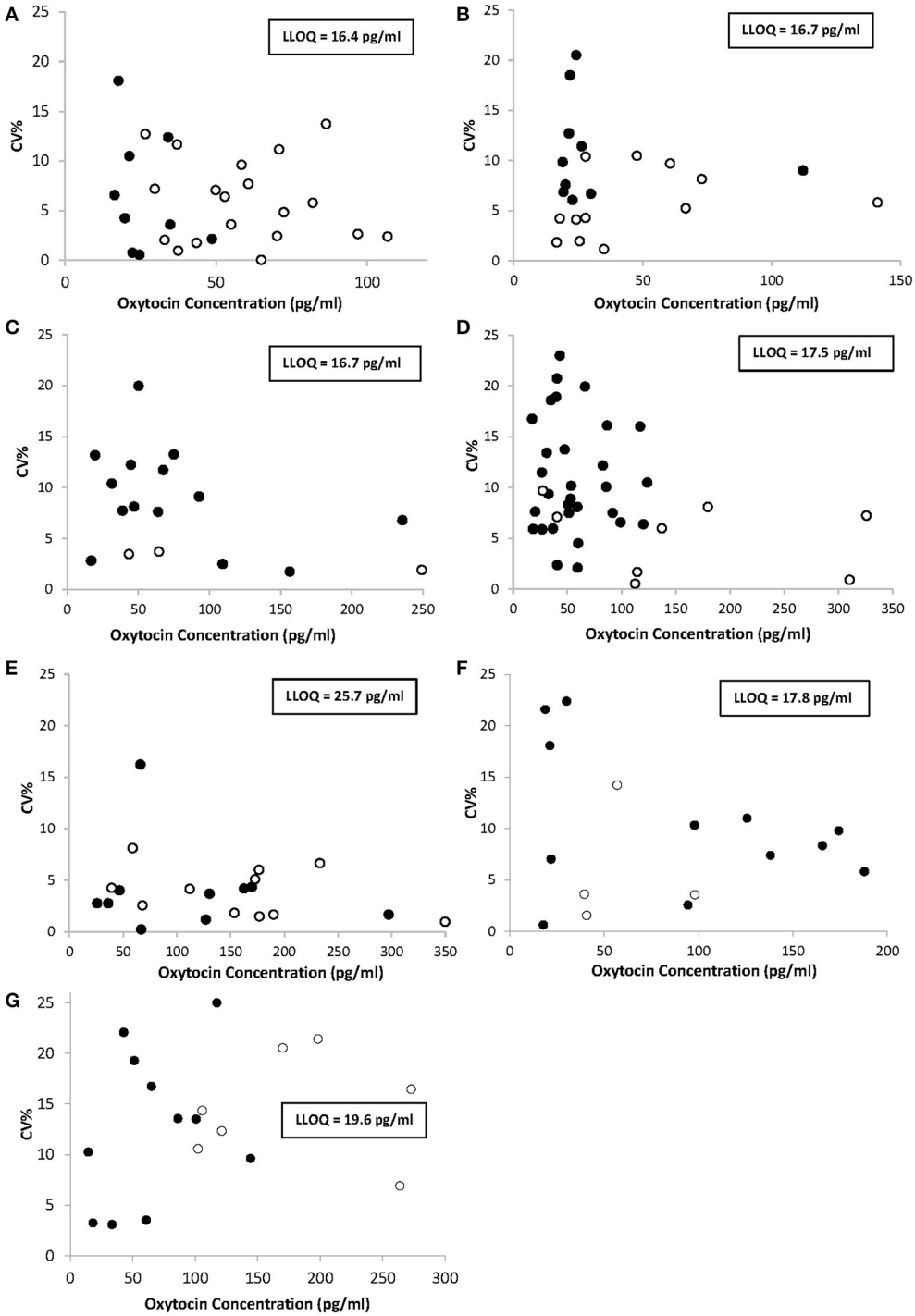

FIGURE 1 | Precision profiles of oxytocin concentrations determined from triplicate measures of multiple runs in neat samples (•) or spiked samples (○) from dogs **(A)**, cats **(B)**, horses **(C)**, cows **(D)**, sheep **(E)**, goats **(F)**, and pigs **(G)**. %CV indicates the percent coefficient of variation. Oxytocin concentrations stand for the concentrations measured in the wells of the EIA plate and not in the animals' plasma, for which specified concentration factors must be applied. LLOQ, lower limit of quantification.

satisfactory results, with a total error ≤30% and intermediate levels of precision and extraction efficiency with acceptable values, except VS2 in dog and VS3 in cow. In horse VS, VS2 exhibited a total error >30% although intermediate levels of within-run precision and extraction efficiency were acceptable on their own, while VS1 and VS3 gave satisfactory results. Overall,

TABLE 1 | Within-run precision, extraction efficiency and total error in dog VS.

	Precision			Extraction efficiency						± Total error (%)	Total error acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Expected concentration after spiking (pg/ml)	Mean of measured concentration after spiking (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	% Spiking recovery	± Relative error (%)		
VS1	17.6	18.1	≤25	67.6	54.9	3.6	≤20	81	19	22.6	≤30
VS2	22.2	0.8	≤25	55.5	70.1	2.5	≤20	126	**26**	28.5	≤30
VS3	48.6	2.2	≤25	90.1	72.4	4.9	≤20	80	20	24.9	≤30

Acceptance criteria in this species were as follows: the absolute mean bias % relative error (RE) and the %CV should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ, i.e., 16.4–49.2 pg/ml). The total error should not exceed 30% (40% at the interval around the LLOQ). Values out of the acceptance range are indicated in bold.

TABLE 2 | Within-run precision, extraction efficiency and total error in cat VS.

	Precision			Extraction efficiency						± Total error (%)	Total error acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Expected concentration after spiking (pg/ml)	Mean of measured concentration after spiking (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	% Spiking recovery	± Relative error (%)		
VS1	21.8	18.5	≤25	41.8	47.8	10.5	≤20	114	14	24.5	≤40
VS2	26.4	11.4	≤25	45.6	34.9	1.2	≤25	77	23	24.2	≤40
VS3	112.2	9.0	≤20	145.2	141.1	5.8	≤20	97	3	8.8	≤30

Acceptance criteria in this species were as follows: the absolute mean bias % relative error (RE) and the %CV should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ, i.e., 16.7–50.1 pg/ml). The total error should not exceed 30% (40% at the interval around the LLOQ).

TABLE 3 | Within-run precision, extraction efficiency and total error in horse VS.

	Precision			Extraction efficiency						± Total error (%)	Total error acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Expected concentration after spiking (pg/ml)	Mean of measured concentration after spiking (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	% Spiking recovery	± Relative error (%)		
VS1	17.0	1.1	≤25	79.5	64.4	3.7	≤20	81	19	22.7	≤30
VS2	50.1	3.3	≤25	158.1	128.1	14.9	≤20	81	19	**33.9**	≤30
VS3	235.5	15.7	≤20	297.9	249.1	1.9	≤20	84	16	17.9	≤30

Acceptance criteria in this species were as follows: the absolute mean bias % relative error (RE) and the %CV should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ, i.e., 16.7–50.1 pg/ml). The total error should not exceed 30% (40% at the interval around the LLOQ). Values out of the acceptance range are indicated in bold.

TABLE 4 | Within-run precision, extraction efficiency and total error in cow VS.

	Precision			Extraction efficiency						± Total error (%)	Acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Expected concentration after spiking (pg/ml)	Mean of measured concentration after spiking (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	% Spiking recovery	± Relative error (%)		
VS1	26.9	1.3	≤25	43.7	40.4	7.1	≤25	92	8	15.1	≤40
VS2	59.9	3.0	≤20	126.6	137.1	6.0	≤20	108	8	14.0	≤30
VS3	91.8	4.6	≤20	154.3	114.5	1.7	≤20	74	**26**	27.7	≤30

Acceptance criteria in this species were as follows: the absolute mean bias % relative error (RE) and the %CV should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ, i.e., 17.5–52.5 pg/ml). The total error should not exceed 30% (40% at the interval around the LLOQ). Values out of the acceptance range are indicated in bold.

TABLE 5 | Within-run precision, extraction efficiency and total error in sheep VS.

	Precision			Extraction efficiency						± Total error (%)	Acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Expected concentration after spiking (pg/ml)	Mean of measured concentration after spiking (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	% Spiking recovery	± Relative error (%)		
VS1	65.9	16.2	≤25	128.4	111.8	4.2	≤20	87	13	17.2	≤30
VS2	130.3	3.7	≤20	189.7	176.3	6.0	≤20	93	7	13.0	≤30
VS3	297.0	1.7	≤20	356.3	349.5	1.0	≤20	98	2	3.0	≤30

Acceptance criteria in this species were as follows: the absolute mean bias % relative error (RE) and the %CV should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ, i.e., 25.7–77.1 pg/ml).

TABLE 6 | Within-run precision, extraction efficiency and total error in goat VS.

	Precision			Extraction efficiency						± Total error (%)	Acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Expected concentration after spiking (pg/ml)	Mean of measured concentration after spiking (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	% Spiking recovery	± Relative error (%)		
VS1	21.5	18.4	≤25	48.1	39.5	3.6	≤25	82	18	21.6	≤40
VS2	23.5	17.2	≤25	50.1	40.7	1.6	≤25	81	19	20.6	≤40
VS3	34.0	11.0	≤25	96.4	97.9	3.6	≤20	102	2	5.6	≤30

Acceptance criteria in this species were as follows: the absolute mean bias % relative error (RE) and the %CV should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ, i.e., 17.8–53.4 pg/ml).

TABLE 7 | Within-run precision, extraction efficiency and total error in pig VS.

	Precision		Extraction efficiency						± Total error (%)	Acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Expected concentration after spiking (pg/ml)	Mean of measured concentration after spiking (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	% Spiking recovery	± Relative error (%)	
VS1	19.6	6.0	≤25	86.2	89.6	6.3	≤20	104	4	10.3	≤30
VS2	34.6	1.4	≤25	101.2	99.9	1.5	≤20	126	1	2.5	≤30
VS3	52.3	0.9	≤25	119.0	134.1	7.3	≤20	113	113	20.3	≤30

Acceptance criteria in this species were as follows: the absolute mean bias % relative error (RE) and the %CV should be ≤20% of the nominal value at each concentration level (≤25% at the interval between 1x and 3xLLOQ, i.e., 19.6–58.8 pg/ml).

100% of VS from dogs, cats, cows, sheep, pigs and goats and 67% of VS from horses reached the acceptance criteria.

Between-Run Precision

As shown in **Tables 8–14**, in each species, one VS did not provide satisfactory results regarding between-run precision and this is almost always observed in the VS displaying the lowest concentration (except in pigs' VS), and is sometimes even comprised within the 1x-3xLLOQ interval. Some intermediate levels of within-run precision were also unsatisfactory, particularly in run 2. Of note, results obtained in run 3, from a different kit lot, were not remarkably different from those obtained in runs 1 and 2. Finally, in all species, 67% of VS reached the acceptance criteria for between-run precision.

Dilutional Linearity

In **Figure 2**, the dilutional linearity was shown in canine and feline plasma samples by reporting the measured values vs. the expected theoretical values, calculated on the basis of the spike amount in the initial samples and the following serial dilutions. The resemblance of the measured and expected values of OT concentration was demonstrated by the slope of the linear regression curve, which is close to 1 in every case. In addition, the coefficient of correlation R^2 of the linear regression curve was also meaningful as its value nearby 1 showed the concentrations of the diluted samples were not scattered but along the linear regression curve.

DISCUSSION

In this study, we assessed the suitability of an EIA kit (from Enzolifesciences) for accurately and precisely assaying OT concentrations in solid-phase extracted plasmas from seven domestic animal species. As results obtained in at least 67% of the tested VS were satisfactory regarding the acceptance criteria established in agreement with the EMA Guidelines and the Fit-For-Purpose principle (Lee et al., 2006) for within-run precision and extraction efficiency, we can conclude that the use of this method has been validated for the measurement of plasma OT levels in dog, cat, horse, cow, sheep, goat and pig samples. Furthermore, 100% of VS fulfilled all acceptance criteria in cats, sheep, goats, and pigs. Between-run precisions were also satisfactory and yet highlighted substantial variations in the measurements near the LLOQ values. This was observed in almost all studied species and consequently the authors recommend cautiously interpreting the results obtained in these lowest ranges of concentrations, especially when several runs are needed. For this reason, and because of the sensitivity limit of the kit at 15 pg/ml, we decided to perform sample concentration during the extraction steps in almost all species, except for dogs and cats for which it was not necessary to do so. Indeed, in these species, the basal OT values found in this study were above the kit's LOD. For the other species, assay attempts were made without concentrating their plasma, but were unsuccessful as we found out afterwards that their basal peripheral levels of OT were below the kit LOD. Sample concentration was thus necessary to reach OT values in the dynamic range of the EIA kit and

TABLE 8 | Between-run precision in dog VS, established from three independent runs on different days, using two different lots of Enzolifesciences EIA kits.

	Run 1			Run 2			Run 3			Between-run mean concentration (pg/ml)	Between-run precision (%CV)	Precision acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)			
VS A	26.1	3.7	≤25	39.7	**31.9**	≤25	52.7	**23.0**	≤20	39.5	**33.7**	≤25
VS B	73.5	10.5	≤20	67.3	**23.8**	≤20	79.9	**27.7**	≤20	73.5	8.6	≤20
VS C	132.8	20.0	≤20	124.7	**24.0**	≤20	125.9	14.3	≤20	127.8	3.4	≤20

Values out of the acceptance range (i.e., if CV >20%, or >25% at the interval between 1x and 3xLLOQ) are indicated in bold.

TABLE 9 | Between-run precision in cat VS, established from three independent runs on different days, using two different lots of Enzolifesciences EIA kits (EIA kit lot used in run 3 was different from EIA kit lot used in run 1 and 2).

	Run 1			Run 2			Run 3			Between-run mean concentration (pg/ml)	Between-run precision (%CV)	Precision acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)			
VS A	361.2	17.6	≤20	338.2	9.1	≤20	312.6	9.3	≤20	337.4	7.2	≤20
VS B	220.8	6.9	≤20	191.0	8.4	≤20	196.4	17.2	≤20	202.8	7.8	≤20
VS C	43.0	24.7	≤25	50.2	**22.8**	≤20	66.0	**21.0**	≤20	53.0	**22.2**	≤20

Values out of the acceptance range (i.e., if CV >20%, or >25% at the interval between 1x and 3xLLOQ) are indicated in bold.

TABLE 10 | Between-run precision in horse VS, established from three independent runs on different days, using two different lots of Enzolifesciences EIA kits (EIA kit lot used in run 3 was different from EIA kit lot used in run 1 and 2).

	Run 1			Run 2			Run 3			Between-run mean concentration (pg/ml)	Between-run precision (%CV)	Precision acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)			
VS A	26.1	3.7	≤25	39.7	**31.9**	≤25	52.7	**23.0**	≤20	39.5	**33.7**	≤25
VS B	73.5	10.5	≤20	67.3	**23.8**	≤20	79.9	**27.7**	≤20	73.5	8.6	≤20
VS C	132.8	20.0	≤20	124.7	**24.0**	≤20	125.9	14.3	≤20	127.8	3.4	≤20

Values out of the acceptance range (i.e., if CV >20%, or >25% at the interval between 1x and 3xLLOQ) are indicated in bold.

TABLE 11 | Between-run precision in cow VS, established from three independent runs on different days, using two different lots of Enzolifesciences EIA kits (EIA kit lot used in run 3 was different from EIA kit lot used in run 1 and 2).

	Run 1			Run 2			Run 3			Between-run mean concentration (pg/ml)	Between-run precision (%CV)	Precision acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)			
VS A	64.5	**31.9**	≤20	67.2	19.5	≤20	101.3	**32.7**	≤20	77.7	**26.4**	≤20
VS B	251.5	**27.3**	≤20	294.8	10.9	≤20	277.7	15.2	≤20	274.7	7.9	≤20
VS C	268.5	12.8	≤20	210.4	19.5	≤20	246.7	18.9	≤20	241.9	12.1	≤20

Values out of the acceptance range (i.e., if CV >20%, or >25% at the interval between 1x and 3xLLOQ) are indicated in bold.

TABLE 12 | Between-run precision in sheep VS, established from three independent runs on different days, using two different lots of Enzolifesciences EIA kits (EIA kit lot used in run 3 was different from EIA kit lot used in run 1 and 2).

	Run 1			Run 2			Run 3			Between-run mean concentration (pg/ml)	Between-run precision (%CV)	Precision acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)			
VS A	425.1	9.2	≤20	419.1	11.4	≤20	386.2	13.4	≤20	410.1	5.1	≤25
VS B	88.9	**32.9**	≤20	77.2	**45.3**	≤20	119.8	**41.6**	≤20	95.3	**23.1**	≤20
VS C	137.1	10.1	≤20	146.6	**37.9**	≤20	170.1	**34.0**	≤20	151.3	11.2	≤20

Values out of the acceptance range (i.e., if CV >20%, or >25% at the interval between 1x and 3xLLOQ) are indicated in bold.

TABLE 13 | Between-run precision in goat VS, established from three independent runs on different days, using two different lots of Enzolifesciences EIA kits (EIA kit lot used in run 3 was different from EIA kit lot used in run 1 and 2).

	Run 1			Run 2			Run 3			Between-run mean concentration (pg/ml)	Between-run precision (%CV)	Precision acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)			
VS A	246.5	15.7	≤20	219.6	36.5	≤20	244.2	31.2	≤20	236.7	6.3	≤20
VS B	127.2	14.2	≤20	126.3	**23.8**	≤20	162.6	**31.6**	≤20	138.7	14.9	≤20
VS C	43.2	**38.0**	≤25	61.5	**52.5**	≤20	71.2	**28.8**	≤20	58.6	**24.2**	≤20

Values out of the acceptance range (i.e., if CV >20%, or >25% at the interval between 1x and 3xLLOQ) are indicated in bold.

TABLE 14 | Between-run precision in pig VS, established from three independent runs on different days, using two different lots of Enzolifesciences EIA kits (EIA kit lot used in run 3 was different from EIA kit lot used in run 1 and 2).

	Run 1			Run 2			Run 3			Between-run mean concentration (pg/ml)	Between-run precision (%CV)	Precision acceptance criteria (%)
	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)	Mean concentration (pg/ml)	Within-run precision (%CV)	Precision acceptance criteria (%)			
VS A	494.8	25.3	≤20	408.2	9.6	≤20	566.0	25.4	≤20	498.7	16.1	≤20
VS B	63.8	**40.9**	≤20	57.9	11.9	≤25	95.6	**3.8**	≤20	72.4	**28.0**	≤20
VS C	58.5	11.8	≤25	75.5	**31.9**	≤20	72.4	**28.0**	≤20	66.3	13.0	≤20

Values out of the acceptance range (i.e., if CV >20%, or >25% at the interval between 1x and 3xLLOQ) are indicated in bold.

concentration factors were adapted to each species after several analyses. Nevertheless, sample concentration required higher amounts of plasma, and thus a greater volume of blood, which must be drawn without jeopardizing animal health. This has to be taken into account from an ethical point of view and considering the 3Rs rule (Franco and Olsson, 2014). In the case of domestic animals enrolled in this study, they were of sufficient size to safely supply the volume of blood required.

Here, the authors have chosen to perform a partial validation since the kit was of a commercial nature and already validated for use in human plasma. Indeed, the EMA Guidelines state that a full validation is not required when a change in biological matrix occurs, which is the case in this study, with plasma originating from domestic animals. A partial validation can range from as little as the determination of the within-run precision and accuracy to a nearly full validation. In our partial validation, we established the precision profile of OT measurements in each studied species and assessed the extraction efficiency (reflecting the global procedure accuracy as explained before), within-run and between-run precisions. Importantly, despite precision and extraction efficiency yielded satisfactory results, we were not able to assess matrix effects. Even if the SPE method and the use of assay buffer as diluent to reconstitute samples are expected to remove interfering substances and minimize the risk of matrix effects (Tate and Ward, 2004), we cannot be sure that all potentially interfering substances are indeed removed, especially after concentration of the plasma samples. So, results should still be interpreted with caution, as perhaps discrepancies might be found because of putative persisting matrix effects in some cases. Despite their importance, parallelism studies between the calibration standard curve and serially diluted study samples could not be performed due to very low basal levels of OT in each species, which made it impossible to obtain the needed serial dilutions for this kind of study, even after concentrating the samples many times. As already stated, the sample concentration required a large amount of plasma to be taken, which was a real constraint depending on the studied species, notably in the smallest animals. In addition, handling large volumes of plasma is a source of increasing errors and practical complications entailed with the use of HyperSep C18 columns, which can clog. So further increasing the concentration factors to artificially elevate the sample OT concentrations in order to carry out parallelism studies was not a desirable option, considering that it would also have alienated the aim from using real samples reflecting the actual study populations, as recommended by Valentin et al. (2011). Finally, a parallelism study can also provide doubtful evidence, as highlighted by McCullough et al. (2013) about the results obtained without extraction steps in Kramer et al. (2004), which were subsequently questioned by Szeto et al. (2011). Besides, because of the prior extraction steps, we did not purposely include lipemic or hemolytic samples in our study. Indeed, in a series of experiments, we observed that extraction steps were effective enough to prevent any discrepancies in the measurements. In the present study, dilution was never required, and samples from 5/7 species even had to be concentrated. However, to provide data on the resemblance between the endogenous analyte and the standard, we performed dilutional

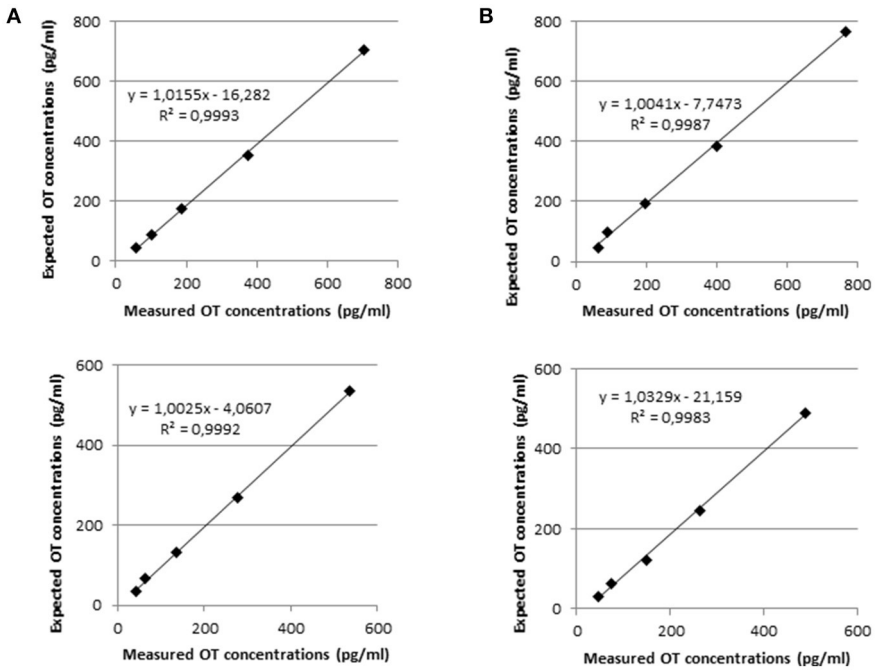

FIGURE 2 | Linearity under dilution of two samples of canine plasma **(A)** and feline plasma **(B)**. The above and below panels show the linearity under dilution was demonstrated in samples spiked with 800 and 500 pg of OT standards, respectively.

linearity tests on dogs and cats' samples, where no concentration was required. The linearity under dilution was ascertained in both samples origin, throughout the linear range of the kit. Despite the differences of biological matrix and the putative outcomes, it is also interesting to note that the dilutional linearity was demonstrated by the manufacturer in human serum (ENZO Life Sciences, 2015). As stated by Lee et al. (2006), the proven dilutional linearity could support parallelism.

Furthermore, the fit-for-purpose principle states that "assay validation should be tailored to meet the intended purpose of the biomarker study, with a level of rigor commensurate with the intended use of data" (Lee et al., 2006). In the present context of research on the link between OT and multiple behaviors or emotional status in domestic animals, OT can be considered as an exploratory biomarker. This is why the analytical validation performed here comprised 3 VS levels, as well as 3 independent runs with 3 replicates per run for precision and accuracy assessment, as supported by the literature (Lee et al., 2006; Valentin et al., 2011). In addition, we chose triplicate analysis over the "classical" duplicate analysis since we observed a significant within-run variability in some cases (DeSilva et al., 2012). In particular, we noticed that insoluble material after sample reconstitution in assay buffer could heavily influence the calculated concentrations, which may be falsely overestimated due to a decrease in OD in the wells where insoluble material has been observed throughout the EIA procedure. The presence of insoluble material was especially noticeable in dog plasma samples and proved very difficult to remove. To resolve this issue, we added a step to the procedure for all species; samples were spun briefly after the reconstitution in assay buffer and were then carefully pipetted into the plate wells.

Interestingly, the precision profiles of neat samples showed that the basal plasma levels of OT observed in every species involved in this study were coherent with those previously described in the literature. For dogs, when samples were extracted, OT mean concentrations using RIA were found to be approximately 63 pg/ml in bitches in anestrus (Olsson et al., 2003) and 45 $\pm$ 40 or 65 $\pm$ 82 pg/ml (in two different breed populations) using the same EIA method (Hollinshead et al., 2010). There are however marked differences with dogs plasma OT concentration measurements from studies in which the plasma was not extracted (Handlin et al., 2011; Rehn et al., 2013); this discrepancy is to be expected since such discrepancies have already been extensively documented in the literature from human samples (Szeto et al., 2011; McCullough et al., 2013; Christensen et al., 2014) where higher OT concentrations in unextracted plasmas have been demonstrated to be due to the presence of non-OT immunoreactive products. In cats, the authors found only one study dealing with plasma OT concentrations in the species that reported a basal level between 20 and 30 pg/ml in solid-phase extracted samples and assayed with "in-house" EIA (Fieni et al., 2006). In three studies (Ginther and Beg, 2009, 2011, 2012) using horse samples and a very similar method of extraction and assay comprising a concentration factor of 13, Ginther and Beg found OT concentrations between 10 and 35 pg/ml, which are close to the values we found once the concentration factors have been applied. In cows, studies using RIA methods (Mačuhová et al., 2004; Belo and Bruckmaier, 2010)

found basal levels of plasma OT between 1–4 and 3–6 pg/ml, which are similar to our results. Again, predictably, authors (Sutherland et al., 2012) using EIA methods with unextracted cow plasma exhibited OT concentrations 100-times higher than in the present study and studies using RIA. In sheep, basal plasma OT concentration was measured using RIA in the 5–10 pg/ml interval in extracted samples from ewes (Dwyer et al., 2004). In goats, reports using extracted samples and RIA or "in-house" EIA found basal plasma OT concentrations around 10 pg/ml (Payne and Cooke, 1998) or respectively in the interval of 14–47 pg/ml (Hernandez et al., 2002; Olsson and Högberg, 2009). Finally, basal plasma OT levels in pigs were described around 5–20 pg/ml in experiments using RIA following extraction (Castrén et al., 1993; Gilbert et al., 1996). This thorough comparison of our findings with the literature in every species supports the use of the method described and analytically validated here as a suitable means of measuring relevant OT measurements in the plasma of the domestic animal species involved in the study.

Of note, basal plasma OT levels among the seven studied species varied greatly. To the best of our knowledge, the reason for this species differences is not known, although our main assumption relates to the animals' size and the total volume of blood in each species: indeed, we experimentally noticed that the smaller the species is, the higher the basal levels of OT are. This would be interesting to further investigate it. Likewise, the question of sex differences in OT plasma levels is noteworthy when considering the increasing number of reports stating the sex importance in relation to the OT system (Neumann, 2008; Bakermans-Kranenburg and van IJzendoorn, 2013; Crockford et al., 2014; Alves et al., 2015), notably through the influence of steroid hormones, such as estrogen (Murakami et al., 2011; Acevedo-Rodriguez et al., 2015). Unfortunately, in this study, we did not specifically record the sexes of the enrolled animals as our purpose was to describe and validate a method which could be useful for numerous domestic species, regardless of the sex. Because the method has been especially validated on individuals from both sexes, we indeed think the method presented here could be helpful in studies investigating the sex effect on OT plasma levels. However, our recommendation would be to assay the samples from different animals' sexes on the same plate because the possible between-run variability found here may not permit to highlight small putative differences between sexes.

The procedure described in this study for assaying OT is mainly based on the procedure assessed by Szeto et al. (2011) for human plasma samples. These authors suggested that even extracted plasma contained non-OT immunoreactive products that could come from OT degradation. Thus, when we refer to OT, it should be understood as "oxytocin-like immunoreactive products." Recently, some authors (Brandtzaeg et al., 2016) have described a new procedure to assay the total fraction of OT in plasma, in contrast with all previously published methods, including the one presented here, which aim to measure the free fraction of OT in plasma. They found that OT could bind to plasma proteins and thus be precipitated with them during the extraction procedure. In this regard, non-extracted protocols of OT assay may have partial immunoreactivity with bound OT, that could explain the method discrepancies. Treating the plasmas by reduction/alkylation could release OT molecules from plasma proteins, hence allowing higher amounts of OT to be measured, i.e., the total plasma OT fraction, notably in dog plasma. This could eliminate some drawbacks of the present method, such as the need to considerably increase the concentration of domestic animal samples, which requires drawing substantial amounts of blood. However, the biological activity of the bound fraction needs further clarification. Likewise, the interest of OT total fraction as a behavioral/emotional biomarker remains to be investigated.

CONCLUSION

Method validation is a fastidious but critical step to providing reliable measurements and supporting strong scientific findings. The field of OT research is rapidly expanding. In human science, elegant advancements have been made to improve OT measurements and to establish standard, repeatable, reliable and validated methods to achieve them. Applying them to plasma samples of seven domestic animal species, the authors hope this study will help to provide reliable tools for measuring peripheral OT and supporting future studies on domestic animals in OT research fields.

AUTHOR CONTRIBUTIONS

CB, AC, and PP wrote the manuscript and conceived the study. CB and CC analyzed the data, conceived, designed and performed the experiments. EC performed and supervised the blood sampling procedures in all species.

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Social Behavior of Pet Dogs Is Associated with Peripheral OXTR Methylation

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Oxytocin is a key modulator of emotional processing and social cognitive function. In line with this, polymorphisms of genes involved in oxytocin signaling, like the oxytocin receptor (*OXTR*) gene, are known to influence social behavior in various species. However, to date, no study has investigated environmental factors possibly influencing the epigenetic variation of the *OXTR* gene and its behavioral effects in dogs. Pet dogs form individualized and strong relationships with their owners who are central figures in the social environment of their dogs and therefore might influence the methylation levels of their *OXTR* gene. Here we set out to investigate whether DNA methylation within the *OXTR* promoter region of pet dogs is linked to their owner's interaction style and to the social behavior of the dogs. To be able to do so, we collected buccal epithelial cells and, in Study 1, we used pyrosequencing techniques to look for differentially methylated CpG sites in the canine *OXTR* promoter region on a heterogeneous sample of dogs and wolves of different ages and keeping conditions. Four identified sites (at positions −727, −751, −1371, and −1383 from transcription start site) showing more than 10% methylation variation were then, in Study 2, measured in triplicate in 217 pet Border Collies previously tested for reactions to an adverse social situation (i.e., approach by a threatening human) and with available data on their owners' interaction styles. We found that CpG methylation was significantly associated with the behavior of the dogs, in particular with the likelihood that dogs would hide behind their owner or remain passive when approached by a threatening human. On the other hand, CpG methylation was not related to the owners' behavior but to dog sex (at position −1371). Our findings underpin the complex relationship between epigenetics and behavior and highlight the importance of including epigenetic methods in the analysis of dog behavioral development. Further research is needed to investigate which environmental factors influence the epigenetic variation of the *OXTR* gene.

Keywords: dog, DNA methylation, epigenetics, social behavior, oxytocin receptor gene, ownership style, oxytocin

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INTRODUCTION

Social interactions are central to the life of all social species, and genetic variation across individuals has been associated with mechanisms regulating these interactions. In particular, associations have been found between the genetic variation of different genes involved in the oxytocinergic system and a variety of social phenotypes in different mammalian species, e.g., mice (see Caldwell et al., 2016 for a review), primates (Staes et al., 2014), cats (Arahori et al., 2015), humans (see Ebstein et al., 2012 for a review), and dogs (Kis et al., 2014). For example, polymorphisms in the oxytocin receptor (*OXTR*) gene were associated with dogs' proximity seeking with the owner (Kis et al., 2014), rough temperament in cats (Arahori et al., 2015), and sociability in humans (Li et al., 2015). Furthermore, oxytocin has been associated with social fear (Kirsch et al., 2005), aggression toward unfamiliar individuals (Stallen et al., 2012) and social anxiety (Grillon et al., 2013) in humans, and friendliness toward a threatening human in dogs (Hernádi et al., 2015). In particular, Hernádi et al. (2015) showed that dogs, after intranasal oxytocin administration, showed less friendliness toward the owner approaching them in a threatening way (in the so-called Threatening Approach test, originally developed and validated by Vas et al. (2005, 2008) and looked more at their owners standing behind them than a control group of dogs who received a placebo. These results, taken together with the associations between *OXTR* polymorphisms and dog friendliness and proximity seeking toward the owner found by Kis et al. (2014) during the same test, highlight a potential dual role of the oxytocinergic system: regulating a dog's behavioral response toward a social threat and expressing the relationship between the dog and the owner present in such a situation.

The relationship dogs build with their owners (at least in western societies) has been defined as analogous to the infant-mother attachment bond (Topál et al., 1998), and it has been shown that the presence of the owner influences the coping strategy of a dog exposed to such social threats (Horváth et al., 2007) and attachment-related behaviors like proximity seeking (Gácsi et al., 2013). This latter behavior has been interpreted as dogs seeing their owners as a "safe haven," a concept introduced by Bowlby (1969) in the frame of the attachment theory. The safe haven effect is activated by distress and fear, when a child (or a dog) seeks for proximity to the caregiver in order to find protection and safety. However, it is important to notice that not all dogs seek proximity in the same way, suggesting that individual differences might play a role in shaping the relationship between a dog and its owner. In fact, it has been shown that the reactions of dogs during the Threatening Approach test are strictly associated with the interaction styles of their owners (Cimarelli et al., 2016), supporting the idea that owners can indeed serve as a safe haven for their dogs, but only if they show specific behavioral characteristics. Specifically, only if previous experiences provided the dog with the information that the caregiver was present and responsive.

Similarly, in human infants, the caregivers' parenting style strongly influences the children's attachment styles (that is, their behavioral reaction to separation from and reunion with the caregiver), and it has been proposed that epigenetic modifications of the genome are the biological mechanisms that mediate this link between caregiver and child behavior (Champagne and Curley, 2009). In fact, epigenetic modifications of the DNA, that affect gene expression but do not alter the primary sequence itself, are known to be influenced by various biological and environmental factors (Powledge, 2011; Tammen et al., 2013). One of their best known mechanisms is DNA methylation that in mammals occurs predominantly on cytosine residues that are followed by guanine (CpG sites). Although DNA methylation can exert opposite effects on transcription efficiency depending on the genomic context and extent of methylation, basically it represses gene expression. This is especially true for promoter regions where DNA methylation is considered as a major factor influencing gene expression (e.g., in tissue-specific transcriptional inactivation, Goldberg et al., 2007; Portela and Esteller, 2010). Studies in rodents show that the social environment in which individuals grow up, and as such, also caregiving quality, has a high impact on DNA methylation (Weaver et al., 2004; McGowan et al., 2011). In child development it has been suggested that epigenetic modifications of specific genes caused by environmental factors result in changes in emotion regulation and, in turn, in behavior (van Ijzendoorn et al., 2011). For example, it has been shown that methylation levels in the hippocampus of suicidal victims who had experienced abuse was higher than in individuals who committed suicide but had no history of abuse (McGowan et al., 2009). Also, children with mothers reporting a warmer and more affectionate caregiving style had lower methylation levels in the glucocorticoid receptor gene (Bick et al., 2012). Regarding the role of the oxytocinergic system in the epigenetic modification of social behavior, it seems that this system is influenced by early experience (e.g., Unternaehrer et al., 2015), and it has been suggested that methylation of the *OXTR* gene mediates the effect of parental care on psychosocial development in humans (MacDonald, 2012; MacDonald and Feifel, 2013; Shalev and Ebstein, 2013; Feldman, 2015) and in rodents (Zhang et al., 2010; Bales and Perkeybile, 2012). In humans, a possible role of *OXTR* methylation in behavioral neuroscience is also underpinned by functional gene expression studies (Kusui et al., 2001) and by observations on the relationship between *OXTR* methylation and psychosocial traits (Kumsta et al., 2013). In particular, *OXTR* methylation has been linked to autism (Gregory et al., 2009), social perception (Jack et al., 2012), callous-unemotional traits (Dadds et al., 2014), anxiety/depression (Chagnon et al., 2015) as well as anger and fear perception (Puglia et al., 2015). *OXTR* methylation levels have also been shown to change dynamically upon acute psychosocial stress (Unternaehrer et al., 2012).

Here we suggest that epigenetic mechanisms are also likely to play a role in mediating the effects of the owner behavior on the social behavior of pet dogs. We have shown that dog owners' interaction styles vary along three components that are analogous to components of human parenting styles and that they are associated with how dogs cope with a socially stressful situation (Cimarelli et al., 2016). We hypothesize that methylation of the *OXTR* gene may play a role in mediating such a link between owner and dog behavior. Investigating possible

causes and effects of differential methylation patterns in the dog *OXTR* gene is not only relevant for the field of anthrozoology or canine behavior but could represent a valid model of human caregiving behavior and its effects on the social behavior of cared individuals (either children or dogs). In fact, the vast majority of animal studies have so far focused mainly on laboratory rodents that live in environments not comparable to that of humans. Also, rodent maternal behavior, albeit possibly analogous the human parenting, still has a rather different biological function. It has been pointed out that it is difficult to retrieve useful information from comparing the "reproductive and parenting strategies of humans and other species" (Galbally et al., 2015, p. 2). In contrast, in pet dogs we can directly investigate the effects of human caregiving on dog social behavior. Furthermore, dogs share their environment with humans, not only in terms of habitat and nutrition but also of communication and social interactions (Hare and Tomasello, 2005; Tomasello and Kaminski, 2009; Miklósi and Topál, 2013). Therefore, pet dogs might provide a more relevant animal model than laboratory rodents for studying associations between epigenetic variables and behavior. Finally, this species is also genetically uniquely suited to investigate the genetic background and gene-related associations of various behavior traits (Hejjas et al., 2007). Purebred dogs show a genetic diversity that is intermediate between two extremes represented by the genetically highly variable humans and the genetically nearly homogeneous laboratory animal strains. This intermediate genetic diversity can facilitate the identification of genetic factors underlying phenotypic variation (Ostrander, 2005; Boyko, 2011; Parker, 2012). Despite of these advantages, to date few studies have investigated epigenetic variation in the domestic dog (Maeda et al., 2014; Tomiyasu et al., 2014; Berglund et al., 2015; Montrose et al., 2015; Yamaya et al., 2015) and none of them focused on associations between the *OXTR* gene and behavioral phenotype.

Here, in Study 1, we explored differentially methylated CpG sites within the canine *OXTR* promoter region in a heterogeneous sample of dogs and wolves living in different social environments in order to describe the epigenetic variation of the canine *OXTR* promoter. Then, in Study 2, considering the hypothesis that different owner interaction styles might have an effect on dog behavior through *OXTR* methylation, first we investigated possible relationships between methylation levels on specific regions of the *OXTR* promoter and the dogs' behavioral reactions to an unpleasant social situation (including experimenter-directed behaviors and owner-directed behaviors, e.g., proximity seeking) in a large sample of pet Border Collies. Second, in the same dogs we investigated whether dog-directed interaction styles of the owners are associated with DNA methylation levels of *OXTR* gene promoter of the dogs.

MATERIALS AND METHODS

Ethics Statement

No special permission for non-invasive sample taking and socio-cognitive testing of animals is required either in Austria (Tierversuchsgesetz 2012 – TVG 2012) or in Hungary (Act XXVIII of 1998 on the protection and welfare of animals). In accordance with GPS guidelines and national legislation, the experimental procedures of Study 2 were approved by the Ethical Committee for the use of animals in experiments at the University of Veterinary Medicine Vienna (Ref: 09/10/97/2012 and 10/10/97/2012). Owners of the pet dogs participated in the study on a voluntary basis and gave their consent to the genetic analyses as well as the behavioral testing of their dogs.

Sample Collection and DNA Isolation

DNA samples were collected from the inner cheek of the animals using cotton-tipped swabs. Genomic DNA was isolated by a traditional, salting-out procedure as described earlier (Boor et al., 2002). Briefly, collection swabs were incubated overnight at 56°C in 450 μl cell lysis buffer (0.2 g/l Proteinase K, 0.1 M NaCl, 0.5% SDS, 0.01 M Tris buffer pH = 8.0), RNase treated at room temperature, protein precipitated with saturated NaCl (6 M) and centrifuged. DNA was obtained by precipitating the supernatants with isopropanol. Following ethanol purification, pellets were resuspended in 50 μl of Tris-EDTA (0.01 M Tris, 0.001 M EDTA, pH = 8.0) and stored at −20°C prior to bisulfite conversion.

DNA Methylation Analysis

Two hundred nanograms genomic DNA quantified by a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific, Wilmington, DE, USA) was bisulfite converted using the EZ DNA Methylation-Gold™ Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's protocol. Bisulfite converted DNA was kept at −80°C until further used. Primers were designed to bisulfite converted regions of an approximately 1000 base pairs (bp) long CpG island shore stretch at the canine *OXTR* promoter/ 5′ untranslated region (UTR) by the PyroMark Q24 Software (Qiagen NV, Venlo, Netherlands). CpG island localization was determined by an in-house MS-DOS application using the traditional definition of a CpG island as $a \geq 200$ bp long region with a GC percentage >50% and an observed-to-expected (O/E) CpG ratio greater than 60%. The *OXTR* promoter was located according to genome assembly CanFam 3.1 (GCA_000002285.2) and CpG sites investigated were numbered according to transcription start site (+1) of transcript variant NM_001198659.1 (ENSCAFT00000008950.3; genomic coordinate Chr20:9358932) (Aken et al., 2016). For polymerase chain reaction (PCR) amplification, the 25 μl reaction mixture contained 0.625 units EpiMark Hot Start Taq DNA Polymerase (New England Biolabs, Ipswich, MA, USA), 1x EpiMark Hot Start Taq Reaction Buffer (New England Biolabs, Ipswich, MA, USA), 0.2 mM deoxynucleotide trisphosphate (dNTP), 10 μM of an unmodified forward primer and a biotin-labeled reverse primer (for sequences see **Table 1**) and about 15–20 ng bisulfite converted DNA template. All samples were amplified in triplicate on the same PCR machine (Bio-Rad T100™). Cycling conditions were as follows: Step 1: (95°C/ 1 min)/1 cycle; Step 2: (95°C/30 s, 58°C/1 min, 68°C/45 s)/45 cycles; Step 3: (68°C/5 min)/1 cycle; Step 4: 8°C hold. Successful PCR amplification of a single fragment was verified using agarose gel electrophoresis for each sample and replicate. Pyrosequencing was performed on a PyroMark Q24 platform using sequencing

TABLE 1 | Primers used for the exploration of differentially methylated CpG sites in the canine *OXTR* promoter.

Primer Name	Sequence	Genomic coordinates (Chromosome 20)	Type	Quality score
P1_F	5′ TGA TGT AAT TTT TAA GGG TAA GAA AAA GAT A 3′	9357389 : 9357419	Amp	–
P1_R	5′ TTT AAA TAC ATT CTT CCT CCT AAC ATT TCC TTT C 3′	9357608 : 9357641	Amp	–
P1_S1	5′ AAT TTT TAA TTT TTT TTA ATG TTG T 3′	9357419 : 9357442	Seq	74
P1_S2	5′ TTA ATT AGA ATT TTG GGA TT 3′	9357476 : 9357495	Seq	76
P1_S3	5′ GGT ATA GGG TTG TAA TTG 3′	9357530 : 9357547	Seq	79
P2_F1	5′ AGG GTG ATG AAG TTG TAA AAG T 3′	9358139 : 9358160	Amp	–
P2_F2	5′ AGG GAA AGA TTT TAA GAA AAG ATA AGA AAG 3′	9357913 : 9357938	Amp	–
P2_R	5′ ACA TTT CAT CTT CCT TTA ACA TCA TAT A 3′	9357788 : 9357815	Amp	–
P2_S1	5′ ATG AAG TTG TAA AAGTAT TTA ATT G 3′	9358130 : 9358154	Seq	71*
P2_S2	5′ TAA GTA AAT GTT TGT TTT GGA GTA 3′	9358026 : 9358049	Seq	68*
P2_S3	5′ AAT TTA TTT TTA TTT TAA AGT GAT T 3′	9357875 : 9357899	Seq	80#
P3_F	5′ GG TTT TTG GAT GGG GAT AGG A 3′	9358485 : 9358505	Amp	–
P3_R	5′ ACT TCA TCA CCC TCT TCT CA 3′	9358148 : 9358167	Amp	–
P3_S1	5′ TTT TTG GAT GGG GAT AGG 3′	9358486 : 9358503	Seq	68
P3_S2	5′ GGT AGG AGG TAA AAA AAA G 3′	9358450 : 9358468	Seq	68
P3_S3	5′ GTT GGG GAG AGT TTT TTT GTA GT 3′	9358416 : 9358438	Seq	69
P3_S4	5′ GTA TAG TTT TAA GGG GTT ATT GGG 3′	9358378 : 9358401	Seq	72
P3_S5	5′ ATT TTT AGA TTA GGG TTA GTT TGG A 3′	9358330 : 9358354	Seq	72
P3_S6	5′ AAT TAG TAG TTT TAT TTT ATT TAA G 3′	9358288 : 9358312	Seq	69
P3_S7	5′ GGT TTT TTT TTT TTT TGG TTT AGA A 3′	9358217 : 9358241	Seq	63

*Genomic coordinates are according to CanFam3.1 (GCA_000002285.2). Quality scores (<40: poor; 40–59: low; 60–87: medium; > 88: high quality) are assigned as by the PyroMark Assay Design Software and refer to primer sets (including forward, reverse and sequencing). Amp: amplifying; Seq: sequencing. *For amplification, P2_F1 forward primer was used. #For amplification, P2_F2 forward primer was used.*

primers indicated in **Table 1** with PyroMark Gold Q24 Reagents (Qiagen NV, Venlo, Netherlands). Totally methylated and absolutely unmethylated control DNA were obtained by SSSI methyltransferase treatment (New England Biolabs, Ipswich, MA, USA) and whole genome amplification (REPLI-g Mini Kit, Qiagen NV, Venlo, Netherlands), respectively, according to the manufacturers' protocols. Measurements reported as unreliable by the PyroMark software were removed from the database. Epigenotypes reported are an average of triplicate measurements (outliers, i.e., values deviating more than 3% were removed).

Study 1: Identification of Differentially Methylated CpG Sites in the Canine *OXTR* Promoter

The aim of Study 1 was to identify differentially methylated CpG sites in the promoter of the *OXTR* that show a variation between individuals higher than 10%. As no prior information was available regarding localization of differentially methylated CpG sites in the canine *OXTR* gene, the DNA methylation profiles needed to be explored first. A diverse sample set including both wolves and dogs of different breeds, sex, age and keeping conditions were used during this pilot study to gain more insight into the methylation levels of the canine *OXTR* promoter region. The rationale behind choosing a heterogeneous population for this goal was that in a homogeneous sample, potential variably methylated sites are more likely to be missed, especially if the sample size is small. Given that methylation status of promoter-associated CpG islands and their immediate flanking regions, the CpG island shores, often influences gene expression and because the latter have been shown to be frequently differentially methylated (Doi et al., 2009; Portela and Esteller, 2010; Deaton and Bird, 2011), we focused on identifying differentially methylated CpGs at near promoter CpG island shore.

Subjects

Twelve animals (nine dogs and three timber wolves, six females and six males, mean age ± SD = 47.94 ± 37.84 months; see **Table 2** for all details about the subjects of Study 1) were involved in the present study. All wolves and two dogs were born in captivity and were hand-raised in peer-groups at the Wolf Science Center[1] after being separated from their mothers before they were 10 days old. Among the remaining seven dogs, one lived at a Hungarian dog school as guarding dog, six were kept as pet dogs and among them four lived inside the house and two lived mainly outside.

Data Analysis

We used the traditional CpG island definition (at least 200 bp long DNA stretch with a G+C content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6) to identify CpG islands.

Results

We identified a CpG island located right at the 5′ UTR of the canine *OXTR* gene (**Figure 1**). Accordingly, an 1117 bp long segment located at a CpG island shore in the canine *OXTR*

[1]www.wolfscience.at

TABLE 2 | Animals involved in the identification of the differentially methylated CpG sites (Study 1).

Sub-species	Breed	Living conditions	Sex	Age
Wolf	Timber	Hand-raised at the WSC	Male	6 years
Wolf	Timber	Hand-raised at the WSC	Male	2 years
Wolf	Timber	Hand-raised at the WSC	Female	4 years
Dog	Mix breed	Hand-raised at the WSC	Female	2 years
Dog	Mix breed	Hand-raised at the WSC	Female	3 weeks
Dog	Mix breed	Pet dog (kept inside)	Female	7 years
Dog	Shetland Sheepdog	Pet dog (kept inside)	Female	2 years
Dog	Caucasian Shepherd	Guard dog at dog school	Male	7 years
Dog	Boxer	Pet dog (kept inside)	Male	6 months
Dog	Central Asian Shepherd	Pet dog (kept outside)	Male	2 weeks
Dog	West Highland White Terrier	Pet dog (kept inside)	Male	9 years
Dog	Beagle	Pet dog (kept outside)	Female	5 years

promoter was investigated for variably methylated CpG sites. Range of methylation levels for given CpG sites are shown in **Figure 2** (not all CpG sites were covered by pyrosequencing analysis due to difficulties of designing effective primers for bisulfite converted DNA). Out of the 26 CpG sites analyzed, four were found that showed at least 10% variation in their methylation levels among the subjects, presented with accurate methylation levels for the 0 and 100% methylated controls

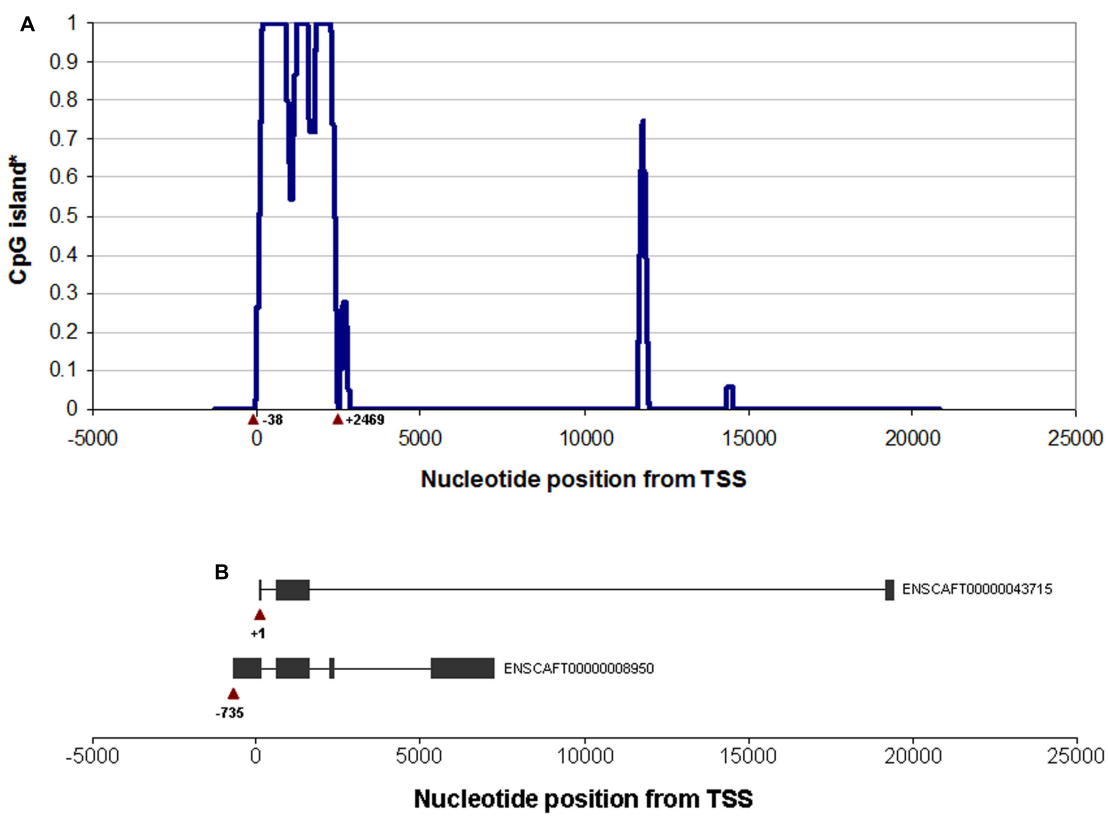

FIGURE 1 | CpG island structure of the canine OXTR gene. (A) Nucleotide positions according to transcription start site of transcript variant NM_001198659.1 (ENSCAFT00000008950.3) to which the traditional CpG island definition applies ("an at least 200 bp long DNA stretch with *a* ≥ 50% G+C content and *a* ≥ 0.6 observed-to-expected CpG ratio"). *Y axis shows the proportion of all possible different 200 bp long DNA stretches containing the same nucleotide to which the described CpG island definition applies. Positions of nucleotides at the beginning and the end of the CpG island located right at the beginning of the OXTR gene are indicated with respect to +1 as the transcription start site of transcript variant NM_001198659.1 (ENSCAFT00000008950.3). **(B)** Schematic structure of the transcript variants ENSCAFT00000008950.3 (NM_001198659.1) and ENSCAFT00000043715.1. Boxes represent exons, lines represent introns. Nucleotide positions of the transcription sites are indicated relative to transcription start site of NM_001198659.1 (ENSCAFT00000008950.3).

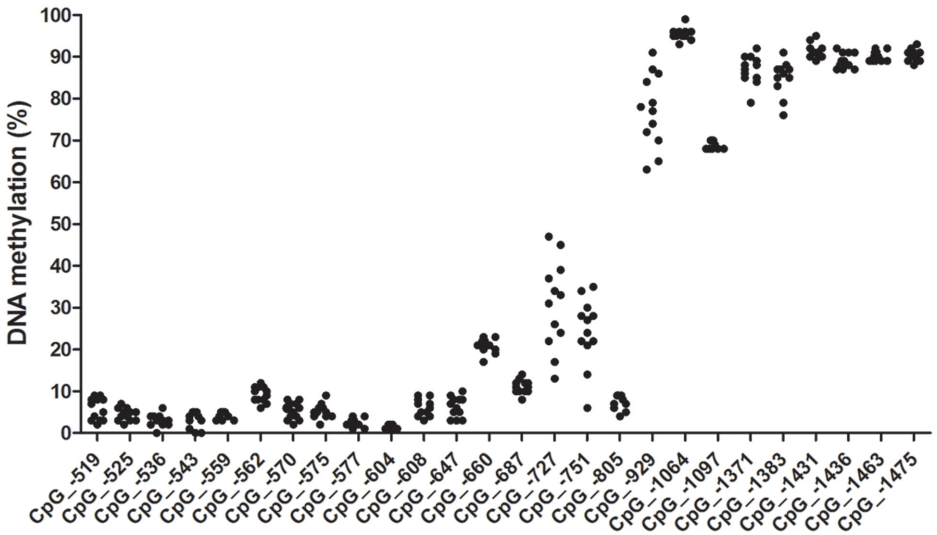

FIGURE 2 | CpG methylation levels in the canine *OXTR* promoter. Methylation ranges indicated are as observed in the exploratory sample set of 12 animals of different subspecies, breed, sex, age and keeping conditions. CpG numbering is according to transcription start site of +1 of transcript variant NM_001198659.1. Methylation levels for CpGs –451 to –489 are not indicated due to poor sequence quality, but apparently they were all in the low methylation level range. CpG –590 was not covered by any sequencing primers.

and their different ratio mixtures (between 10 and 90%) as well as gave high peaks in the chromatogram even in the case of low (10–12 ng) initial (pre-PCR) DNA quantities. These four CpG sites were located −727, −751, −1371, and −1383 bp relative to transcription start site of transcript variant NM_001198659.1 (ENSCAFT00000008950.3), see **Figure 3**, with their genomic coordinates being Chr20:9358205, Chr20:9358181, Chr20:9357561, and Chr20:9357549, respectively, according to genome assembly CanFam3.1 (GCA_000002285.2). These four CpG sites were then analyzed in Study 2 (see below). Genomic alignment of the canine *OXTR* promoter segment investigated to the corresponding human sequence is shown in Supplementary Figure 1.

Study 2: Associations between Owner Interaction Style, Dog Behavior, and Methylation of the *OXTR* Promoter

Subjects

Study 2 originally involved 220 pure bred Border Collies but three individuals were excluded from the present analyses since it was not possible to obtain DNA samples from them. A single breed was involved to minimize background genetic variability. The 217 dogs (135 females (45 neutered) and 95 males (32 neutered); mean age ± SD = 48.07 ± 42.43 months) involved in the study were all kept as pets in Vienna (Austria) and surroundings. These subjects, together with their owners, participated in a behavioral test battery accompanied by buccal DNA sample collection. All subjects were tested at the Clever Dog Lab (Vienna, Austria) between September 2010 and November 2013. The owners were recruited from the database of volunteer participants of the Clever Dog Lab.

Behavioral Test Battery

The behavioral data were collected as part of a bigger project, and the methods and some of the results have been described by Cimarelli et al. (2016). In summary, the pet Border Collies (N = 217) participated in a modified version of the Threatening Approach test (Vas et al., 2005; Gácsi et al., 2013; Hernádi et al., 2015): the owner stood motionless behind the dog and held the leash. The experimenter (E), initially standing five meters away from the owner-dog dyad, started walking toward the dog slowly (approximately 1 step/4 s) with the upper body bent toward the dog and staring in the eyes of the dog. The test was over when E reached the dog, the dog approached E in a friendly manner, or when the dog showed strong signs of aggression and/or fear (i.e., snapped at E or hid behind the owner). At the end of the test, E crouched down and talked gently to the dog to resolve the situation. We analyzed whether the dog showed any of the following behaviors before E crouched down (recorded as binomial variables): friendly (approaching E wagging the tail), appeasing (approaching E with the tail between the legs, ears pulled back and tense body posture), aggressive (growling, snarling or snapping at E), passive (no visible reaction) and hiding behind the owner (withdrawing in a way that the owner would be positioned between the dogs and E). In addition, we also scored the final reaction of the dogs showed when E made the last step toward them (1 = retreat behind the owner; 2 = passive behavior; 3 = appeasing/friendly approach; 4 = aggressive approach).

Aside from analyzing the dogs' behavior in this test, the owners' interaction style with their dog was also characterized (Owner interaction style test). The behavior of the owner toward her/his dog was observed and coded in a set of 8 experimental tasks. The tasks included: (1) showing a preference toward one

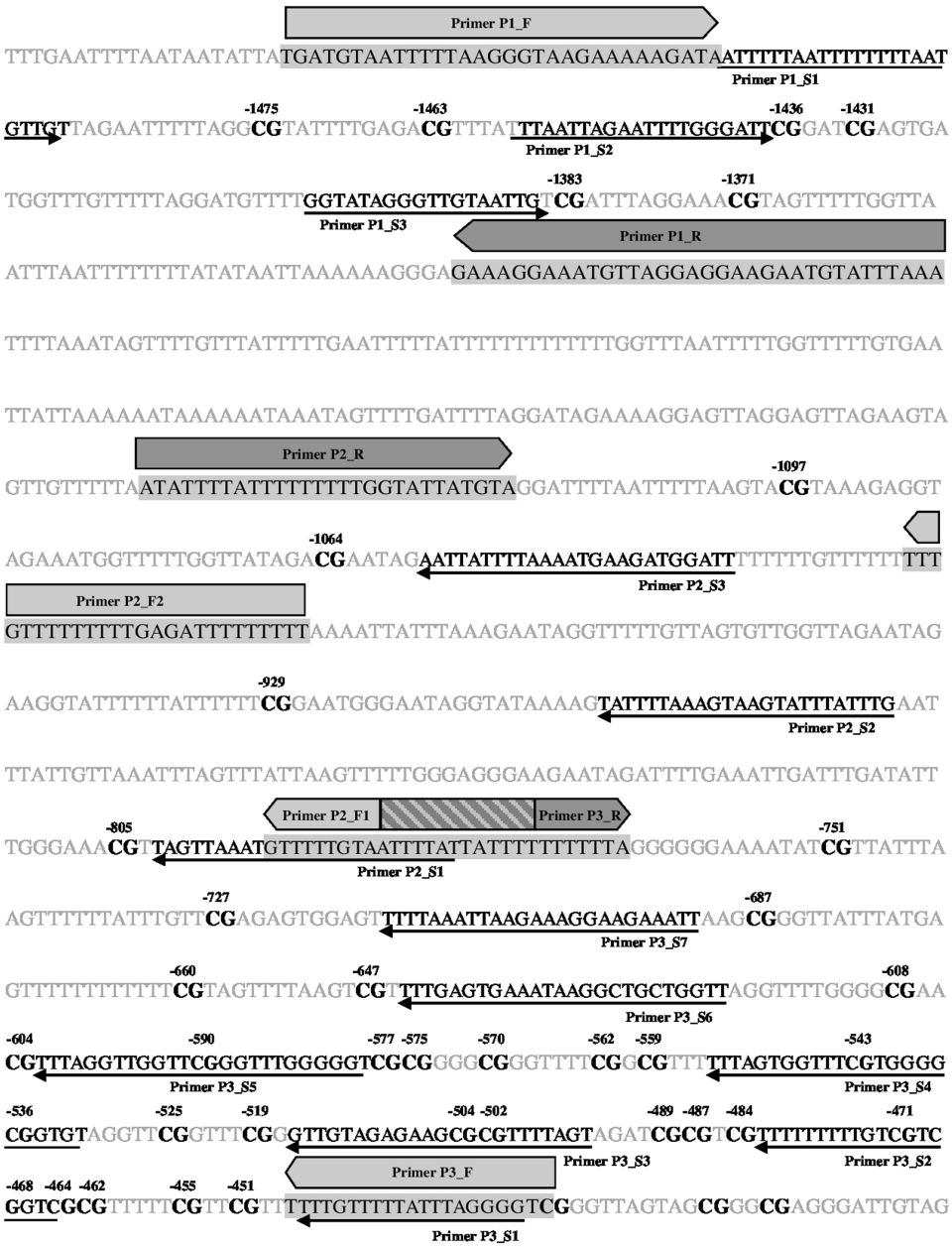

FIGURE 3 | Localization of primers and CpG sites covered. Bisulfite converted sequence of forward strand is indicated. Primer pairs P2_F1/ P2_F2 & P2_R as well as P3_F & P3_R were designed to the reverse strand. Forward and reverse amplifying primers are indicated by filled light and darks arrows, respectively, as well as by black letters in the sequence highlighted by a gray background; sequencing primers are indicated by thin arrows as well as black italics letter in the sequence. Overlap region of forward primer P2_F1 and reverse primer P3_R is indicated by a striped box. CpG sites are shown in bold black letters. CpGs covered by sequencing primers are numbered according to transcription start site of +1 of transcript variant NM_001198659.1 [ENSCAFT00000008950.3; genomic coordinate: CanFam3.1 (GCA_000002285.2) Chr20:9358932].

of two plates to the dog ("Food choice"); (2) holding the dog while the experimenter was taking a buccal sample from the inner mouth of the dog ("DNA sample"); (3) greeting after a short period of separation ("Greeting"); (4) playing with the dog using a rope in a tug-of-war game ("Tug-of-war"); (5) putting a T-shirt on a dog ("T-shirt"); (6) commanding the dog to perform three simple behaviors (i.e., sit, lay down, stay; "Commands"); (7) demonstrating the dog how to remove the lid from a bin to get a piece of food ("Teaching"); (8) playing a retrieval game using a ball ("Ball"). The following variables were measured: communication style (4-point scale, in Food choice and Teaching tests), warmth (4-point scale, in the Greeting test), enthusiasm (4-point scale, in the Tug-of-war and Ball test), social support (4-point scale, in DNA sample and T-shirt test),

TABLE 3 | Factors affecting the methylation levels of the CpG sites analyzed in Study 2.

Dependent variable	Predictor	Estimate ± *SE*	*DF*	*t* value	*p*-value	Effect size (Pearson's *r*)
−727	Age	0.01 ± 0.02	1	0.55	0.58	0.01
	Sex	−3.53 ± 1.66	1	−2.12	0.03	0.15
	Neutered status	1.55 ± 1.87	1	0.83	0.41	0.05
	Sex*Neutered status	−2.38 ± 3.86	1	−0.62	0.54	0.04
	Owner Warmth	−0.75 ± 1.16	1	−0.65	0.52	0.06
	Owner Social Support	−1.12 ± 1.10	1	−1.06	0.29	0.08
	Owner Control	−1.14 ± 1.11	1	−1.03	0.30	0.05
−751	Age	0.00 ± 0.01	1	0.43	0.67	0.03
	Sex	−1.22 ± 0.73	1	−1.67	0.09	0.12
	Neutered status	0.69 ± 0.75	1	0.92	0.36	0.06
	Sex*Neutered status	−1.90 ± 1.53	1	−1.24	0.22	0.08
	Owner Warmth	0.30 ± 0.50	1	0.06	0.95	0.02
	Owner Social Support	−0.49 ± 0.47	1	−1.06	0.29	0.05
	Owner Control	0.18 ± 0.48	1	0.38	0.71	0.03
−1383	Age	0.00 ± 0.01	1	0.86	0.39	0.06
	Sex	0.37 ± 0.46	1	0.81	0.42	0.03
	Neutered status	−0.73 ± 0.49	1	−1.51	0.13	0.06
	Sex*Neutered status	0.03 ± 0.95	1	0.04	0.97	0.00
	Owner Warmth	0.27 ± 0.31	1	0.88	0.38	0.07
	Owner Social Support	−0.06 ± 0.30	1	−0.20	0.84	0.02
	Owner Control	0.00 ± 0.31	1	0.01	0.99	0.04
−1371	Age	0.00 ± 0.00	1	0.03	0.97	0.08
	Sex	0.81 ± 0.24	1	3.31	0.001*	0.22
	Neutered status	−0.49 ± 0.26	1	−1.90	0.06	0.12
	Sex*Neutered status	−0.34 ± 0.58	1	−0.58	0.56	0.04
	Owner Warmth	0.03 ± 0.17	1	0.16	0.87	0.07
	Owner Social Support	0.19 ± 0.16	1	1.18	0.24	0.02
	Owner Control	−0.07 ± 0.17	1	−0.42	0.67	0.04

**Significant after post hoc sequential Bonferroni correction for multiple testing.*

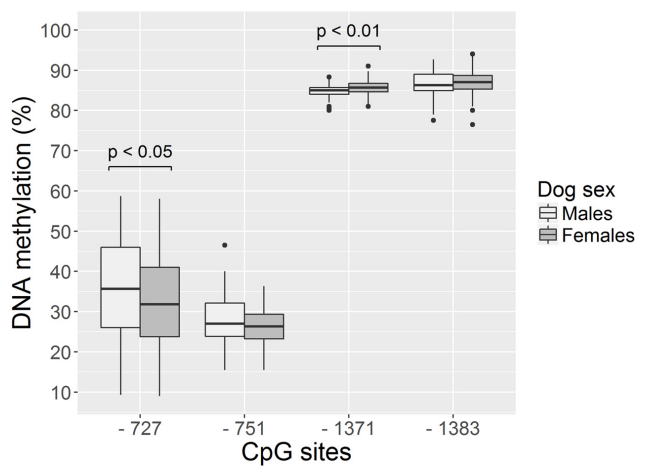

FIGURE 4 | Sex differences for methylation levels –727, –751, –1371, –1383 in dogs involved in Study 2. Females have higher methylation levels than males at position –1371 while males have higher methylation levels than females at position –727. Horizontal bars represent medians, the bottom and the top of the boxes represent the lower and the upper quartiles, respectively, whiskers represent the interquartile range and filled circles represent outliers.

authoritarian behaviors (0 = none, 1 = the owner raises the tone of voice, 2 = the owner forces the dog in a determined position in the Commands test). Furthermore, the number of commands, attention sounds (e.g., clapping the hands), vocal praises and petting were counted in the DNA sample, Tug-of-war, Commands and Ball tests. Previous analyses showed that the behavioral variables analyzed during this test grouped in three factors, namely the "Owner Warmth" (characterized by a positive and warm communication and interaction style showed in positive contexts, e.g., play), "Owner Social Support" (characterized by the number of petting and praising given by the owner in stressful situations, e.g., DNA sample test) and "Owner Control" [mainly characterized by the number of commands; for a detailed description see (Cimarelli et al., 2016)].

Statistical Analysis

In order to estimate whether the methylation levels of the adjacent sites identified in Study 1 were correlated, Pearson correlations between sites were calculated. To investigate whether the Owner interaction styles and the demographic characteristics of the dog (i.e., sex, age, and neutered status) were associated with the methylation levels of the CpG sites identified in

Study 1, we ran Generalized Least Squares models (GLSs) with the methylation levels as dependent variable and the Owner interaction styles and the dog demographic variables as predictors [R package nlme (Pinheiro et al., 2007), function *gls*]. Furthermore, to investigate associations between the methylation levels and dog behavior during the Threatening Approach test, we ran Generalized Linear Models (GZLM) with binomial distributions. We ran models with the methylation levels of the different CpG sites as predictors and the following variables as response variables: "Aggression," "Appeasing," "Friendly," "Hide behind the owner," and "Passive." Furthermore, we ran a Multinomial Regression Model with the "Reaction at the end of the test" as dependent variable and the methylation levels as predictors. We selected the best model using model reduction based on *p*-values. Non-significant predictors ($p > 0.05$) were removed from the model and are not reported in the results. Model residuals were tested for normality using the Shapiro-Wilk normality test and homoscedasticity was assessed via plots of residuals against fitted values. We accounted for multiple testing using *post hoc* sequential Bonferroni (Holm, 1979). All statistical tests were conducted using R version 3.1.1 (R Development Core Team). See Supplementary Materials for a

TABLE 4 | Factors affecting male and female dogs' reaction during the Threatening Approach test (Study 2).

Dependent variable	Predictor	Sex	Estimate + *SE*	*DF*	*z* value	*p*-value	Effect size (Pearson's *r*)
Aggression	−727	Males	−0.00 ± 0.05	1	−0.02	0.99	0.07
		Females	0.00 ± 0.03	1	0.05	0.96	0.07
	−751	Males	−0.05 ± 0.06	1	−0.92	0.36	0.08
		Females	−0.00 ± 0.08	1	−0.04	0.97	0.04
	−1371	Males	−0.17 ± 0.16	1	−0.01	0.31	0.13
		Females	0.21 ± 0.16	1	1.31	0.19	0.10
	−1383	Males	0.01 ± 0.11	1	0.09	0.93	0.06
		Females	−0.12 ± 0.10	1	−1.18	0.24	0.08
Friendly	−727	Males	−0.04 ± 0.06	1	−0.70	0.48	0.14
		Females	0.05 ± 0.03	1	1.50	0.13	0.15
	−751	Males	0.01 ± 0.41	1	0.03	0.98	0.10
		Females	0.05 ± 0.09	1	0.54	0.59	0.16
	−1371	Males	0.14 ± 0.46	1	0.31	0.75	0.03
		Females	0.33 ± 0.19	1	1.71	0.09	0.13
	−1383	Males	−0.11 ± 0.45	1	−0.24	0.81	0.11
		Females	−0.03 ± 0.16	1	−0.19	0.85	0.12
Appeasing	−727	Males	−0.00 ± 0.04	1	−0.07	0.94	0.14
		Females	0.02 ± 0.02	1	1.21	0.23	0.18
	−751	Males	−0.01 ± 0.04	1	−0.19	0.85	0.08
		Females	−0.06 ± 0.06	1	−1.01	0.31	0.05
	−1371	Males	0.03 ± 0.16	1	0.18	0.86	0.06
		Females	0.05 ± 0.14	1	0.39	0.69	0.02
	−1383	Males	−0.16 ± 0.08	1	−2.09	0.04	0.28
		Females	0.14 ± 0.07	1	1.97	0.05	0.22
Passive	−727	Males	−0.02 ± 0.04	1	−0.51	0.61	0.11
		Females	−0.03 ± 0.03	1	−1.09	0.27	0.04
	−751	Males	0.03 ± 0.11	1	0.30	0.76	0.09
		Females	0.07 ± 0.09	1	0.78	0.43	0.01
	−1371	Males	−0.37 ± 0.24	1	−1.55	0.12	0.04
		Females	−0.09 ± 0.21	1	−0.44	0.66	0.04
	−1383	Males	0.43 ± 0.15	1	2.81	0.005*	0.31
		Females	0.17 ± 0.11	1	1.54	0.12	0.13
Hide behind	−727	Males	−0.03 ± 0.03	1	−0.91	0.36	0.20
		Females	−0.01 ± 0.02	1	−0.33	0.74	0.04
	−751	Males	0.13 ± 0.05	1	2.59	0.009*	0.35
		Females	0.03 ± 0.07	1	0.4	0.69	0.00
	−1371	Males	−0.14 ± 0.19	1	−0.72	0.47	0.10
		Females	−0.02 ± 0.13	1	−0.16	0.87	0.05
	−1383	Males	0.03 ± 0.13	1	0.22	0.82	0.04
		Females	0.05 ± 0.10	1	0.51	0.61	0.03

**Significant after post hoc sequential Bonferroni correction for multiple testing.*

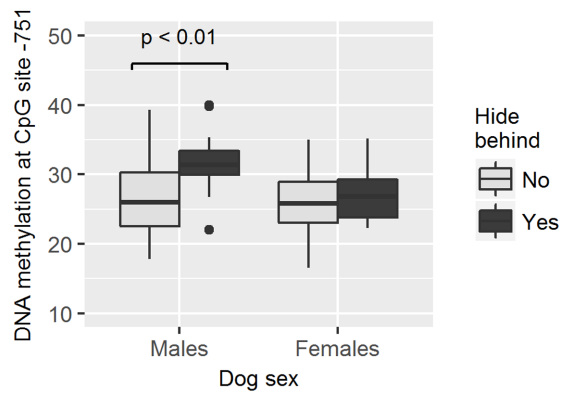

FIGURE 5 | Association between methylation levels at position –751 and the likelihood of dogs to hide behind the owner during the Threatening Approach test. Males hiding behind the owner had higher methylation levels than males not hiding behind the owner. Horizontal bars represent medians, the bottom and the top of the boxes represent the lower and the upper quartiles, respectively, whiskers represent the interquartile range and filled circles represent outliers.

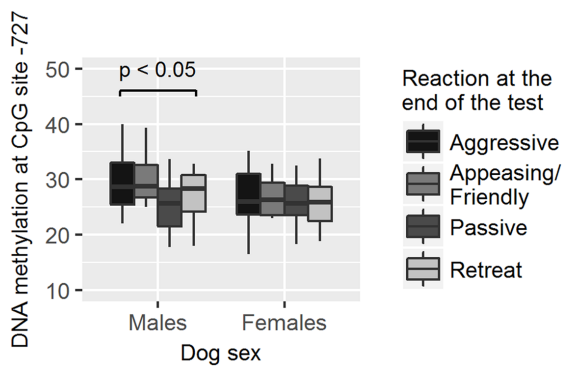

FIGURE 6 | Association between methylation levels at position –727 and their reaction at the end of the Threatening Approach test. Male dogs approaching the experimenter either in an aggressive or in an appeasing/friendly manner had higher methylation levels than male dogs that remained passive or retreated. Horizontal bars represent medians, the bottom and the top of the boxes represent the lower and the upper quartiles, respectively, whiskers represent the interquartile range and filled circles represent outliers.

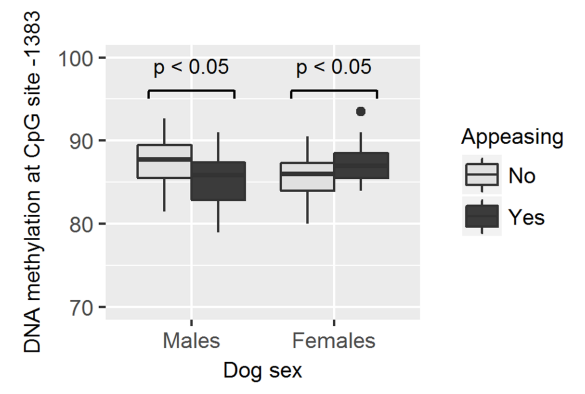

FIGURE 7 | Association between methylation levels at position –1383 and the likelihood of dogs to approach the experimenter in an appeasing way. Males approaching the experimenter in an appeasing way had lower methylation levels than males who did not, while the opposite was true for female dogs. Horizontal bars represent medians, the bottom and the top of the boxes represent the lower and the upper quartiles, respectively, whiskers represent the interquartile range and filled circles represent outliers.

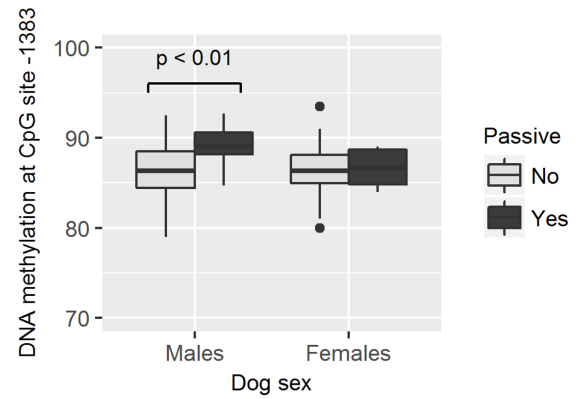

FIGURE 8 | Association between methylation levels at position –1383 and the likelihood of dogs to stay passive during the Threatening Approach test. Males remaining passive had higher methylation levels than males showing any other reaction. Horizontal bars represent medians, the bottom and the top of the boxes represent the lower and the upper quartiles, respectively, whiskers represent the interquartile range and filled circles represent outliers.

complete correlation matrix between all variables included in the present study.

Results

Characteristics of OXTR Promoter CpG Site Methylation

The four sites identified in Study 1 were further investigated in the Border Collie group ($N = 217$). The degree of methylation of these CpGs in the Border Collie population ranged between 9.0 and 58.7% (−727), 15.5 and 46.5% (−751), 80.5 and 89.7% (−1371), and 76.5 and 94.0% (−1383). Sites −1383 and −1371 were found to be moderately correlated ($r_{210} = 0.23$, $p < 0.01$) while sites −727 and −751 were strongly correlated ($r_{210} = 0.69$, $p < 0.01$).

*Associations of Methylation Levels with Sex, Age, Neutered Status, Sex*Neutered Status Interaction and Owner Interaction Scores*

The three Owner interaction style factors, together with the dogs' sex, age, neutered status and sex*neutered status were investigated as predictors for methylation levels of the three CpG sites. We found that the none of the predictors was significantly associated with the methylation level of sites –751 and −1383 ($p > 0.05$, **Table 3** and **Figure 4**). On the other hand, the sex of the dog was associated with the methylation level in site −1367 and −723. In particular, female dogs had higher methylation levels than males in position −1371 (GLS, estimate $\pm$ $SD = 0.81 \pm 0.24$, $t_{210} = 3.31$, $p < 0.01$, significant after correcting for multiple

TABLE 5 | Factors affecting dog's reaction at the end of the Threatening Approach test (Study 2).

Dependent variable	Predictor	Sex	Level: Estimate ± *SE*	*DF*	X^2	*p*-value	Effect size (Pearson's *r*)
Reaction at the end of the test	−727	Males	(1) – 0.01 ± 0.04	3	8.30	0.04	0.19
			(2) – 0.05 ± 0.03				
			(3) – 0.06 ± 0.02				
		Females	(1) 0.01 ± 0.03	3	0.81	0.85	0.06
			(2) 0.00 ± 0.02				
			(3) 0.01 ± 0.02				
	−751	Males	(1) – 0.03 ± 0.08	3	7.22	0.07	0.18
			(2) – 0.08 ± 0.05				
			(3) – 0.13 ± 0.05				
		Females	(1) 0.00 ± 0.07	3	1.18	0.76	0.07
			(2) – 0.05 ± 0.06				
			(3) – 0.03 ± 0.05				
	−1371	Males	(1) – 0.03 ± 0.09	3	1.99	0.57	0.10
			(2) 0.07 ± 0.15				
			(3) – 0.15 ± 0.14				
		Females	(1) – 0.02 ± 0.09	3	0.69	0.87	0.06
			(2) – 0.02 ± 0.09				
			(3) 0.07 ± 0.09				
	−1383	Males	(1) 0.20 ± 0.18	3	3.63	0.30	0.13
			(2) – 0.11 ± 0.10				
			(3) – 0.02 ± 0.09				
		Females	(1) 0.12 ± 0.12	3	3.04	0.39	0.12
			(2) 0.12 ± 0.10				
			(3) – 0.01 ± 0.09				

testing; **Table 3** and **Figure 4**), while males seemed to have higher methylation levels than females in position −727 (GLS, estimate ± SD = −3.53 ± 1.66, t_{210} = −2.12, p = 0.03; no longer significant when correcting for multiple testing; **Table 3** and **Figure 4**).

Associations of Methylation Levels with Dog Reaction in Males and Females

As methylation levels were found to differ by dog sex, the association between different methylation levels and the dog behavior was analyzed separately in female and male dogs. We found that males who hid behind the owner had higher methylation levels in site −751 than those who did not hide behind the owner (GZLM, estimate ± SD = 0.13 ± 0.05, z_{70} = 2.59, p < 0.01, significant after correcting for multiple testing; **Table 4** and **Figure 5**) and that males remaining passive or retreating at the end of the test tended to have lower methylation levels in site −727 than males approaching the experimenter in an appeasing or aggressive manner (Multinomial Regression Model, X^2 = 8.30, df = 3, p = 0.04; no longer significant when correcting for multiple testing; **Table 5** and **Figure 6**). Furthermore, females who approached the experimenter in an appeasing way tended to have higher levels of methylation in site −1383 (GZLM, estimate ± SD = 0.14 ± 0.07, z_{104} = 1.97, p = 0.04, no longer significant after correcting for multiple testing; **Table 4** and **Figure 7**) than those who did not show any sign of appeasement, contrary to the males who approached the experimenter in an appeasing manner which tended to have lower methylation levels in site −1383 than those who did not (GZLM, estimate ± SD = −0.16 ± 0.08, z_{70} = −2.09, p = 0.04, no longer significant after correcting for multiple testing; **Table 4** and **Figure 7**). On the other hand, males who remained passive till the end of the Threatening Approach test had higher methylation levels in site −1383 than those who showed any other reaction (GZLM, estimate ± SD = 0.43 ± 0.15, z_{77} = 2.81, p < 0.01, significant after correcting for multiple testing; **Table 4** and **Figure 8**). All non-significant associations are reported in **Tables 4**, **5**.

DISCUSSION

The present study explored for the first time the DNA methylation patterns in canids and their associations with pet dogs' social behavior. Specifically, four CpG sites in the *OXTR* promoter were identified where at least 10% of inter-individual variation in their methylation level was observed. The methylation levels of these CpG sites were different in female and male dogs and were associated with the behavioral reaction dogs showed when exposed to social stress. These results provide the first evidence of an association between epigenetic modifications of *OXTR* and dog social behavior. In particular, we found higher methylation levels in females than in males at site −1383 and we found a tendency to have lower methylation level at site −727 in females than in males. Moreover, lower methylation levels in

this position tended to be associated with a higher likelihood to approach a threatening unfamiliar person (either in an aggressive, appeasing or friendly manner) in males and a lower likelihood to remain passive or hide behind the owner. Regarding the two sites −751 and −1383, males with higher methylation levels were more likely to remain passive or to hide behind the owner than those having lower methylation levels.

It is not surprising that we found different methylation patterns in female and male dogs. Oxytocin is a hormone also with sex-related functions, therefore its receptors are expressed differently in males and females (Alves et al., 2015). It has also been shown that oxytocin administration and oxytocin level influence the social behavior of prairie voles in a sex-specific way (Bales and Carter, 2003; Bales et al., 2007). Similarly, in our study, a tendency for a different association was found in male and female dogs in regards to the appeasing behavior; while females were more likely to approach the experimenter in an appeasing manner if their methylation levels were higher in site −1383, in males we found the opposite relationship. These results might be explained by a differential interplay between the methylation of the *OXTR* gene and other biological mechanisms (e.g., the expression of sex hormones) and/or reflect a sex-specific response strategy to social threat.

Furthermore, our results suggest that different CpG sites might be differently involved in behavioral regulation. Higher promoter methylation levels generally lead to lower *OXTR* gene expression, which, in turn, leads to fewer available receptors for the oxytocin to bind. The present study suggests that the different sites might regulate *OXTR* expression in different manners: for instance, CpG sites −727 might be located in a transcription inhibitory region, where suppression of inhibition by methylation would potentially lead to higher gene expression (Portela and Esteller, 2010), or methylation of this site could trigger the use of an alternate, potentially more active, promoter (Maunakea et al., 2010). In fact, higher methylation levels on −751 or on −1383 were associated with more owner-directed behaviors or a passive state while higher methylation levels on −727 tended to lead to the opposite behavioral outcome.

Naturally, the identified associations can only be genuine if the analyzed biomarker – *OXTR* promoter methylation at the investigated CpG sites in canine buccal epithelia – reliably refers to neural processes, regulating the *OXTR* gene expression in the brain. A direct experimental verification of such a biological connection is unfortunately highly challenging, mainly because of the limited accessibility of brain tissues of (pet) dogs. Still, indirect evidence suggests that *OXTR* promoter methylation levels as measured in buccal epithelium could indeed be of physiological relevance for behavior. Human-related studies identified strong correlation between brain and surrogate tissue DNA methylation levels regarding functionally important *OXTR* promoter CpG sites (Gregory et al., 2009; Jack et al., 2012; Bell et al., 2015; Chagnon et al., 2015; Puglia et al., 2015). As buccal epithelium is of the same germ layer origin as neural tissues (Tam and Behringer, 1997), it is plausible that the inherited component of DNA methylation states remains relatively similar during embryonic development, when basic DNA methylation patterns are established (Reik and Walter, 2001). In later life, these patterns are modified both by environmental factors and stochastic effects (Kaminsky et al., 2009; Aguilera et al., 2010; Choi and Friso, 2010). How different tissues could react to environmental stimuli in similar manners in terms of DNA methylation is yet to be elucidated. However, it has been reported in humans that, even in the case of white blood cells, dynamic changes in *OXTR* promoter methylation can be observed in response to social stimuli (Unternaehrer et al., 2012). Given that *OXTR* protein is also expressed in squamous epithelial cells according to The Human Protein Atlas (Uhlen et al., 2010, 2015), it is feasible that nerves innervating the oral epithelium directly mediate epigenetic communication between brain and buccal tissues (Kress et al., 2006; Kim et al., 2009; da Silva et al., 2015). It is important to mention, however, that it has been shown that *OXTR* promoter methylation in rodents brain affects transcription efficiency in a region-specific manner (Harony-Nicolas et al., 2014). Future studies should investigate associations between *OXTR* methylation in brain tissues and in buccal cells and tissue-specific oxytocin expression in order to fully inform the psychophysiological role of *OXTR* methylation in the buccal epithelium in dogs.

Contrary to our predictions, in the present study, we could not find any association between owner behavior and methylation levels of the *OXTR* gene of their dogs. It might be that the methylation profiles of the CpG sites investigated in the present study are mostly inherited (Reik and Walter, 2001) and/or not be representative of the methylation levels of the *OXTR* gene in brain tissues that could still be potentially affected by the environment. In addition, it is possible that the owner interaction styles analyzed in the present study were factors not strong enough for such methylation changes. Indeed, the present analyses were carried out in a rather uniform population of purebred Border Collies kept as pets, and it would be necessary to investigate different breeds and/or dogs living in different social environments (e.g., in shelters or as stray dogs) in order to further investigate the role of the environment in shaping dog social behavior through epigenetic modifications. Further on, in this study we focused on the promoter of the *OXTR* gene, and we cannot exclude the possibility that owners' interaction styles might affect other regulatory regions in other genes.

It is important to notice that the *OXTR* methylation might not be the only factor to influence dog behavior, it is possibly also mediated by which SNPs the dog was carrying (Smearman et al., 2016). In fact, some studies highlighted a correlation between degree of methylation and SNPs (Bell et al., 2012; Smith et al., 2015). In our study, we did not take into account the genetic background of our subjects, but future studies should address the interaction between environment, SNPs, DNA methylation and behavior in order to have a better understanding of the mechanisms regulating dog social behavior. Epigenetic modifications other than DNA methylation should also be investigated. It is also important to note that the pyrosequencing technique used for DNA methylation assessment is not suitable for differentiating between 5-methylcytosines and 5-hydroxymethylcytosines (Guibert and Weber, 2013), so it cannot be ascertained yet if (some of) the observed relationships are not linked to hydroxymethylation. Another important issue is

that even when considering only a single epigenetic mark (DNA methylation) and a single gene, it would be useful to obtain data regarding the whole gene and all of its regulatory regions, i.e., not only a limited number of CpG sites in the promoter region. The present tissue choice (buccal epithelia) is unfortunately not suitable for such a comprehensive analysis mainly because of the obtainable DNA yield. Though buccal tissue has the major advantage of offering non-invasive sample taking and thus easy accessibility and keeping physiological effects of the sample taking procedure itself to a minimum, future studies should consider the use of other tissues as well in order to ensure investigation of a larger number of CpG sites within the same population.

Social behavior is a complex and multi factorial phenotype regulated by various interacting mechanisms: genetic background as well as inherited and environmentally induced epigenetic modifications of the individuals. The present study focused on only one of the possible mechanisms, namely the methylation of a single gene promoter, without clearly disentangling between inherited or environmentally influenced epigenetic patterns. As such, our results can provide an initial contribution to shedding light on the complex processes shaping social behavior. In particular, by indicating epigenetic analyses as a novel tool for the understanding of the mechanisms regulating dog behavior and ultimately suggesting pet dogs as good models for the field of human epigenetics. Future studies would need to investigate the interactions between the methylation levels and the polymorphisms of *OXTR*, the correspondence between buccal DNA methylation states of the CpG analyzed here and those in different regions of the brain, the effect of methylation in those areas on nervous system functions and on dog behavior, and other environmental factors possibly influencing epigenetic modifications.

AUTHOR CONTRIBUTIONS

GC, ZB, ZV, ZR, and MS-S designed the study. GC, ZB, BT, ZV, ZR, and MS-S prepared the study material and data acquisition. GC, ZB, and BT entered the data and prepared it for statistical analyses. GC, ZB, and BT analyzed the data. GC, ZB, BT, and ZV interpreted the data. ZV, ZR, and MS-S obtained funding. GC and ZB wrote the first draft of the manuscript. GC, BT, ZB, and ZV critically revised the manuscript for important intellectual content. All authors gave final approval of the manuscript version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

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frontiers in Psychology

ORIGINAL RESEARCH
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Dog-Owner Attachment Is Associated With Oxytocin Receptor Gene Polymorphisms in Both Parties. A Comparative Study on Austrian and Hungarian Border Collies

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Variations in human infants' attachment behavior are associated with single nucleotide polymorphisms (SNPs) in the oxytocin receptor (OXTR) gene, suggesting a genetic component to infant-mother attachment. However, due to the genetic relatedness of infants and their mothers, it is difficult to separate the genetic effects of infants' OXTR genotype from the environmental effects of mothers' genotype possibly affecting their parental behavior. The apparent functional analogy between child-parent and dog-owner relationship, however, offers a way to disentangle the effects of these factors because pet dogs are not genetically related to their caregivers. In the present study we investigated whether single nucleotide polymorphisms of pet dogs' OXTR gene (−213AG,−94TC,−74CG) and their owners' OXTR gene (rs53576, rs1042778, rs2254298) are associated with components of dog-owner attachment. In order to investigate whether social-environmental effects modulate the potential genetic influence on attachment, dogs and their owners from two different countries (Austria and Hungary, $N = 135$ in total) were tested in a modified version of the Ainsworth Strange Situation Test (SST) and questionnaires were also used to collect information about owner personality and attachment style. We coded variables related to three components of attachment behavior in dogs: their sensitivity to the separation from and interaction with the owner (Attachment), stress caused by the unfamiliar environment (Anxiety), and their responsiveness to the stranger (Acceptance). We found that (1) dogs' behavior was significantly associated with polymorphisms in both dogs' and owners' OXTR gene, (2) SNPs in dogs' and owners' OXTR gene interactively influenced dog-human relationship, (3) dogs' attachment behavior was affected by the country of origin, and (4) it was related to their owners' personality as well as attachment style. Thus, the present study provides evidence, for the first time, that both genetic variation in the OXTR gene and various aspects of pet dogs' environmental background are associated with their attachment to their human caregivers.

Keywords: oxytocin, dog *(Canis familiaris)*, owner, attachment, relationship, personality

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INTRODUCTION

Attachment is an organizational construct that serves to organize the development of emotional bond between human infants and their caregivers (Bowlby, 1958). In early infancy its function is to obtain protection and care from another person by adapting one's behavior to characteristics of the key attachment figure (Bowlby, 1969). This early development results in different attachment styles that can be assessed in terms of two dimensions of security/insecurity: attachment-related anxiety and attachment-related avoidance (e.g., Ainsworth et al., 1978; Brennan et al., 1998; Fraley and Spieker, 2003). Attachment styles have well-documented cognitive, physiological, and neurological correlates (e.g., Diamond, 2001; Gillath et al., 2005), and behavioral and psychological consequences that last into adulthood, including self-regulation of stress and emotions, influence on relationship quality with romantic partners, sexual motivation, and reactions to relationship breakups or losses (see Shaver and Clark, 1994; Mikulincer et al., 2003; for reviews).

Most studies have focused on the environmental effects shaping attachment (such as parental behavior, Fearon et al., 2014). more recently however, candidate gene studies have reported associations between attachment styles of human infants and polymorphisms in their dopamine D4 receptor, serotonin transporter, and oxytocin receptor (OXTR) genes (Lakatos et al., 2000; Barry et al., 2008; Chen et al., 2011; Spangler, 2011), suggesting that genetic polymorphisms may moderate the links between parental behavior and other environmental effects and infant attachment. Therefore, it has become obvious that attachment styles are shaped by a combination of genetic factors and social experiences (Fonagy, 2001). This is well-demonstrated by the fact that carrying a specific genetic polymorphism can be associated with developing a particular attachment style in one kind of social environment but not in another (Gillath et al., 2008). For instance, Chen et al. (2011) found that the A allele, as compared to the G allele, of *OXTR* rs2254298 was more likely associated with secure attachment in a non-Caucasian sample but not in a Caucasian sample. Such results in human infants allow for limited conclusions however, because of the genetic relatedness of the infants and their parents. Allelic variations associated with different attachment styles of infants have been shown to affect also various characteristics of the parents, allowing for an alternative, indirect, link between genotype and infant attachment.

In human subjects variations of oxytocin-related genes have been found in association with various personality traits, their components and neurological correlates, such as agreeableness extraversion, social loneliness, anxiety, and amygdala volume (Lucht et al., 2009; Saphire-Bernstein et al., 2011; Haram et al., 2014; Wang et al., 2014). Nonhuman examples also can be found, namely cats with the A allele in the SNP G738A show significantly higher "Roughness" personality scores than cats without the A allele (Arahori et al., 2016). Parental personality affects parenting style (Council et al., 1988; Kendler et al., 1997; Metsäpelto and Pulkkinen, 2003), thereby personality traits have an impact on the quality of parent-child relationships, and on the children's attachment style (Kochanska et al., 2004). However, as infants and their parents likely carry similar alleles in the polymorphic regions of their OXTR, in humans it is difficult to dissect whether and how infant genotypes, parent genotypes and other characteristics of parents (e.g., their personality or their own attachment style) affect infant attachment. The domestic dog, however, provides a unique opportunity to investigate this question.

Dogs have been part of human societies for longer than any other domestic species (Clutton-Brock, 1999). Their ability to form attachment with humans is one of the most widely recognized consequences of domestication (Topál and Gácsi, 2012). Topál et al. (1998) were the first to reveal that dogs develop attachment to their owners analogous to the infant-mother attachment in humans (for a replication see Prato-Previde et al., 2003).

Similarly to human infants, the most widely used paradigm to investigate dogs' attachment behavior toward their owners is the Strange Situation Test (SST; Topál et al., 1998). The test consists of seven episodes, each lasting 2–3 min, when the dog is either with the primary caregiver (owner), with a stranger, or alone in an unfamiliar place. The essential element of the test is that separation from the human caregiver in an unfamiliar environment evokes moderate stress, which manifests in proximity seeking while the reunion with the caregiver evokes contact-seeking behaviors. Multivariate analysis of Topál et al. data (1998) (factor and cluster analyses) separated three key aspects of dogs' behavioral structure. These major factors revealed that dogs' behavior during the test was affected by: (i) their sensitivity to the separation from the owner (Attachment), (ii) the degree of stress the unfamiliar environment evoked from them (Anxiety), and (iii) their responsiveness to the stranger (Acceptance). Their study demonstrated that adult dogs show specific patterns of attachment behavior toward their owners, and dogs' individual behavior patterns can be explained by the different combinations of these determining factors. Many studies have reported that dogs show a great variability in their behavior in the SST (Topál et al., 1998; Gácsi et al., 2001; Naderi et al., 2002; Prato-Previde et al., 2003; Mariti et al., 2014; Scandurra et al., 2016) suggesting that adult dogs are particularly suitable subjects for studying the phenomenon of animal-to-human attachment.

Pet dogs also offer a good model for investigating to what extent attachment patterns are shaped by the independent genetics of the dogs and their owners and by environmental factors, such as the owners' personality, attachment style, or the country they live in. Drawing a parallel between infant-mother and dog-owner attachment has recently gained further support from the finding that ownership styles seem to be composed of components similar to those of human parenting behavior, suggesting that owners largely use their parenting repertoire when interacting with their dogs (Cimarelli et al., 2017). Importantly, however, only little (and indirect) data is available on how different genetic and environmental factors influence dogs' attachment to human.

Similarly to the finding that polymorphism in the OXTR gene is related to security/insecurity of mother-infant attachment in

humans (Chen et al., 2011), it has been suggested that oxytocin plays an important role in the relationships between dogs and their owners, with higher oxytocin levels being associated with a more positive relationship from perspective of the owner (Thielke and Udell, 2015). Thielke and Udell even suggested that intranasal OXT administration can be combined with behavioral therapies for dogs with behavioral problems related to separation anxiety. Somewhat contrasting this view, van Rooy et al. (2015) in a study examining several candidate genes (OPRM1, AVPR1A, DRD2, OXTR) and their associations with separation-related distress in 42 Australian golden retrievers, found no significant associations between separation-related behavior scores and OXTR gene.

The main purpose of the present study was to explore environmental and genetic influences on dogs' attachment behavior. In order to benefit from the genetic unrelatedness of dogs and their owners, in separate analyses we investigated (1) whether various OXTR polymorphisms of dogs as well as their owners are associated with the attachment behavior of the dogs in two different countries, (2) whether such effects of the dogs' genotypes are affected by the age, sex, and neutering of the dogs. Finally, to tackle potential mechanisms that may mediate the effects of owner genotypes on dog attachment, we analyzed (3) if owner personality, attachment style, and attachment to pets have an effect on dogs' attachment behavior.

MATERIALS AND METHODS

Ethics Statement

The procedures were approved in accordance with GPS guidelines and national legislation by the Ethical Committees at the University of Veterinary Medicine Vienna and the Medical University of Vienna in Austria (Ref No. 04/12/97/2012 and 2073/2012, respectively) and the University Institutional Animal Care and Use Committee (UIACUC) of Eötvös Loránd University in Hungary (Ref No. XIV-I-001/531-4-2012). Owners of the pet dogs participated in the study on a voluntary basis and gave their consent to the genetic analyses as well as the behavioral testing of their dogs.

Subjects

Border Collies ($N = 135$; mean age $\pm$ *SD*: 4.17 $\pm$ 3.01 years, range: 10 months−14 years) kept as pet dogs were recruited in two countries, Austria and Hungary (Austria: male/female: 34/37, neutered/intact: 40/24; Hungary: male/female: 29/35, neutered/intact: 43/19). Dogs and their owners (Austria: male/female: 17/54, mean age $\pm$ *SD*: 35.80 $\pm$ 10.65 years, age range: 15.99–65.99 years; Hungary: male/female: 7/57, mean age $\pm$ *SD*: 34.32 $\pm$ 15.18 years, age range: 13.35–54.33 years) participated in the behavioral testing (modified version of the SST test—Horn et al., 2013, see later). Owners and their dogs were recruited for SST on a volunteer basis and there were no specific inclusion/exclusion criteria.

Experimental Set-Up

Dogs' attachment to their human caregivers was tested using the same protocol and experimental set-up in both countries (Horn et al., 2013). Testing took place in an experimental room that was unknown to the dog (5 × 6 m; **Figure 1**). The experimental room contained four cameras linked to monitoring and recording equipment in an adjacent room. The room contained two chairs (Chair 1, Chair 2), several toys placed on the floor, two elevated locations out of the dog's reach (i.e., windowsill, table; Location 1, Location 2), building blocks placed in Location 1, and a water bowl with fresh water. Three areas with 1 m radius were marked with tape on the floor for later video coding: "close to Chair 1," "close to Chair 2," "close to Door." Additionally, there were three lines indicating the quartiles between the table and the location with the building blocks. The experimental rooms were cleaned with liquid disinfectant to eliminate odors.

Procedure

Before the start of the experiment, the experimenter explained the procedure in detail to the owner while the dog was sitting in an adjacent room with a helper otherwise uninvolved in the study. The test consisted of seven episodes of ∼3 min each. In three episodes a stranger was present in the room. The stranger was of the same gender as the dog's owner and has never been seen by or interacted with the dog prior the experiment.

Episode 1 (Owner and Dog)

The owner entered the experimental room with the dog on leash and sat down on Chair 1. After letting the dog off the leash and placing the leash on the floor next to the chair, the owner first sat

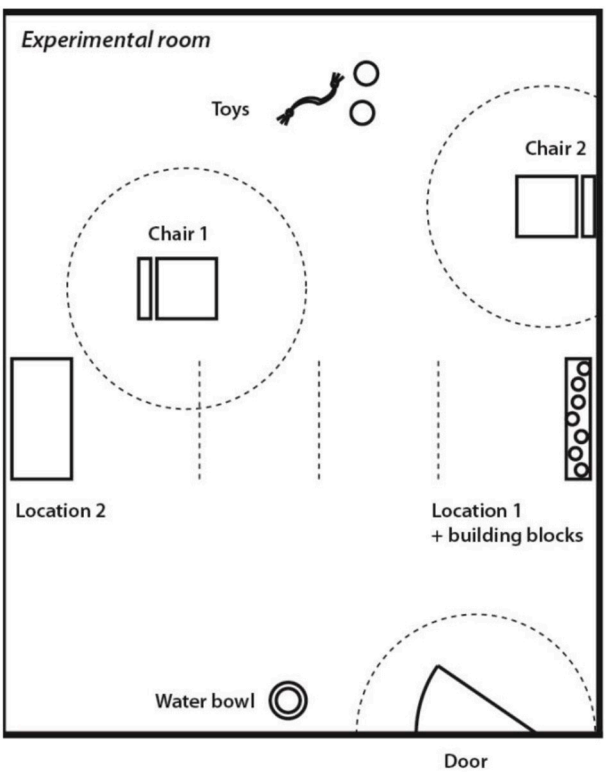

FIGURE 1 | Set-up used in the "Strange Situation Test".

quietly and started to fill the questionnaires in without interacting with the dog for 2 min. After that the owner carried building blocks from Location 1 to Location 2 in order to build a tower without interacting with the dog for 1 min. Then the owner sat back on Chair 1 and continued filling the questionnaires.

Episode 2 (Owner, Stranger, and Dog + Owner Leaving)

A stranger entered the room quietly and sat down on Chair 2 opposite of the owner without interacting with the dog for 1 min. Then the stranger got up and initiated play with the dog. After the first minute of the play phase the owner left the room quietly and the stranger continued to play with the dog for another minute.

Episode 3 (Stranger and Dog + Stranger Leaving)

The stranger returned to Chair 2 and did paperwork without interacting with the dog for 2 min. After that the stranger carried all the building blocks from Location 2 back to Location 1 without interacting with the dog for 1 min. At the end of this phase the stranger left the room quietly.

Episode 4 (Dog Alone)

The dog was left alone in the room for 3 min. This episode was curtailed, if the dog was too distressed by the separation.

Episode 5 (Owner and Dog + Owner Leaving)

The owner entered the room, paused next to the door without interacting with the dog (∼5 s), then greeted the dog shortly (∼5 s), and finally sat back on Chair 1. The owner continued filling the questionnaire in without interacting with the dog for 3 min, and at the end of this phase left the room quietly again.

Episode 6 (Dog Alone)

The dog was left alone in the room for 3 min. This episode was curtailed, if the dog was too distressed by the separation.

Episode 7 (Stranger and Dog)

The stranger entered the room, paused next to the door without interacting with the dog (∼5 s), then greeted the dog shortly (∼5 s), and finally sat back on Chair 2. The stranger continued doing paperwork without interacting with the dog for 3 min and at the end of this phase put the leash on the dog and left the room together with the dog.

Questionnaires

Owners both from Austria and Hungary were additionally asked to fill in three questionnaires assessing their personality (BFI), romantic relationships (ECR-R), and dog-owner relationship (modified ECR-R).

The 44–item Big Five Inventory (BFI; Supplementary 1) was developed by John and Srivastava (1999). The questionnaire includes 8 questions related to extraversion (e.g., "Is full of energy"); 9 questions for agreeableness (e.g., "Can be cold and aloof"); 9 questions for conscientiousness (e.g., "Tends to be lazy"); 8 questions for neuroticism (e.g., "Is emotionally stable, not easily upset"); and 10 questions for openness (e.g., "Is curious about many different things"). Each item was rated on a Likert scale from 1 ("strongly disagree") to 5 ("strongly agree").

The relationship between the owner and his/her partner was measured by the 36-item Experiences in Close Relationship-Revised Questionnaire (ECR-R; Supplementary 2; Fraley et al., 2000). Each item was rated on a Likert scale from 1 ("strongly disagree") to 7 ("strongly agree"). The questionnaire includes 18 questions related to bond-related anxiety (e.g., "I worry a lot about my relationships") and 18 questions related for bond-related avoidance (e.g., "I tell my partner just about everything"). The trait scores were calculated by averaging the scores of the variables representing each trait.

The relationship between owner and his/her dog was measured by the modified Experiences in Close Relationship-Revised Questionnaire (ECR-R; Supplementary 3). This questionnaire was developed by Beck and Madresh (2008) based on the 36-item ECR-R for humans (Fraley et al., 2000). Each item was rated on a Likert scale from 1 ("strongly disagree") to 7 ("strongly agree"). The questionnaire includes 8 questions related to pet-related anxiety (e.g., "My pet makes me feel confident.") and 8 questions related for pet-related avoidance (e.g., "I prefer not to show a pet how I feel deep down"). The trait scores were calculated by averaging the scores of the variables representing each trait.

Owners were asked to start filling the questionnaires in during the test. As the number of questions in the 3 questionnaires were rather high, they were given the opportunity to finish the questionnaires at home, which lead however to relatively low response rates that varied between 49.6 and 74.8% (owner personality: $N = 97$ in total, Austria: 42, Hungary: 55; romantic relationships: $N = 101$ in total, Austria: 40, Hungary: 61; and dog-owner relationship: $N = 67$ in total, Austria: 34, Hungary: 33).

DNA-Sampling

Before the behavioral test we collected buccal cell samples from those owners who agreed to provide DNA samples ($N = 66$, Austria: 33, Hungary: 33) and from those dogs whose owners agreed to provide genetic information about their dogs ($N = 130$, Austria: 69, Hungary: 61) with a non-invasive method, by swabbing the upper gum area with 4 cotton tips (Wan et al., 2013; Kis et al., 2014). The cotton tips were then sealed in a tube and preserved in the freezer until genotyping (Bence et al., 2016). DNA purification was initiated by incubating the buccal samples at 56°C overnight in 0.2 mg/ml Proteinase K cell lysis buffer. It was followed by protein denaturation using saturated NaCl solution. Finally, DNA was precipitated using isopropanol and ethanol by standard procedures and DNA pellet was resuspended in 100 µl 0.5× TE (1× TE: 10 mM Tris pH = 8, 1 mM EDTA) buffer. Typical DNA concentration of the genomic DNA samples isolated from buccal swabs was around 20 ng/µl and measured by NanoDrop® 2000 Spectrophotometer (Thermo Fisher Scientific, Wilmington, Delavare).

SNP Genotyping

−213AG,−94TC and−74CG canine SNPs are located in the 5′ flanking region. The Qiagen Hot-StarTaq polymerase kit (Qiagen, Hilden, Germany) was used for PCR amplification. The reaction mixture contained 1 µM of each primer, approximately 5 ng of DNA template, 200 µM dNTP, 0.025 U HotStarTaq DNA

polymerase, 1× buffer, and 1× Q-solution supplied together with the enzyme. The PCR cycle consisted of an initial denaturation at 95°C for 15 min, 40 cycles of 1-min denaturation at 95°C, 1-min annealing at various temperatures, a 1-min extension at 72°C, and a 10-min final extension at 72°C. The PCR reaction was performed in a total volume of 10 μl. The −213AG and the −74CG polymorphisms were genotyped by PCR-RFLP method using the primers described in **Table 2**. PCR products were incubated for 3 h at 37°C in a restriction enzyme mixture containing 0.5 U/μl Hpy99I restriction enzyme (NEB, Ipswich, Massachusetts, USA) for −213 SNP and 0.5 U/μl BsiEI restriction enzyme (NEB, Ipswich, Massachusetts, USA) for −74CG SNP with 1xBSA and 1x NEB4 buffer. Total reaction volume was 16 ml after adding the restriction enzyme mix to the PCR products. The −94TC SNP was genotyped by allele specific amplification (ASA) using the forward and reverse primers described in **Table 2**. The PCR products were analyzed by conventional submarine agarose gel electrophoresis (Biocenter, Szeged, Hungary), using 2.5% agarose gel and visualized by ethidium bromide staining. Genotype frequencies have been determined and Hardy–Weinberg Equilibrium analyses were carried out. The genotype frequencies were in Hardy–Weinberg equilibrium in both countries. Rare homozygote (AA) genotypes were grouped together with heterozygotes (AA+AG) (**Table 1**).

The rs53576 and the rs2254298 polymorphisms were located in intron 3 and the rs1042778 in exon 4 of the human OXTR gene. OXTR rs53576 and rs2254298 SNPs were genotyped by PCR-RFLP method using the primers described in **Table 2**. PCR products were incubated for 3 h at 37°C in a restriction enzyme mixture containing 0.5 U/μl AvaII restriction enzyme (NEB) for rs53576 SNP and 0.5 U/μl DdeI restriction enzyme (NEB) for rs2254298 SNP, 1x BSA and 1x NEB4 buffer. The PCR products were analyzed by conventional submarine agarose gel electrophoresis (Biocenter, Szeged, Hungary), using 2.5% agarose gel and visualized by ethidium bromide staining.

Genotype frequencies have been determined and Hardy-Weinberg Equilibrium analyses were carried out. The genotype frequencies of any SNPs were in Hardy–Weinberg equilibrium in both country (Austria: $p = 0.0761$, Hungary: $p = 0.0704$). Rare homozygote (AA) genotypes were grouped together with heterozygotes (AA+AG). For detailed information about the SNPs see **Table 2**.

Behavior Coding

Multivariate analysis of Topál et al. data (1998) (factor and cluster analyses) separated three key aspects of dogs' behavioral structure; Attachment, Anxiety, and Acceptance. In our study we grouped our variables based on these three aspects of the dogs' behavior in the SST. All three composite scores we created were built from several independently coded scores (**Table 3**). This method of evaluation, in contrast to the previously applied independent behavior variables (e.g., Topál et al., 1998), allowed us to separate the three factors that characterize the dogs' behavior in the SST. By scoring a list of behaviors in different contexts (**Table 3**) and summing these scores up for each composite score, each dog received a score of Attachment, Anxiety, and Acceptance ranging from −1 to 11.

Inter-rater reliability for dogs' behavior was calculated by coding 30% of the sample by four independent coders. Intra-class correlation coefficient (ICC) was used to assess reliability [$ICC_{(2, 4)} = 0.976$, $p = 0.002$ for Attachment, $ICC_{(2, 4)} = 0.923$, $p = 0.018$ for Anxiety and $ICC_{(2, 4)} = 0.993$, $p < 0.001$ for Acceptance].

Statistical Analysis

Three Generalized Estimating Equation models using restricted maximum likelihood estimation were used. The first one ($N = 66$) tested the effects of dog (−213AG,−94TC,−74CG) and owner (rs53576, rs1042778, rs2254298) SNPs, Country (Austria or Hungary) and two-way interactions between dog and owner

TABLE 1 | Allele frequencies (%) and number of individuals (N) for Border collies and Owners from Austria and Hungary.

Dog SNPs		**−213AG**				**−94TC**				**−74CG**		
Austria	AA	AG	GG	HWE	CC	CT	TT	HWE	CC	CG	GG	HWE
%	0.06	0.22	0.72	$p = 0.977$	0.13	0.58	0.29	$p = 0.982$	0.15	0.27	0.58	$p = 0.945$
N	4	15	49		9	40	20		10	18	38	
Hungary	AA	AG	GG	HWE	CC	CT	TT	HWE	CC	CG	GG	HWE
%	0.13	0.32	0.55	$p = 0.973$	0.44	0.34	0.21	$p = 0.963$	0.24	0.32	0.44	$p = 0.948$
N	8	19	33		27	21	13		14	19	26	
Owner SNPs		**rs53576**				**rs1042778**				**rs2254298**		
Austria	CC	CT	TT	HWE	AA	AC	CC	HWE	CC	CT	TT	HWE
%a	0.52	0.22	0.26	$p = 0.872$	0.42	0.42	0.15	$p = 0.997$	0.83	0.17	0.00	$p = 0.996$
N	14	6	7		14	14	5		25	5	0	
Hungary	CC	CT	TT	HWE	AA	AC	CC	HWE	CC	CT	TT	HWE
%	0.39	0.36	0.24	$p = 0.968$	0.13	0.25	0.63	$p = 0.946$	0.84	0.16	0.00	$p = 0.996$
N	13	12	8		4	8	20		26	5	0	

Statistical tests for Hardy–Weinberg Equilibrium (HWE) are also provided.

TABLE 2 | Summary of the SNPs included in the analysis.

Polymorphism/position	Primer	Sequence (5′-3′)	T_A (°C)	Product size (base pairs)	Restriction enzyme	Product size (base pairs) after digestion
DOG SNPs						
−213AG/5′ flanking region	Forward	CCA TTG GAA TCC GCC CCC T	56	635	Hpy99I	C allele: 180+ 201 +254
	Reverse	CAC CAC CAG GTC GGC TAT G	56			G allele: 41 +160+180+254
−94TC/5′ flanking region	Forward	CCA TTG GAA TCC GCC CCC T	60	635		
	Reverse	CAC CAC CAG GTC GGC TAT G	60			
	C allele specific	CCG ATC TGC TGG TCC CGG	60	295		
	T allele specific	CCG ATC TGC TGG TCC CGA	60			
−74CG/5′ flanking region	Forward	CCA TTG GAA TCC GCC CCC T	56	635	BsiEI	C allele: 180+201+254
	Reverse	CAC CAC CAG GTC GGC TAT G	56			G allele: 41+160+180+254
OWNER SNPs						
rs53576/intron 3	Forward	ACT GGG GCA ACC AAA CAT CT	56	304	AvaII	G allele: 133 + 61 + 110
	Reverse	ACT CTT CAT GGC CCA GAG TG	56			A allele: 194 + 110
rs2254298/intron 3	Forward	CTG TCT TTG CAC CTT TGC TA	56	347	DdeI	C allele: 276 + 71
	Reverse	ATG AAA GCA GAG GTT GTG TG	56			T allele: 246 + 30 + 71
rs1042778/exon 4	Forward	GCT CCA GCC AGA GGA G	60	283		
	Reverse	AGT GGG TTC AGG GTG GTA	60			
	A allele specific	AGC CAC CCC AAG GAG T	60	182		
	C allele specific	AGC CAC CCC AAG GAG G	60			

SNP-s on the behavioral scales. The second model ($N = 130$) tested the effects of dog OXTR SNPs (−213AG,−94TC,−74CG), Country (Austria or Hungary), Dogs' Sex (male or female), Neutering (Intact or Neutered), Age (covariant), as well as all two-way interactions of these with the behavioral scores measured in the SST test (Attachment, Anxiety, Acceptance), except the SNPs' interactions with each other. The third model ($N = 67$) tested the effects of the owners' questionnaire scales (BFI, ECR-R_Partner, ECR-R Dog) on the three behavioral scores of the dogs.Levels of significance (p) were corrected using FDRbh method to adjust for multiple comparisons (see Benjamini and Yekutieli, 2001).

An overview of the study is shown in **Figure 2**.

RESULTS

The Interactive Effect of Dog and Owner OXTR Polymorphisms (Table 4)

Our analysis showed that dog and owner OXTR SNPs had both main and interactive effects on dog behavior in the SST (**Table 4**). Attachment composite score was associated with both dog and owner OXTR SNPs (−213AG: $p < 0.01$−74CG: $p < 0.01$, rs1042778: $p < 0.01$, rs2254298: $p < 0.01$, respectively). Interestingly, however, dog and owner OXTR SNPs had also interactive effects (−213AG × rs2254298: $p < 0.01$,−213AG × rs53576: $p < 0.05$,−74CG × rs53576: $p < 0.01$). The effect of Country was confirmed in interaction with dog OXTR SNPs (Country ×−213AG: $p < 0.01$, Country ×−74CG: $p < 0.01$).

The same holds true for the Anxiety score. Apart from the main effect of dog −74CG SNP ($p < 0.05$), interactive effects of the dog and human OXTR genotypes were also found (-213AG × rs53576: $p < 0.01$,−74CG × rs53576: $p < 0.05$). We also found a significant interactions between Country and dog OXTR SNPs (Country × −213AG, Country × −74CG, both $p < 0.01$).

Similarly, Acceptance of the stranger was also associated with the OXTR polymorphisms in dogs (−74CG: $p < 0.05$) and in the owners (rs53576, $p < 0.05$), and there were also significant interactions between dog- and owner OXTR SNPs (-213AG × rs1042778: $p < 0.01$,−213AG × rs53576: $p < 0.01$,−94TC × rs1042778: $p < 0.01$,−74CG × rs1042778: $p < 0.01$,−74CG × rs53576: $p < 0.01$).

The Effect of Dog OXTR Polymorphisms and Dog Characteristics (Table 5)

Two OXTR polymorphisms (−213AG,−74CG) and other dog characteristics (such as country, age, sex, and neuter status) have been found to influence the dogs' behavior in the SST as a main effect or in interaction with each other. Attachment was most notably associated with Country ($p < 0.01$) and Country × Neuter status interaction was also significant ($p < 0.05$). Anxiety was associated with the −213AG SNP ($p < 0.05$) and Country ($p < 0.05$). There were significant interactions between Sex and dog OXTR SNPs (Sex ×−213AG: $p < 0.05$; Sex ×−74CG: $p < 0.05$) and Neuter status ×−213AG was also significant ($p < 0.05$). The analysis of the Acceptance of the stranger showed no main effects of OXTR SNPs, but the Neuter status ($p < 0.05$) and the interaction between Neuter status and Age ($p < 0.05$) were significant.

The Effects of Owner Personality and Relationship Experiences (Table 6, Supplementary 4)

Our analysis indicates that dogs' behavior in the SST is related to several aspects of owner personality and to the owner's experience

TABLE 3 | Behavior variables observed in Strange Situation Test (D, dog; S, stranger; O, owner).

Attachment		Score
Owner PRESENT	D is mostly close to O (closest bodypart is within 1 m) when does not explore or play	1
	D does not stand at the door (within 1 m) for more than a few seconds	1
	during the cube-carrying the D mostly watches or follows O	1
	when O first leaves, D follows O to door (within 1 m)	1
	when O leaves the second time, D follows O to door (within 1 m)	1
	when O enters, D approaches (within reaching distance) at once and wags tail	1
Owner ABSENT	D plays with S (at least for 2 s)	−1
	any vocalization	1
	D stands by or orients at door (at least for 2 s−1, most of the time−2)	2
	when S enters, D does not great and tries to sneak out the door	1
	D is mostly at the chair of O (within 1 m) if not at the door	1
ANXIETY		
Owner PRESENT	D stands at door (within 1m; at least 2 s−1, most of the time−2)	2
	D does not explore or play at least for 2 s	1
	D positions himself (hides) under/behind O's chair (relative to door or S) for at least 2 s	1
	as soon as O stands up D approaches door within 1 m (before O)	1
	D watches or approaches door while O is carrying cubes (for at least 2 s)	1
	any vocalization (if not clearly asking for the ball)	1
Owner ABSENT	any contact seeking behavior with O before the separation	1
	at 1st separation D vocalizes or runs around up and down or scretches door	1
	at 2nd separation D vocalizes or runs around up and down or scretches door	1
	D follows S to the door when she leaves (within 1 m)	1
	D plays or lies down comfortably (head down) but not at door for at least 2 s	−1
ACCEPTANCE		
Owner PRESENT	D approaches S when she 1st enters (at once, within reaching distance)	1
	D gets in physical contact and wags when S 1st enters	1
At any time	D takes toy to S (not during play)	1
	D seeks physical contact (jumps on, snuggles up to, nudges) during the episodes	1
	D avoids S during play (stands off, avoids her touch)	−1
Owner ABSENT	D gets in physical contact and wags when the S enters 2nd time	1
	during cube carrying D mostly watches (1) and also follows (2) S	2
	D plays with S also during separation (at least for 2 s−1, most of the time−2)	2
	D is close (closest bodypart is within 1 m) to S during separation (at least for 2 s−1, most of the time−2)	2

with romantic partners and dogs. The dogs' Attachment score was significantly associated with their owners' relationship both with their romantic partners and their dogs. Namely, higher Attachment scores in dogs were associated with lower Bond-related avoidance ($p < 0.01$) and higher Pet-related avoidance ($p < 0.01$) of their owners.

Higher Anxiety scores in dogs were in association with higher Extraversion ($p < 0.05$) and Openness ($p < 0.01$), as well as with higher Bond-related anxiety ($p < 0.01$), Pet-related anxiety ($p < 0.01$), and Pet-related avoidance scores ($p < 0.01$), and lower Bond-related avoidance ($p < 0.01$) of their owners.

As regards Acceptance, higher scores in dogs were in association with lower Openness ($p < 0.01$) and higher Bond-related avoidance ($p < 0.01$), Pet-related anxiety ($p < 0.01$), and Pet-related avoidance scores ($p < 0.01$) of their owners. See **Figure 3** for the overview of the results.

DISCUSSION

Chen et al. (2011) had suggested that one source of the variation in human infants' attachment to their mothers is the polymorphism of their OXTR gene but they remained cautious about this conclusion due to the genetic relatedness of infants and their parents. Based on the analogy between infant-mother and dog-owner attachment, our findings seem to confirm their suggestion, as the present study provides the first evidence that genetic variations in dogs' OXTR gene are associated with their attachment behavior to their owners. All behavioral aspects measured in the SST (Attachment, Anxiety, and Acceptance) showed significant association with all three dog OXTR SNPs investigated in this study (as a main or an interaction effect). All 3 canine OXTR SNPs have proved to have behavioral associations also in former studies. Behavioral associations of the −213AG polymorphism have already been reported in other

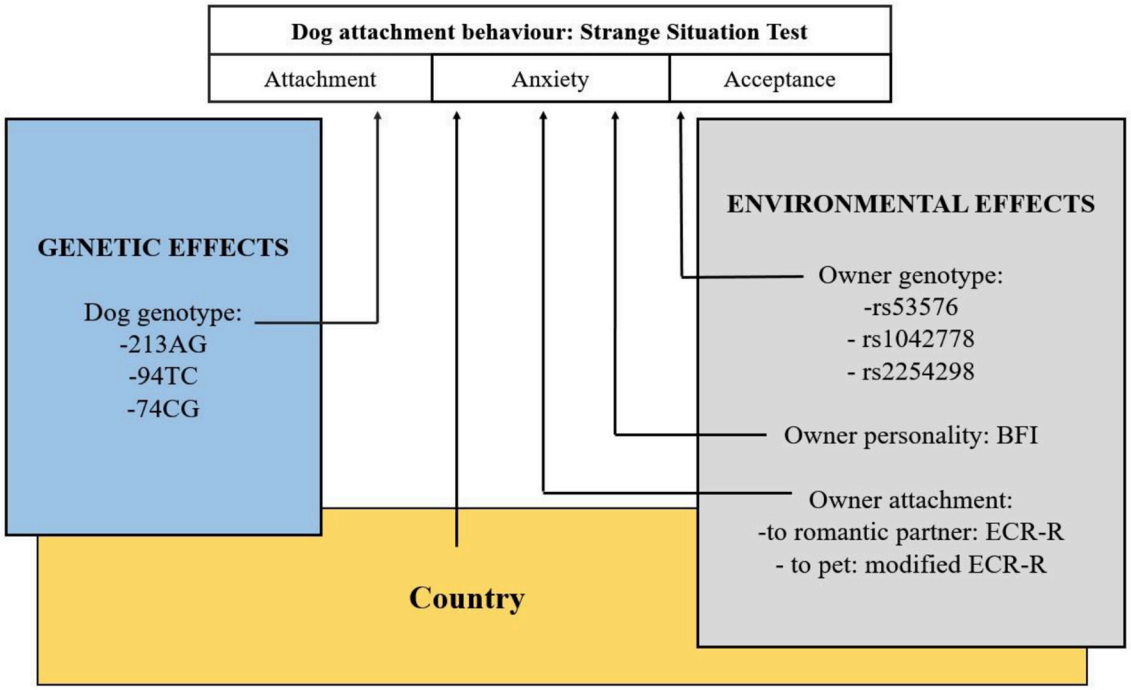

FIGURE 2 | Overview of the study. Examination of environmental and genetic associations of dogs' attachment behavior to their owners. Another study (Kovács et al., 2016b) investigating dogs' social sensitivity has also found significant main effect of the same OXTR polymorphism.

studies. The −213AG polymorphism had been shown to be associated with proximity seeking not only in Border Collies but also in German Shepherds (Kis et al., 2014). Another study (Kovács et al., 2016b) investigating dogs' social sensitivity has also found significant main effect of the same OXTR polymorphism (−213AG) on readiness to look at the human face in two test situations. Moreover, Turcsán (2017) found that this SNP was associated with greeting behavior, especially when the dog had no prior negative experience with the experimenter. Oláh et al. (2017) also found that dogs' first reaction and their friendliness in the threatening approach test were significantly modulated by −213AG and −74GC polymorphisms. In line with our finding that −213AG polymorphism is associated with Stranger acceptance, Romero et al. (2014) also found that oxytocin modulates social motivation to approach and affiliate with conspecifics and human partners. Earlier studies (Windle et al., 1997; Neumann, 2002; Bello et al., 2008) had also shown, that oxytocin can regulate the activity of the hypothalamic–pituitary–adrenal axis, thereby modifying the stress response. This mechanism may explain the associations we found between the OXTR polymorphisms and Anxiety composite score.

We found some differences between the results of different models. For example −74CG has significant main effects on dogs' Attachment, Anxiety as well as Acceptance scores in our first model (when testing its effects in interaction with the owners' genotypes), but in the second model it has an influence only on dogs' Anxiety and only when tested in interaction with dogs' sex. Moreover, while −74CG (and not −213AG) has a main effect on dogs' Anxiety in the first model,−213AG (and not −74CG) has a main effect on the Anxiety in our second model. This seems to result from the facts that sex of the dogs influenced the associations of two canine OXTR SNPs, namely the effects of −213AG and −74CG on dogs' Anxiety. It has been shown both in humans (e.g., Herzmann et al., 2013) and dogs (Nagasawa et al., 2015; Kovács et al., 2016a; Turcsán, 2017) that oxytocin can have differential effects on males and females. In prairie voles more is known about the underlying mechanisms of such sex differences, as Smeltzer et al. (2006) found higher binding by the oxytocin receptors in the medial prefrontal cortex in female than in male prairie voles. Another possible explanation is that steroid hormones, such as estradiol and progesterone, can modulate the affinity of the oxytocin receptors (estradiol enhances OXT receptor affinity, while progesterone has been shown to decrease receptor binding; Gimpl et al., 2002; Choleris et al., 2008).

Another important finding of the study is that both dog and owner OXT genetic variation shapes the dog-owner attachment in an interactive manner. Earlier research has also shown a mutual effect of both dogs and their owners on the peripheral oxytocin levels of both parties (Nagasawa et al., 2009, 2015). This is the first study, however, providing evidence that the oxytocin system of both parties impacts on dogs' attachment behavior. We found significant effects of two human OXTR SNPs (rs2254298 and rs1042778) on the Attachment composite score and one SNP (rs53576) on Acceptance, and several significant interactions of the effects of the human and dog OXTR gene on the attachment behavior of dogs (e.g., −213AG × rs2254298,−74CG × rs53576,−94TC × rs1042778,−74CG × rs1042778). Dog owners' behavior is likely to be one of the

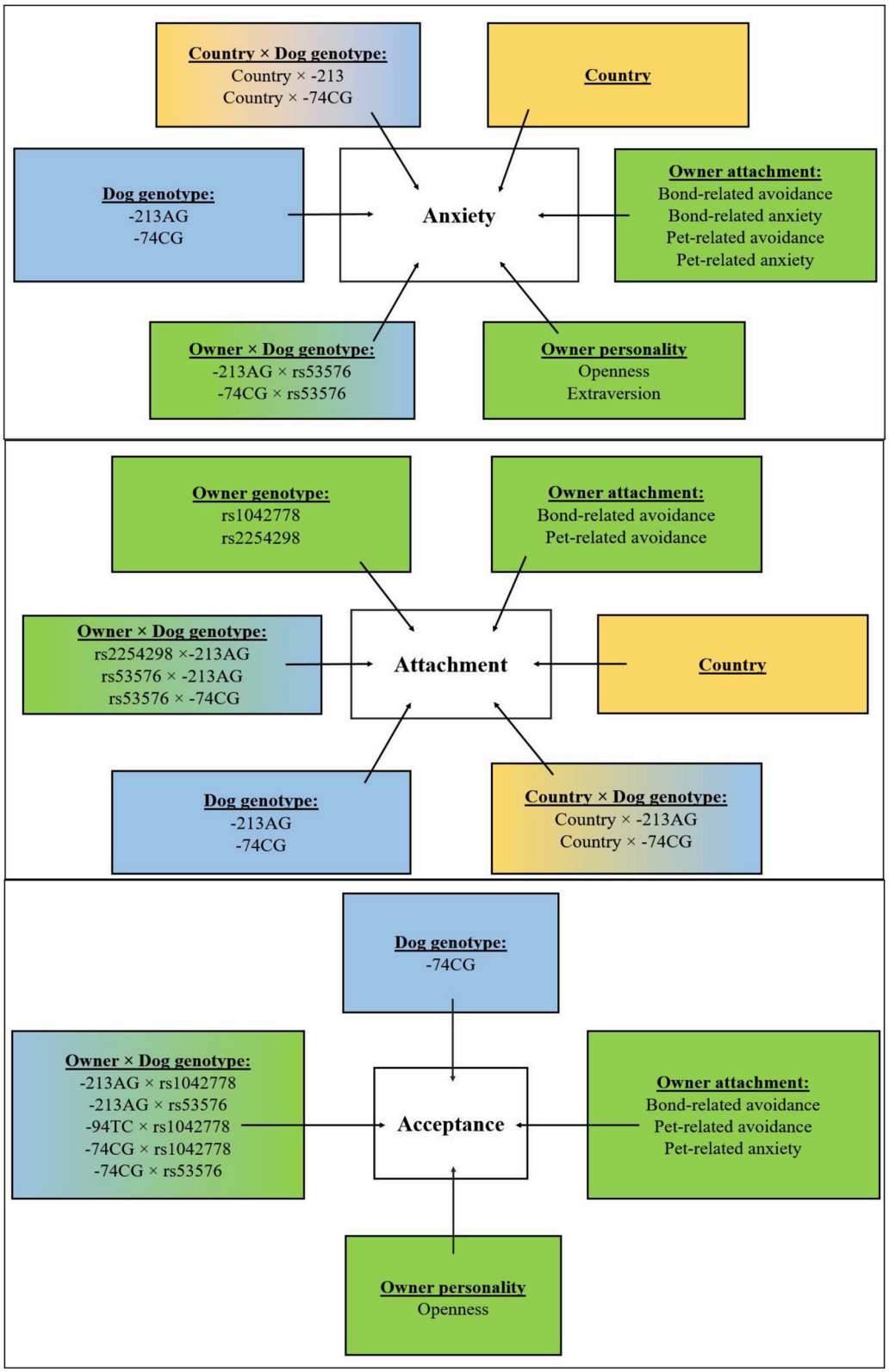

FIGURE 3 | Overview of the results. Effects of country (yellow boxes), environmental (green boxes), and genetic (blue boxes) associations of dogs' behavior (attachment, anxiety, and acceptance composite scores) and their interactions.

TABLE 4 | Summary of the effects of dog and owner OXTR polymorphisms on dogs' Attachment, Anxiety, and Acceptance composite scores as measured in the Strange Situation Test.

Composite score	Effect	WCS	*p*	Detail
Attachment				**Main effects**
	−213AG	**20.735**	**<0.01**	**AA+AG > GG**
	−94TC	0.458	>0.1	
	−74CG	**16.086**	**<0.01**	**CC+CG > GG**
	rs1042778	**11.573**	**<0.01**	**CC > AA+AC**
	rs2254298	**14.190**	**<0.01**	**CT+TT > CC**
	rs53576	0.000	>0.1	
	Country	1.887	>0.1	
				Significant pairwise interactions
	−213AG × rs2254298	**12.340**	**<0.01**	**AA+AG (−213AG) +CC (rs2254298) > GG (−213AG) + CC (rs2254298)**
	−213AG × rs53576	**7.772**	**<0.05**	**AA+AG (−213AG) + CT+TT (rs53576) > GG (−213AG) + CT+TT (rs53576)**
	−74CG × rs53576	**10.904**	**<0.01**	**GG (−74CG) + CT+TT (rs53576) > CC+CG (−74CG) + CT+TT (rs53576)**
	Country × −213AG	**15.817**	**<0.01**	**Austria AA+AG > Austria GG**
	Country × −74CG	**24.791**	**<0.01**	**Hungary GG > Austria GG**
Anxiety				**Main effects**
	−213AG	4.868	>0.1	
	−94TC	0.000	>0.1	
	−74CG	**7.808**	**<0.05**	**CC+CG > GG**
	rs1042778	1.988	>0.1	
	rs2254298	1.369	>0.1	
	rs53576	1.222	>0.1	
	Country	3.954		
				Significant pairwise interactions
	−213AG × rs53576	**14.826**	**<0.01**	**AA+AG (−213AG) + CT+TT (s53576) > AA+AG (−213AG) + CC (rs53576)**
	−74CG × rs53576	**5.955**	**<0.05**	**GG (−74CG) + CT+TT (rs53576) > CC+CG (−74CG) + CT+TT (rs53576)**
	Country × −213AG	**20.088**	**<0.01**	**Austria AA+AG > Austria GG**
	Country × −74TC	**10.421**	**<0.01**	**Hungary GG > Austria GG**
Acceptance				**Main effects**
	−213AG	4.562	>0.1	
	−94TC	0.101	>0.1	
	−74CG	**6.233**	**<0.05**	**CC+CG > GG**
	rs1042778	0.548	>0.1	
	rs2254298	1.480	>0.1	
	rs53576	**6.331**	**<0.05**	**CT+TT > CC**
	Country	1.592	>0.1	
				Significant pairwise interactions
	−213AG × rs1042778	**13.561**	**<0.01**	**GG (−213AG) + CT+TT (rs53576) > GG (−213AG) + CC (rs53576)**
	−213AG × rs53576	**12.347**	**<0.01**	**CC (−94TC) + CT+TT (rs53576) > CT+TT (−94TC) + CT+TT (rs53576)**
	−94TC × rs1042778	**12.695**	**<0.01**	**CT+TT (−94TC) + CC (rs1042778) > CC (−94TC) + CC (rs1042778)**
	−74CG × rs1042778	**12.018**	**<0.01**	**CC+CG (−74CG) + AA+AC (rs1042778) > GG (−74CG) + AA+AC (rs1042778)**
	−74CG × rs53576	**16.541**	**<0.01**	**CC+CG (−74CG) + CT+TT (rs53576) > GG (−74CG) + CT+TT (rs53576)**

Significant effects are highlighted in bold.

greatest environmental factors influencing the dogs' attachment behavior. It is possible that the owners' behavior mediates this link between the owners' genotype and the dogs' relationship to them, particularly given Bakermans-Kranenburg's and van Ijzendoorn (2008) finding that OXTR is related to parenting style in humans. As dogs are unrelated to their human caregivers,

TABLE 5 | The effects of dog OXTR polymorphisms and dog characteristics on Attachment, Anxiety, and Acceptance composite scores as measured in the Strange Situation Test.

Composite score	Effect	WCS	*p*	Details
Attachment		**Main effects**		
	−213AG	4.854	>0.1	
	−94TC	0.000	>0.1	
	74CG	1.325	>0.1	
	Country	**16.868**	**<0.01**	**Hungary > Austria**
	Age	4.207	>0.1	
	Sex	1.762	>0.1	
	Neuter status	0.137	>0.1	
		Significant pairwise interactions		
	Country × Neuter status	**8.486**	**<0.05**	**Neutered Hungary > Neutered Austria**
Anxiety		**Main effects**		
	−213AG	**12.349**	**<0.05**	**AA+AG > GG**
	−94TC	0.099	>0.1	
	−74CG	4.105	>0.1	
	Country	**10.516**	**<0.05**	**Hungary > Austria**
	Age	0.228	>0.1	
	Sex	3.498	>0.05	
	Neuter status	0.619	>0.1	
		Significant pairwise interactions		
	Sex × −213AG	**11.027**	**<0.05**	**Male AA+AG > Male GG**
	Sex × −74CG	**7.723**	**<0.05**	**Female CC+CG > Female GG**
	Neuter status × −213AG	**8.975**	**<0.05**	**Neutered AA+AG > Neutered GG**
Acceptance		**Main effects**		
	−213AG	0.179	> 0.1	
	−94TC	1.469	> 0.1	
	−74CG	1.161	>0.1	
	Country	0.055	>0.1	
	Age	0.167	>0.1	
	Sex	3.098	>0.05	
	Neuter status	**8.411**	**<0.05**	**Neutered > Intact**
		Significant pairwise interactions		
	Neuter status × Age	**11.880**	**<0.05**	**Intact young > Neutered young**

Significant effects are highlighted in bold.

the owner's genetic background may have an influence on their parenting style or other relevant behavior that, in turn, through epigenetic processes, affects the dogs' attachment behavior or the effects of the dogs' own OXTR genotype on it. However, given that in this study a considerable number of human DNA samples were missing, future studies with larger sample sizes need to consolidate these results.

As a further confirmation of owner influences on dog attachment, we have also found that the owners' Bond-related avoidance to their partners and Pet-related avoidance influenced all the three composite scores of their dogs' attachment, Openness and Pet-related anxiety affected dogs' Anxiety and Acceptance, while Extraversion personality trait influenced the Anxiety composite score. Previously, Konok et al. (2015) examined in a questionnaire study whether owners' attachment style and personality traits influence the occurrence of separation-relation disorder in the dogs. They found that owners scoring higher on self-reported attachment avoidance are more likely to have dogs with separation-related disorder. They suggested that owners' attachment style influences their caregiving behavior toward their dogs, and owners with attachment avoidance may show less consistent responsiveness to their dog's needs. Schöberl et al. (2016) also investigated the effects of owner personality and found that

TABLE 6 | The effects of owner personality and relationship experiences with both romantic partners and dogs on dogs' Attachment, Anxiety, and Acceptance composite scores as measured in the Strange Situation Test.

Composite score	Effect	WCS	*p*	Detail
Attachment	Extraversion	3.545	>0.05	
	Agreeableness	0.044	>0.1	
	Conscientiousness	1.671	>0.1	
	Neuroticism	0.525	>0.1	
	Openness	1.430	>0.1	
	Bond-related anxiety	0.545	>0.1	
	Bond-related avoidance	**30.691**	**<0.01**	**lower Bond-related avoidance > higher Bond-related avoidance**
	Pet-related avoidance	**18.539**	**<0.01**	**higher Pet-related avoidance > lower Pet-related avoidance**
	Pet-related anxiety	3.601	>0.01	
Anxiety	**Extraversion**	**4.990**	**<0.05**	**higher Extraversion > lower Extraversion**
	Agreeableness	0.001	>0.1	
	Conscientiousness	4.150	>0.1	
	Neuroticism	1.525	>0.1	
	Openness	**9.577**	**<0.01**	**higher Openness > lower Openness**
	Bond-related anxiety	**8.944**	**<0.01**	**higher Bond-related anxiety > lower Bond-related anxiety**
	Bond-related avoidance	**15.345**	**<0.01**	**lower Bond-related Avoidance > higher Bond-related Avoidance**
	Pet-related avoidance	**46.042**	**<0.01**	**higher Pet-related avoidance > lower Pet-related avoidance**
	Pet-related anxiety	**18.790**	**<0.01**	**higher Pet-related anxiety > lower Pet-related anxiety**
Acceptance	Extraversion	0.896	>0.1	
	Agreeableness	0.393	>0.1	
	Conscientiousness	2.837	>0.05	
	Neuroticism	0.604	>0.1	
	Openness	**11.588**	**<0.01**	**lower Openness > higher Openness**
	Bond-related anxiety	0.396	>0.1	
	Bond-related avoidance	**16.032**	**<0.01**	**higher Pet-related avoidance > lower Pet-related avoidance**
	Pet-related avoidance	**40.075**	**<0.01**	**higher Pet-related avoidance > lower Pet-related avoidance**
	Pet-related anxiety	**20.356**	**<0.01**	**higher Pet-related anxiety > lower Pet-related anxiety**

Significant effects are highlighted in bold.

owners with high neuroticism and agreeableness had dogs with lower cortisol reactivity in the Strange Situation Test. In contrast, in humans, children's insecure attachment, behavior problems and separation anxiety disorders are often associated with mothers' neuroticism and anxiety disorder (Manassis et al., 1994; Biederman et al., 2001; Kochanska et al., 2004). Interestingly, in our study the Neuroticism personality trait associated with none of the dog composite scores. Our finding about the correlation between owners' personality and their dogs' behavior is especially important from an applied perspective, as environmental factors may have the potential to modify oxytocin-related behavioral changes.

Finally, one of the most powerful effects we found was a difference between Austria and Hungary. There were main effects of country on two of the three behavioral components (Attachment and Anxiety composite scores) with dogs in Hungary showing higher Attachment and Anxiety, than dogs in Austria. Country has also influenced the effect of dog genetic background on attachment; similarly to Chen's et al. (2011) study where a certain OXTR genotype was associated with secure attachment in a non-Caucasian sample but not in a Caucasian sample. Oxytocin functions, similarly to serotonin (Yoshida et al., 2009) and dopamine functions (Liu and Wang, 2003), are likely be influenced by multiple factors, including other genetic polymorphisms that vary across ethnic populations (e.g., Chang et al., 1996; Kunugi et al., 1997). These uncontrolled genetic differences in our study could potentially mask the function of oxytocin and thus may have weakened the measurable association between variations in OXTR function and attachment behavior of dogs. Alternatively or additionally, different components of the dogs' social environment may differ between the two countries. It has been suggested that the behavioral expression of certain genotypes is sensitive to input from the social environment (Way and Taylor, 2010). Kim et al. (2010) suggested that the social environment can alter or even reverse the phenotypic expression of different genotypes. They found that culture-specific norms as a form of social input can also affect phenotypic expression of OXTR.

In conclusion, our study provides experimental evidence that genetic variations of the OXTR gene in both dogs and

their owners, as well as various aspects of dogs' environmental background are associated with their attachment to their human caregivers. Based on previous and present results we propose that polymorphism in the oxytocin receptor gene is a potentially important factor in regulating dog-human relationship. Although the complex joint effects of genetic and environmental factors on dogs' human-directed social behavior warrant further investigation, these findings offer a promising approach to studying causes and treatments of separation anxiety in dogs.

AUTHOR CONTRIBUTIONS

KK, ZV, AK, and JT: Designed the study. All authors prepared the study material and data acquisition. KK and DK: Entered the data and prepared it for statistical analyses; KK, ZV, AK, BT, and JT: Analyzed the data; KK, ZV, and JT: Interpreted the data; ZV, ZR, and JT: Obtained funding; KK: Wrote the first draft of the manuscript; KK, ZV, AK, MG, and JT: Critically revised the manuscript for important intellectual content. All authors gave final approval of the manuscript version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2018.00435/full#supplementary-material

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Context and Individual Characteristics Modulate the Association between Oxytocin Receptor Gene Polymorphism and Social Behavior in Border Collies

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Recent studies suggest that the relationship between endogenous oxytocin and social affiliative behavior can be critically moderated by contextual and individual factors in humans. While oxytocin has been shown to influence human-directed affiliative behaviors in dogs, no study investigated yet how such factors moderate these effects. Our study aimed to investigate whether the context and the dogs' individual characteristics moderate the associations between the social affiliative (greeting) behavior and four single-nucleotide polymorphisms (SNPs) of the oxytocin receptor (*OXTR*) gene. We recorded the greeting behavior in three contexts: (1) when the dog first met an unfamiliar experimenter, (2) during a separation from the owner, and (3) after the experimenter approached the dog in a threatening manner. In the latter two contexts (during separation and after threatening), we categorized the dogs into stressed and non-stressed groups based on their behavior in the preceding situations. In line with previous studies, we found that polymorphisms in the *OXTR* gene are related to the greeting behavior of dogs. However, we also showed that the analyzed SNPs were associated with greeting in different contexts and in different individuals, suggesting that the four SNPs might be related to different functions of the oxytocin system. The −213A/G was associated with greeting only when the dog had no prior negative experience with the experimenter. The rs8679682 was found in association with greeting in all three contexts but these associations were significant only in non-stressed dogs. The −94T/C was associated with greeting only when the dog was stressed and had an interaction with the sex of the dog. The −74C/G SNP was associated with greeting only when the dog was stressed during separation and also had a sex interaction. Taken together, our results suggest that, similarly to humans, the effects of oxytocin on the dogs' social behavior are not universal, but constrained by features of situations and individuals. Understanding these constraints helps further clarify how oxytocin mediates social behavior which, in the long run, could improve the application of oxytocin in pharmacotherapy.

Keywords: oxytocin receptor gene, dog, greeting behavior, stress, individual differences, contextual differences

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INTRODUCTION

Encountering an unfamiliar individual can always carry some risk. Initiating interactions (especially affiliative or cooperative interactions) with a stranger without knowing his/her attitude and intentions could lead to unpleasant or even dangerous experiences. Research on humans and non-human animals showed that the oxytocin system plays a prominent role in evaluating the potential benefits and risks of social encounters, and thus, modulating the willingness to approach and engage another individual in social interactions (Young et al., 2001; Depue and Morrone-Strupinsky, 2005; Lim and Young, 2006). Various mechanisms have been proposed to explain this influence of oxytocin, a peptide hormone produced in the hypothalamus. Humans' social interactions with others are associated with greater amygdala activation than non-social interactions (Eiji Nawa et al., 2008), and oxytocin is thought to down-regulate this activation (Tost et al., 2010; Kumsta and Heinrichs, 2013). On the other hand, oxytocin has been proposed to link approach behavior to the rewarding experience of social interactions (Insel, 2003; Campbell, 2008), and to facilitate the categorization of others as in-group members (De Dreu, 2012), thereby promoting positive interactions during social encounters.

Oxytocin shows remarkable evolutionary preservation in structure and function (Donaldson and Young, 2008). Therefore, it is an especially interesting question how it contributes to regulating social behavior in today societies where meeting and interacting with strangers happen extremely frequently. Humans share this open social environment with dogs, and both species, indeed, seem to be strongly motivated to engage in social interactions (Over, 2016; vonHoldt et al., 2017). Despite their genetically based gregariousness, the social behavior of dogs toward humans is not at all uniform. There is considerable variation in this phenotype not only across but also within breeds (Mehrkam and Wynne, 2014; Persson et al., 2015), and recent studies have showed that there is a significant genetic basis for this variation (Persson et al., 2015, 2016). In particular, genetic variation in the oxytocin system has been put forward as a prominent candidate to account for differences in human-directed social behavior, including interactions with strangers (Beetz et al., 2012).

Polymorphisms of the oxytocin receptor (*OXTR*) gene have been most investigated so far, and have indeed been found to be associated with behavioral variation that humans and dogs show when interacting with strangers (see Kumsta and Heinrichs, 2013; Li et al., 2015 for human reviews). Variations in the *OXTR* gene sequence can influence the location, density, distribution pattern, and functioning of the oxytocin receptors (Young et al., 2001; Donaldson and Young, 2008; MacDonald and MacDonald, 2010; Meyer-Lindenberg et al., 2011), and animal research has demonstrated that differences in the neural distribution and expression of the receptors often lead to individual differences in social behavior (Insel and Shapiro, 1992). Taken together, genetic variation of the *OXTR* gene can alter receptor density, affinity, or function in specific brain regions, thereby moderating the subjects' sensitivity to oxytocin, and in turn their behavior.

The behavioral associations of *OXTR* variation are far from uniform, however. In dogs, Kis et al. (2014) found for instance that two out of three single-nucleotide polymorphisms (SNPs) of the *OXTR* gene had opposite associations with stranger-directed behavior in two breeds. The −213AG polymorphism was the only one that was similarly associated with proximity seeking both in Border collies and in German shepherds: dogs carrying the G allele approached and followed a stranger less than AA dogs. The two other polymorphisms investigated (rs8679684 and 19208A/G) were associated with friendliness, but differently in the two breeds: in German shepherds carriers of the A allele for both SNPs were more friendly, whereas in Border collies individuals carrying the A allele were less friendly (Kis et al., 2014). Furthermore, while Kubinyi et al. (2017) reported findings on Siberian huskies similar to the above results in Border collies, they failed to find associations between the same three SNPs and the greeting behavior of Border collies. Only in huskies the G/G homozygotes on the 19208A/G SNP were faster and more persistent in greeting an unfamiliar human than dogs carrying the rare A allele.

The inconsistent findings of both human and non-human studies seem to confirm concerns questioning the explanatory power of individual SNPs (Benjamin et al., 2012). Others argue, however, that much of this inconsistence comes about because the effects of oxytocin depend on the context and on the characteristics of the individual (sex, personality traits, etc.) (Bartz et al., 2011). As an example, behavioral associations of *OXTR* polymorphisms have been shown to depend on the social environment the subjects lived in, as well as on the context their behavior was tested in (Kim et al., 2010; Chen et al., 2011). Kim et al. (2010) showed that in humans the G allele of the rs53576 *OXTR* SNP, relative to the AA genotype, was associated with higher emotional support seeking in American subjects but not in Korean subjects, and even in American subjects only in periods of distress. This finding they explain by the difference that in American culture it is normative to seek emotional support in times of distress but not in Korean culture, and suggest that psychological distress and culture are important moderators that shape behavioral outcomes associated with *OXTR* genotypes. In concert with this finding, Chen et al. (2011) showed that individuals carrying at least one copy of the G allele of the same SNP could benefit more from receiving social support than AA individuals. Subjects with GG or AG genotype showed significantly lower cortisol and subjective stress rating relative to the AA genotype, but only when they received social support while preparing for a stressful encounter with strangers. These results suggest that experiencing stress is an important modulator of the behavioral effects of the genetic variation of *OXTR*. More specifically, when an individual perceives a situation stressful, certain *OXTR* genotypes may be more likely to promote approach and affiliation (as a form of social support seeking, and thereby buffering the stress response), whereas the positive effect of these genotypes in support seeking may not be evident in times and contexts when the individual is not experiencing stress.

In the current study we set out to investigate whether and to what extent the context of the situation and the

dogs' experiencing stress moderate the associations between pet dogs' social approach and affiliation (greeting behavior) to an unfamiliar human and polymorphisms in their *OXTR* gene. Greeting behavior (describing how the dog approaches and interacts with a friendly but unfamiliar experimenter) is frequently assessed in dog studies, and it is related to the sociability personality trait of the individuals (Svartberg and Forkman, 2002). Studies have also showed that the dogs' greeting behavior (and sociability in general) has a genetic background (e.g., van der Waaij et al., 2008; Persson et al., 2015), although its heritability might be breed-dependent (Héjjas et al., 2009; Wan et al., 2013). In the current study, the dogs' greeting behavior was tested in three contexts: when the dog first met the experimenter, when the dog was separated from the owner, and after the experimenter approached the dog in a threatening manner. In order to test for moderating effect of stress, in each of the second and the third contexts, based on their reaction to separation and to a social threat, respectively, we categorized the dogs into two groups: (1) dogs either stressed or not by being separated from the owner when in an unfamiliar place, and (2) dogs either stressed (showing an overt avoidant or aggressive reaction) or not (reacting in a friendly way or passively) by the experimenter, when she approached them in a threatening way. Both the separation from the owner (especially when at the same time also facing a stranger) and the threatening approach had been found to evoke a stress response in dogs both at a behavioral and a physiological level (i.e., increased heart rate and heart rate variability in separation: Palestrini et al., 2005; Maros et al., 2008; and increased cortisol concentrations after the threatening approach: Horváth et al., 2008). Beyond this, however, dogs show a rather diverse sensitivity for both situations (Topál et al., 1998; Vas et al., 2008) that are differently stressful for the different individuals. Gácsi et al. (2013) found that dogs which were behaviorally reactive in these two situations (measured by vocalization) showed increased heart rate/heart rate variability after the test, but no significant cardiac response was found in non-reactive dogs. Due to this variation, these two situations seemed suitable to investigate whether the dogs' stress reactivity interacts with their *OXTR* genotype in affecting behavior during social encounters. Furthermore, we investigated whether the sex and age of the dogs moderated the associations between *OXTR* polymorphisms and greeting behavior. We chose a single breed for the analyses because there are marked differences regarding *OXTR* variations between different breeds (Bence et al., 2017); thus, the genetic constitutions of the breeds could overshadow the effect of one candidate gene, and because previous studies found breed differences both in social behavior and in the associations between *OXTR* polymorphisms and social behavior (Kis et al., 2014; Kovács et al., 2016; Kubinyi et al., 2017).

MATERIALS AND METHODS

Ethics Statement

This study was approved by the institutional ethics and animal welfare committee at the University of Veterinary Medicine Vienna (Approval numbers: 09/04/97/2012, 04/05/97/2012, 09/10/97/2012) in accordance with Good Scientific Practice guidelines and national legislation[1]. The owners participated on a voluntary basis and they all signed an informed consent form before beginning the experiment.

Subjects

Altogether, 217 purebred Border collies, recruited from the Clever Dog Lab database in Vienna, participated in a behavior test battery and were genotyped for *OXTR* polymorphisms. In the current analyses, we excluded dogs younger than 10 months and dogs with missing genotype data. The final sample consisted of $N = 173$ dogs, 72 (41.6%) males and 101 females, and their mean age ($\pm$SD) was 3.87 ± 3.02. From this sample, due to technical reasons, two dogs had missing values for the *Greeting during first encounter*, four dogs for the *Greeting during separation*, and one dog for the *Greeting after threatening approach* context (please see below for descriptions of test contexts).

Procedure

Phenotyping

The test was conducted in an experimental room of the Clever Dog Lab (5 m $\times$ 6 m), and dogs were allowed to explore the room prior to the test. The dogs participated in three situations, all three were presented on the same day with ca. 20 min between them. The order of the test contexts was the same for all subjects, and the experimenter was the same in all three contexts.

(1) Greeting during first encounter (see Héjjas et al., 2009; Wan et al., 2013; Kis et al., 2014).

The owner held the dog on a loose leash in the middle of the test room and was instructed not to talk to or interact with the dog. The experimenter (unfamiliar to the dog) entered the room, verbally greeted the owner and the dog, and then approached the dog in a friendly manner (walking at a normal pace, looking in the direction of the dog, and smiling). The experimenter stopped 1.5 m away from the dog.

- If the dog approached the experimenter and showed "friendly" behaviors (moving toward her and tail wagging) or remained neutral (did not move away from the owner, no tail wagging, and no aggression), then the experimenter petted it while continuously speaking in a friendly way.
- If the dog showed fearful/stress behaviors (avoiding eye contact, low body posture, and low ear position), or actively avoided the experimenter, then she crouched down and called the dog. If the dog approached in a non-aggressive manner, the experimenter petted it while continuously speaking in a friendly way. If the dog did not respond, the experimenter talked continuously to the dog in a friendly manner for 10 s and then terminated the test.
- If the dog growled or barked at the experimenter, then she talked continuously to the dog in a friendly manner for 10 s while avoiding eye contact, and then terminated the test.

(2) Greeting during separation.

[1]http://www.vetmeduni.ac.at/fileadmin/v/z/forschung/GoodScientificPractice_English.pdf

TABLE 1 | Variables coded in the test: approach, enthusiasm, and tail wagging.

Situation(s)	Variable	Definition
	Approach	When E approaches, the dog . . . 0: does not approach her on its own; 1: approaches when called; 2: approaches hesitatingly or after a while; 3: approaches immediately without calling.
(1) Greeting during first encounter (2) Greeting during separation (3) Greeting after threatening approach	Enthusiasm	The dog . . . 0: is not interested, avoids interacting with E (i.e., turns away or withdraws); 1: behaves passively, does not elicit interaction (i.e., stays in one place, may sniff around a bit); 2: behaves friendly (i.e., approaches the E, may cuddle, jump or lick once); 3: is very excited/enthusiastic with intensive searching for contact (i.e., rushes to E, cuddles, jumps up or licks her, tries to stay close and in physical contact with E).
	Tail wagging	The dog . . . 0: shows no or very little tail wagging; 1.5: wags its tail intermittently; 3: wags its tail continuously
Separation	Stress signals	During the 1-min long separation period the dog . . . 0: does not show any (detectable) stress signals; 1: shows signs of stress, including vocalization, pacing, yawning, lip licking, salivation, stretching, self-grooming, shaking, or scratching the door
Threatening approach	Reaction to threat	Behavior shown just before the test is terminated: 0: the dog approaches E with tail wagging or remains passive (i.e., no approach and no avoidance, may wag tail intermittently); 1: the dog hides behind the owner or moves away from the E (with low tail and ear position) or shows signs of aggression (i.e., barking, growling, snapping, or lunging toward E).

These three variables were coded in each of the three greeting contexts, and for each context they were combined into a scale. E, experimenter.

TABLE 2 | Reliability measures of the three greeting scales.

	Internal consistency	Inter-observer reliability	
Situation	**Cronbach's α**	**ICC**	***F*-test**
Greeting during first encounter	0.727	0.878	$F = 8.211, p < 0.001$
Greeting during separation	0.758	0.834	$F = 6.028, p < 0.001$
Greeting after threatening approach	0.675	0.868	$F = 7.549, p < 0.001$

ICC, intraclass correlation coefficient.

The dog was unleashed and left alone in the experimental room. After 1 min, the experimenter entered the room, stood next to the door for 5 s without interacting with the dog, and then followed the protocol of the "*Greeting during first encounter*" test context.

(3) Greeting after threatening approach.

The protocol of the threatening approach was similar to the procedure described in Vas et al. (2005). The owner held the leash of the dog and he/she was not allowed to talk or interact with the dog. The experimenter called the dog's name, and then approached the dog slowly and haltingly, with a slightly bent upper body while staring steadily into the eyes of the dog. The approach was terminated if (a) the experimenter reached the dog's position, (b) the dog approached her in a non-aggressive manner, (c) the dog moved away and hid behind the owner, or (d) the dog reacted aggressively (e.g., excessive growling or barking, snapping, or attacking).

After the threatening approach was terminated, the experimenter stepped a few steps away from the dog while the owner unleashed the dog, crouched down, called the dog in a friendly manner, and then followed the protocol of the "*Greeting during first encounter*" test.

Coded Variables

The dogs' behavior was recorded by four cameras located in the corners of the testing room and the recordings were analyzed at a later date. The same three variables were coded in all three greeting contexts: if, when, and how the dog approached the experimenter, how much enthusiasm the dog showed during the greeting, and if and how much the dog wagged its tail (for the definitions of the variables, see **Table 1**). We created a scale for each context by taking the mean of these three variables. The three scales showed good internal consistency (Cronbach's $\alpha > 0.6$, **Table 2**). To assess the inter-observer reliability of the scales, a subset of 40 videos was coded twice by two of

three independent coders. The intraclass correlation coefficients (ICC 1,*k*, absolute agreement; Weir, 2005) calculated between the coders were >0.8 for all three scales, indicating good inter-observer reliability (**Table 2**).

Additionally, we also coded how the dogs reacted to separation from their owner and to being approached in a threatening way by the experimenter. During separation, we coded whether or not the dog showed any sign of stress. In the threatening approach test, we coded whether the dog showed any sign of fear or aggression just before the test was terminated (when the strongest threat was exposed to the dog, see **Table 1** for details). We used each of these binomial measures to categorize dogs into "stressed" and "non-stressed" groups based on their behavior in each of the *Greeting during separation* and *Greeting after threatening approach* tests.

Genotyping

We collected buccal samples from the dogs in a non-invasive manner by swabbing the upper gum area of the dogs with four cotton tips (see Héjjas et al., 2007). The cotton tips were then sealed in a tube and preserved in the freezer until genotyping.

The procedures of the DNA isolation, and sequencing and genotyping the SNPs were the same as described in Bence et al. (2017). The −213A/G, −74C/G, and the rs8679682 polymorphisms were genotyped by the PCR-RFLP method. PCR amplification was performed using 5′-9-CCA TTG GAA TCC GCC CCC T-3′-9 forward and 5′-9-CAC CAC CAG GTC GGC TAT G-3′9 reverse primers for −213AG and −74CG SNPs and 5′-GAA AGG CCA TTC TCA GGA AA-3′ forward and 5′-CCC CCA TCA TCT TCT ACC A-3′ reverse primers for rs8679682 SNP. Annealing temperature was 56°C and the total reaction volume was 10 ml. The PCR products were incubated for 3 h at 37°C in a restriction enzyme mixture containing 0.5 U/μl Hpy99I restriction enzyme (NEB) for −213A/G SNP, 0.5 U/μl BsiEI restriction enzyme (NEB) for −74C/G SNP, and 0.5 U/μl PshAi restriction enzyme (NEB) for rs8679682 SNP with 1× BSA and 1× NEB4 buffer. The −94T/C SNP was genotyped by allele-specific amplification (ASA) using the primers described above. Allele-specific primers were 5′-CCG ATC TGC TGG TCC CGG-3′ and 5′-CCG ATC TGC TGG TCC CGA-3′ and the annealing temperature was 60°C. The digested PCR products were analyzed by conventional submarine agarose gel electrophoresis (Biocenter, Szeged, Hungary), using 2.5% agarose gel and visualized by ethidium bromide staining.

Of the eight SNPs found in the *OXTR* gene in dogs, only four were polymorph enough (i.e., both homozygotes were present) in Border collies to be included in the current study. The genotype frequencies and the results of the Hardy–Weinberg equilibrium analyses of these four polymorphisms are shown in **Table 3**. In two cases (−213A/G and the rs8679682 SNPs), the rare homozygotes were <15%, therefore we combined them with the heterozygotes.

TABLE 3 | Genotype frequencies and Hardy–Weinberg analyses of the four *OXTR* SNPs analyzed in this study.

Polymorphism	Genotype	*N*	%	χ^2 test for HWE violation
−213A/G	AA	9	5.2	$p = 0.539$
	AG	55	31.8	
	GG	19	63.0	
−94T/C	CC	30	17.3	$p = 0.013$
	CT	102	59.0	
	TT	41	23.7	
−74C/G	CC	39	22.5	$p = 0.001$
	CG	51	29.5	
	GG	83	48.0	
rs8679682	CC	25	14.5	$p = 0.060$
	CT	97	56.1	
	TT	51	29.5	

Strong statistical significance ($p < 0.00001$) would suggest that a given SNP may be subjected to disturbing factors (e.g., mutation, selection, nonrandom mating, genetic drift, or gene flow), which may bias the results of gene–behavior association analyses.

Statistical Analyses

First, in order to provide descriptive analyses, we investigated if the four possible modifying factors: context, sex, age, and stress in the preceding situation *per se* have a significant effect on the greeting behavior of the dogs. The context, sex, and age were included in a generalized linear mixed model as main effects, with the greeting behavior as the dependent variable and the dogs' ID as a random effect. The model also included all two-way and three-way interactions. The effect of being stressed or not in the preceding situation was investigated with independent *t*-tests in the *Greeting during separation* and *Greeting after threatening approach* contexts separately. Next, to investigate whether there was any association between our subjects' different individual characteristics (genotype distributions of the four SNPs, stress during separation, reaction to threatening, and sex of the dogs), we analyzed the relationships between these variables using χ^2 tests.

Second, we used another generalized linear mixed model to analyze if the dogs' individual characteristics (sex, age) and/or the context modified the association between the dogs' *OXTR* genotype and behavior. This model included the greeting behavior as the dependent variable, the four SNPs, sex, and context as fixed factors, age as covariate, and the dogs' ID as random effect. Here we also included all age × SNP, sex × SNP, and context × SNP interactions, as well as all sex × SNP × context and age × SNP × context interactions. Non-significant effects were removed from the model using a backward elimination procedure.

Third, we ran an additional GLM for each of the *Greeting during separation* and *Greeting after threatening approach* tests, in order to investigate how the dogs' reaction to the preceding situation moderated the association between the *OXTR* genotypes and the behavior. The dogs' reaction to the preceding situation was not added as a fixed factor in the previous models because the expected two-way or three-way interactions between SNP and reaction (and sex or age) may not be detected due to the lower sample size ($N = 73$–100 dogs per stress category). Therefore, we analyzed the effect of the SNPs separately in the two reaction categories of dogs using GLM models with the same setup as described

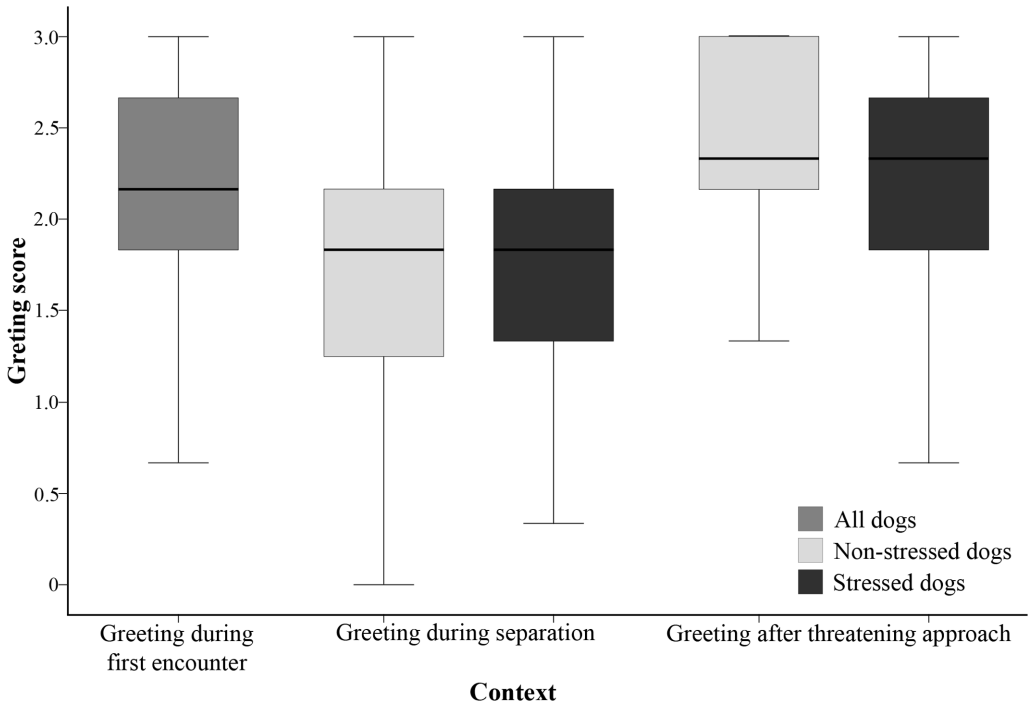

FIGURE 1 | Greeting score of the dogs in the three contexts. Significant differences were found between the *Greeting during separation* and the other two contexts. For the *Greeting during separation* and *Greeting after threatening approach* contexts, the stressed and non-stressed dogs are presented separately, but no differences were found between the stressed and non-stressed dogs in any of these contexts.

TABLE 4 | Relationship between genotype distributions, stress during separation, reaction to threatening, and the dogs' sex.

	Sex			Stress during separation			Reaction to threatening		
	χ^2	*df*	*p*	χ^2	*df*	*p*	χ^2	*df*	*p*
Stress during separation	0.206	1	0.650	–	–	–	0.228	1	0.633
Reaction to threatening	0.553	1	0.457	0.228	1	0.633	–	–	–
−213A/G	3.242	1	0.072	0.426	1	0.514	1.662	1	0.203
rs8679682	0.567	1	0.452	0.849	1	0.357	0.026	1	0.871
−94T/C	0.679	2	0.712	0.238	2	0.888	2.469	2	0.291
−74C/G	2.017	2	0.365	3.074	2	0.215	1.094	2	0.579

above. In all the models, the effect size of each factor was estimated with Partial η^2. SPSS version 22 was used for the analyses.

RESULTS

Descriptive Analyses

Regarding the factors affecting the greeting behavior, neither the dogs' sex, nor the age, nor any of their interactions were significantly related to the greeting behavior ($p > 0.261$ at removal). We found a main effect of the context ($F_{2,511} = 50.760$, $p < 0.001$), pairwise contrast revealed that the dogs greeted the experimenter more when they first met her and after threatening approach than during separation ($p < 0.001$ for both), but there was no difference between the *Greeting during first encounter* and *Greeting after threatening approach* contexts ($p = 0.728$) (**Figure 1**).

We found no significant differences between the stressed and non-stressed dogs either in the *Greeting during separation* or in the *Greeting after threatening approach* contexts ($p = 0.206$; $p = 0.289$, respectively) (**Figure 1**).

Regarding possible correlated effects of the different individual characteristics, we found no significant associations between stress during separation, reaction to the threatening approach, sex of the dog, and the genotype distributions of the four SNPs (**Table 4**).

Overall Analyses of *OXTR* and Context Effects

We found two significant sex × SNP × context and two significant age × SNP × context interactions, as well as a

TABLE 5 | The effects of dog *OXTR* polymorphisms and dog characteristics on behavior in the *Greeting during first encounter* context.

Source	*df*	*F*	*p*	Partial η^2	*Post hoc* comparisons
Corrected model	4	3.339	0.012	0.074	
rs8679682	1	4.422	0.037	0.026	CC+CT > TT
−213A/G	1	6.262	0.013	0.036	
Age	1	4.325	0.039	0.025	
−213A/G × age	1	5.695	0.018	0.033	AA+AG: younger > older ($p = 0.006$); GG: no age effect ($p = 0.841$)
Total	171				

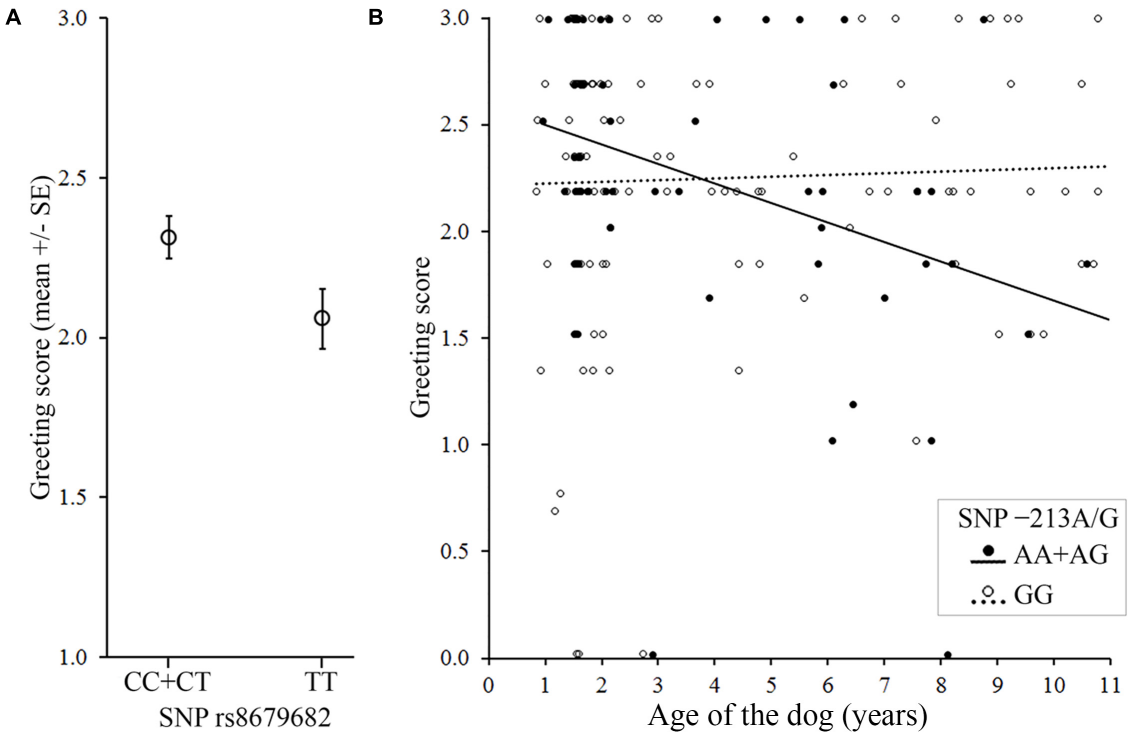

FIGURE 2 | Associations between the *OXTR* SNPs and the behavior score in the Greeting during first encounter. **(A)** Dogs carrying the C allele in the rs8679682 SNP greeted the experimenter more than dogs with TT genotype. **(B)** Older dogs carrying the A allele in the −213A/G SNP greeted the experimenter less than younger dogs, while no age effect was found in GG genotype.

significant two-way interaction between SNP and context. The −213A/G SNP showed a significant three-way interaction with age and context ($F_{2,464} = 3.151$, $p = 0.044$), the −94T/C SNP with sex and context ($F_{4,464} = 3.174$, $p = 0.014$), and the −74C/G with both age and context and sex and context ($F_{4,464} = 3.798$, $p = 0.005$; $F_{4,464} = 2.427$, $p = 0.047$, respectively). The rs8679682 SNP had no interaction with sex or age, but we found a two-way interaction with context ($F_{2,464} = 4.361$, $p = 0.013$): the difference between this SNP's genotypes was larger in the *Greeting during separation* context than in the other two contexts. To further explore and interpret the three-way interactions, we also analyzed the three contexts separately using general linear models (GLM), including the four SNPs, sex, age, and all age × SNP and sex × SNP interactions. Pairwise contrast was used for *post hoc* tests for main effects and sex interactions; the significant age interactions were interpreted by investigating the effect of age in the different genotypes separately.

Context-Specific Analyses

Greeting during First Encounter

We found a significant main effect of the rs8679682 SNP (Partial $\eta^2 = 0.026$), and an interaction between −213A/G SNP and age (Partial $\eta^2 = 0.033$) (**Table 5** and **Figure 2**).

Greeting during Separation

We found significant main effects of the −213A/G and rs8679682 SNPs (Partial $\eta^2 = 0.030$ and 0.076, respectively), and significant sex interactions of the −94T/C and −74C/G SNPs (Partial $\eta^2 = 0.060$ and 0.054, respectively) (**Table 6a** and **Figure 3**).

In this context, we also investigated if the dogs' reaction to separation moderated the associations between the

TABLE 6 | The effects of dog *OXTR* polymorphisms and dog characteristics on behavior in the *Greeting during separation* context.

Source	*df*	*F*	Sig.	Partial η^2	*Post hoc* comparisons
(a) All dogs					
Corrected model	11	2.455	0.007	0.147	
Sex	1	0.052	0.820	0.000	
−213A/G	1	4.877	0.029	0.030	AA+AG > GG
−94T/C	2	0.327	0.722	0.004	
−74C/G	2	0.714	0.491	0.009	
rs8679682	1	12.892	0.000	0.076	CC+CT > TT
Sex × −94T/C	2	4.968	0.008	0.060	females: CC > CT, TT ($p = 0.019$, $p = 0.018$); males: TT > CC ($p = 0.046$)
Sex × −74C/G	2	4.522	0.012	0.054	females: GG > CG, CC ($p = 0.018$, $p = 0.021$); males CC ∼> GG ($p = 0.064$)
Total	169				
(b) Only stressed dogs					
Corrected model	9	2.095	0.041	0.205	
Sex	1	0.168	0.683	0.002	
−94T/C	2	1.743	0.182	0.046	
−74C/G	2	0.315	0.731	0.009	
Sex × −94T/C	2	6.692	0.002	0.155	females: CC > CT, TT ($p = 0.004$, $p = 0.086$); males: CT, TT > CC ($p = 0.046$, $p = 0.011$)
Sex × −74C/G	2	4.231	0.018	0.104	females: GG ∼> CC ($p = 0.074$); males: CC > GG ($p = 0.024$)
Total	83				
(c) Only non-stressed dogs					
Corrected model	2	5.333	0.007	0.114	
−213A/G	1	5.866	0.018	0.066	AA+AG > GG
rs8679682	1	9.738	0.002	0.105	CC+CT > TT
Total	86				

(a) All dogs were analyzed; (b) only dogs that showed stress signals during separation were analyzed; (c) only dogs that did not show stress signals during separation were analyzed.

SNPs and behavior. In the dogs that showed stress signals during separation, we found significant sex interactions of the −94T/C and −74C/G SNPs (Partial $\eta^2 = 0.155$ and 0.104, respectively), but no main effects of the other two SNPs ($p > 0.180$ at removal) (**Table 6b**). In the dogs that did not show stress signals during separation, we found significant main effects of the −213A/G and rs8679682 SNPs (Partial $\eta^2 = 0.066$ and 0.105, respectively), but no sex interactions of the other two SNPs ($p > 0.501$ at removal) (**Table 6c**).

Greeting after Threatening Approach

We found no significant main effects or interactions of any SNPs ($p > 0.141$ at removal) (**Table 7a** and **Figure 4**). In this context, we also investigated if the dogs' reaction to the threatening approach moderated the associations between the SNPs and behavior. In the dogs that reacted with avoidance or aggression to the threatening approach, we found a significant sex interaction of the −94T/C SNP (Partial $\eta^2 = 0.066$) (**Table 7b**). In the dogs that reacted with friendly or passive behaviors to the threatening approach, we found a significant sex interaction of the rs8679682 SNP (Partial $\eta^2 = 0.070$) (**Table 7c**). A summary of the results of the different models can be found in **Table 8**.

DISCUSSION

Our goal in this study was to investigate how the dogs' oxytocin system interacts with the context of the situation, the stress reactivity of the dogs and their other individual characteristics to predict social approach and affiliative behavior in social encounters. To analyze this, we examined four polymorphisms of the dogs' *OXTR* gene, assessed the dogs' greeting behavior in three different contexts, and grouped the dogs based on their reaction to separation and social threat, respectively. Overall, we found that similarly to previous studies (see reviewed in Kis et al., 2017) all four *OXTR* polymorphisms were significantly associated with greeting in at least one context. However, we also showed that both the context of the greeting and the dogs' being stressed had a moderating effect on these associations.

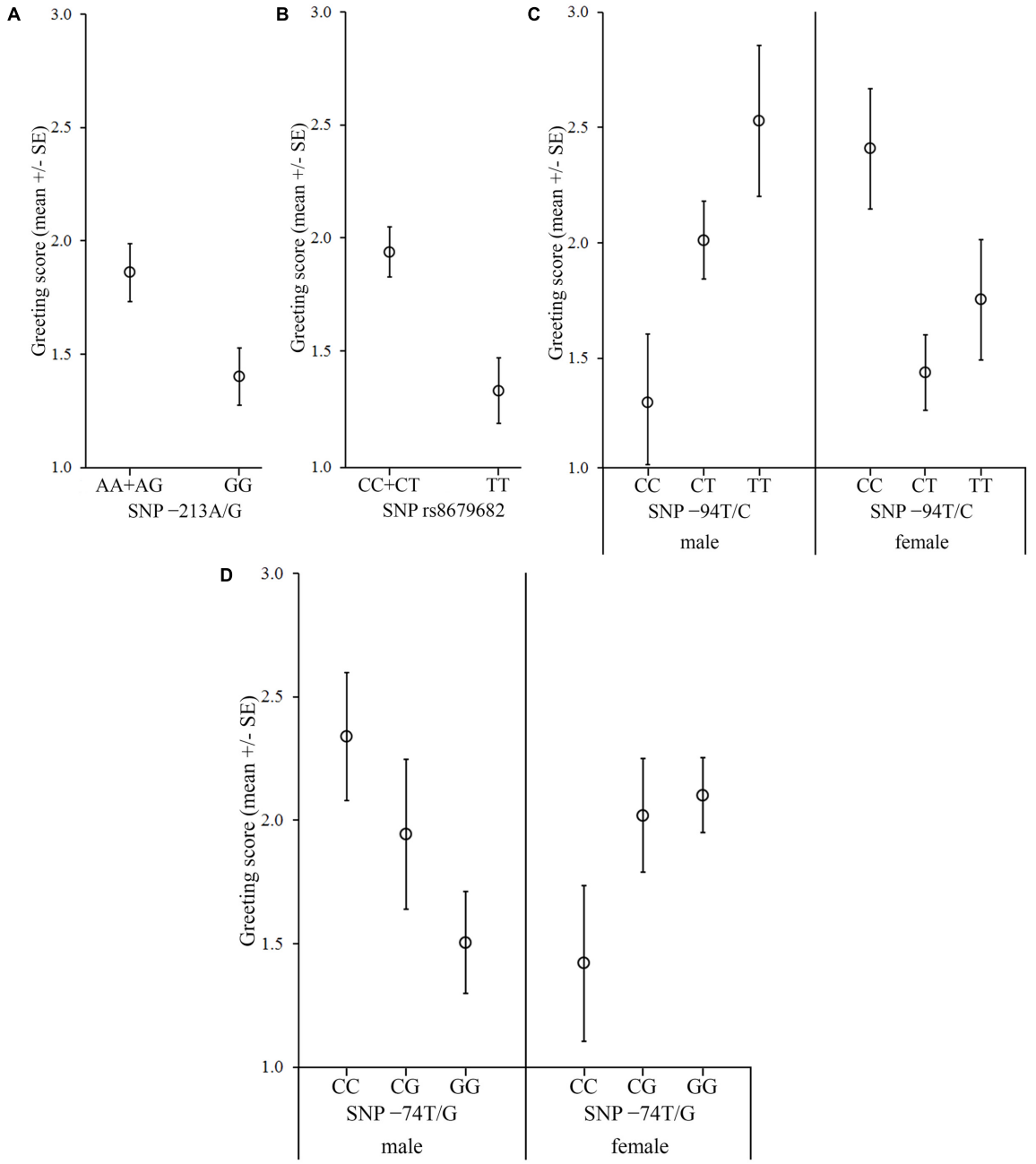

FIGURE 3 | Associations between the *OXTR* SNPs and the behavior score in the Greeting during separation. The dogs were divided into two groups based on the behavior during separation. In the case of dogs, which did not show stress signals during separation, **(A)** dogs carrying the A allele in the −213A/G SNP greeted the experimenter more than dogs carrying the GG genotype, and **(B)** dogs carrying the C allele in the rs8679682 SNP greeted the experimenter more than dogs with a TT genotype. In the case of dogs, which showed stress signals during separation, **(C)** females carrying CC genotype in the −94T/C SNP greeted the experimenter more than the CT and TT genotypes, while the relation was the opposite in males; **(D)** females carrying the GG genotype in the −74C/G SNP received higher scores than those carrying the CC genotype, while the relation was the opposite in males.

Context and Stress in Association with Greeting Behavior

In the first context, upon their *first encounter*, the dogs had the chance to greet the unfamiliar experimenter in the presence of their owner. In the *Greeting after threatening approach* context, the dogs greeted the more or less familiar experimenter also in the presence of the owner; however, in this context the dogs had a clearly negative experience with the experimenter immediately before the greeting situation. Although based on this we expected that dogs would greet the experimenter less enthusiastically after

TABLE 7 | The effects of dog *OXTR* polymorphisms and dog characteristics on behavior in the *Greeting after threatening approach* context.

Source	*df*	*F*	Sig.	Partial η^2	*Post hoc* comparisons
(a) All dogs					
No significant effect					
(b) Only stressed dogs					
Corrected model	5	2.377	0.045	0.112	
Sex	1	5.847	0.018	0.059	
−94T/C	2	3.052	0.052	0.061	
Sex × −94T/C	2	3.321	0.040	0.066	females: no difference; males: CT, TT > CC ($p = 0.011$, $p = 0.010$)
Total	100				
(c) Only non-stressed dogs					
Corrected model	3	2.209	0.095	0.089	
Sex	1	2.398	0.126	0.034	
rs8679682	1	0.000	0.995	0.000	
Sex × rs8679682	1	5.128	0.027	0.070	females: CC+CT > TT ($p = 0.039$); males: no difference
Total	72				

(a) All dogs were analyzed; (b) only stressed dogs (which reacted with avoidance or aggression to the threatening approach) were analyzed; (c) only non-stressed dogs (which reacted with friendly or passive behaviors to the threatening approach) were analyzed.

being threatened than during their first encounter, we found that the dogs greeted the experimenter with a similar intensity in these two contexts. This likely reflects the fact that dogs can flexibly adjust their behavior to the behavior (attitude) of their human partners (as Vas et al., 2005 also showed). This may also explain why no difference was found between the greeting behavior of dogs that had responded with or without overt stress to the social threat (i.e., dogs that showed avoidance or aggression vs. behaved in a friendly manner or remained passive). In contrast to the two contexts above, in the *Greeting during separation* context, the owner was not present during the test, and in this context the dogs greeted the experimenter less intensively than in the other two contexts. Although some of the dogs showed clear signs of stress caused by being separated from their owner and the others did not, the greeting intensity of both groups was uniformly lower in this context than in the presence of the owner. This suggests that the presence of the owner buffers the stress caused by the test situation, probably by providing social support (Gácsi et al., 2013).

Approximately half of the dogs (48.5%) showed stress-related behaviors during separation, and 57.8% of the dogs reacted with avoidance or aggression to the threatening approach (categorized as "stressed" based on Gácsi et al., 2013 and Horváth et al., 2008). It is important to note that we found no association between dogs' signs of stress in response to separation and their behavior in response to a social threat. This means that the stressed vs. non-stressed categorization of the dogs did not reflect their general stress proneness, or willingness to show overt behavioral expression of their stress. Instead, showing behaviors related to stress seems to indicate a negative reaction specific to either test context: either separation anxiety or fear of a threatening person.

OXTR Polymorphism–Behavior Associations

The most important finding of this study is that different *OXTR* SNPs were associated with greeting in stressed dogs and non-stressed dogs in either context. When trying to interpret these results, we should first mention that neither the separation stress nor the reaction to threatening was significantly related to any SNPs (or to the sex of the dog). Therefore, despite numerous studies suggesting the contrary (e.g., Kumsta and Heinrichs, 2013; Buttner, 2016), we cannot conclude that the *OXTR* polymorphisms themselves were directly related to general stress reduction or lower perception of social fear – at least not indiscriminately. Instead, the different SNPs had different behavioral associations depending on how the dog reacted to the context. As a possible explanation, stressed dogs may have approached and greeted the experimenter for different reasons than non-stressed dogs; even if they greeted her to a similar extent, they may have had different motivations to do so. This explanation would indicate that the dogs that did not show a negative reaction to separation or to a social threat maintained the same motivation to greet the experimenter as they had during the first encounter. However, for the dogs that were stressed by either being separated from their owner or threatened, the motivation to affiliate with the experimenter may have changed due to separation stress or social fear. For example, during separation, stressed dogs might look more for social support from the experimenter than non-stressed dogs, as they are more strongly affected by being alone.

In the context of the *Greeting during separation*, the −94T/C and −74C/G SNPs were found in association with the greeting only in stressed dogs, and both had a sex interaction. For the −94T/C, stressed males carrying the TT genotype greeted the

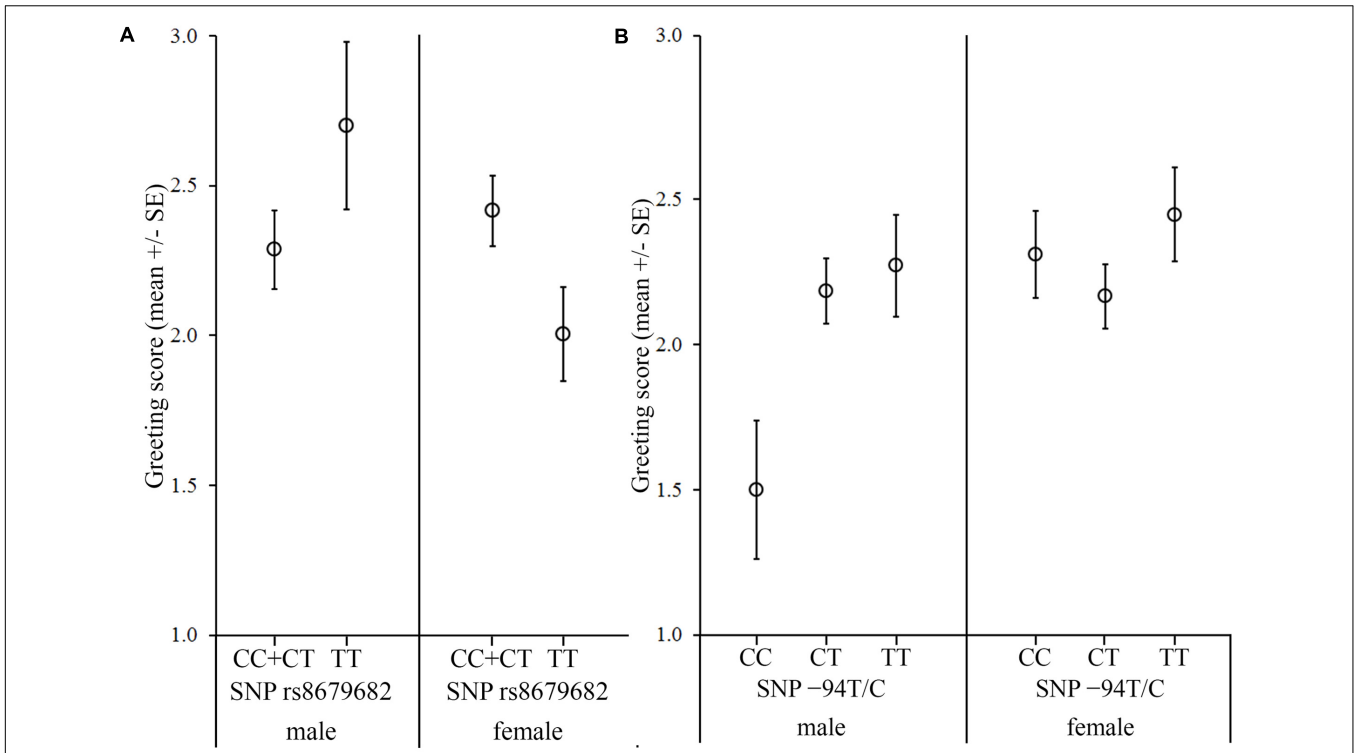

FIGURE 4 | Associations between the *OXTR* SNPs and the behavior score in the Greeting after threatening approach. The dogs were divided into two groups based on the behavior during the threatening approach. **(A)** In the case of dogs, which reacted with friendly or passive behaviors to the threatening approach, we found a significant sex interaction of the rs8679682 SNP female dogs carrying the C allele greeted the experimenter more than females with TT genotype, while no genotype difference was found in male dogs. **(B)** In the case of dogs, which reacted with avoidance or aggression to the threatening approach, we found a sex interaction of the −94T/C SNP; males carrying the CC genotype greeted the experimenter less than the CT and TT genotypes, while no genotype difference was found in females.

TABLE 8 | Overview of the *OXTR* polymorphism–greeting associations found in the three contexts.

		***OXTR* polymorphism**			
Context	**Sample of dogs**	**−213A/G**	**rs8679682**	**−94T/C**	**−74C/G**
First encounter	All dogs	AA+AG > GG	CC+CT > TT	–	–
In absence of owner	Without separation stress	AA+AG > GG	CC+CT > TT	–	–
	With separation stress	–	–	♀: CC > CT, TT ♂: CT, TT > CC	♀: GG > CC ♂: CC > GG
After threat	Passive or friendly	–	♀: CC+CT > TT ♂: –	–	–
	Avoidant or aggressive	–	–	♀: – ♂: CT, TT > CC	–

experimenter more than males with the CC genotype, while the relation was opposite or absent in female dogs. For the −74C/G, stressed males carrying the CC genotype greeted the experimenter more than males carrying the GG genotype, while the relation was the opposite in the case of females. Human studies also found not only stress-dependent *OXTR*–behavior associations, as described in the section "Introduction," but also sex differences in the effects of social support on the stress response (i.e., social support attenuated the stress reaction more for men than women, Kirschbaum et al., 1995; Ditzen et al., 2008).

Interestingly, even though the separation from the owner and a threatening person presented different types of stress for the dogs, the −94T/C SNP had a similar association with the greeting behavior also in the *Greeting after threatening approach* context. That is, stressed males carrying the TT genotype greeted the experimenter more than males with the CC genotype both when their owners left them alone, and when the experimenter had successfully threatened them beforehand. This seems to indicate that the −94T/C has a more general function in regulating the behavior of stressed (especially male) individuals, but, whether this SNP regulates the social fear of stressed dogs or affects their general stress coping ability should be further investigated.

In contrast, the polymorphisms −213A/G and rs8679682 were associated with greeting in non-stressed dogs during separation,

and, in the case of the rs8679682, also after being threatened. Importantly, these same two SNPs were associated with the behavior also in the *Greeting during first encounter* context. This seems to indicate that dogs in general did not perceive the first encounter with the experimenter as stressful.

For the −213A/G, individuals carrying the A allele showed more intense greeting than dogs with the GG genotype. Since this SNP was associated with the dogs' behavior only when the experimenter presented a positive or neutral figure (i.e., *Greeting during first encounter* and *Greeting during separation* in non-stressed dogs), the effect of this SNP might be sensitive to the characteristics of the experimenter. That is, the A allele predicts increased social approach and affiliation but only when the dog had no prior negative experience with the experimenter. Similarly, human studies also found differential effects of oxytocin on social behavior depending on the attitude of the social partner (e.g., Mikolajczak et al., 2010; De Dreu, 2012). Moreover, Kis et al. (2014) also found significant associations between the −213A/G and dogs' proximity seeking (a behavior scale that includes variables assessed in different greeting situations) both in German shepherds and in Border collies, and similarly to our study, they found that in Border collies, carrying the A allele was associated with higher proximity seeking.

For the rs8679682 SNP, non-stressed dogs carrying the C allele showed more intense greeting than dogs with the TT genotype in all three contexts (however, this association might be stronger in females than in males). This seems to indicate that the rs8679682 has a more general function in regulating the social affiliative behavior of dogs toward humans (with the C allele facilitating more positive social behaviors relative to the T allele), but its effects seem to be sensitive to the positive salience of the context. Whether this SNP regulates social motivation, attraction to humans or general curiosity needs further investigation.

Another parallel between our results and other studies is that the sex of the dog also modulates the gene × behavior associations. Sex differences in the function of the oxytocin system are well known (Bos et al., 2012; Chen and Johnson, 2012; Kovács et al., 2016), and can be explained by possible differences in the general hormonal environment (e.g., estrogen level, McCarthy, 1995; Petersson et al., 1999), different patterns of oxytocin release (Jezová et al., 1996), and/or different patterns of amygdala activation between the sexes. For example, oxytocin administration decreases amygdala reactivity to emotional faces in men (Domes et al., 2007) and increases it in women (Domes et al., 2010). Our results suggest that also in dogs, some relationships between polymorphisms of the *OXTR* and social behavior seem to be sex-specific.

CONCLUSION

Taken together, our results show that dogs' greeting behavior depends on the interaction of three factors: the context of the greeting, the individual characteristics of the animal (stress level and sex), and the genotype the dog carries in a given *OXTR* polymorphism. These results seem to be (at least partly) in harmony with the social salience hypothesis suggested in human studies (e.g., Shamay-Tsoory and Abu-Akel, 2016). That is, the way the *OXTR* polymorphisms are associated with behavior seems to depend on how the dogs perceived the situation itself, which, in turn, depends both on the context and the individual inclinations of the dogs.

These results can serve as a starting point for follow-up studies potentially demonstrating that much of the variance observed in the behavioral associations of the *OXTR* polymorphisms is systematic and a function of the context-, stress-, and individual-dependent nature of the effects of *OXTR* variation. In humans, consistent results of multiple studies employing similar procedures have been used to form such a conclusion, and to infer about the psychological and/or biological processes at play (Bartz et al., 2011). Based on our results, we suggest that the four polymorphisms investigated here might be related to different functions of the oxytocin system, as each of them was associated with behavior only in either positively or in negatively perceived situations. Beyond this, the functionality of these SNP variants is still unclear (especially because three of our SNPs are located in non-translating regions), so further molecular studies are warranted to elucidate functional consequences of these variants.

AUTHOR CONTRIBUTIONS

BT, FR, ZR, and ZV designed the study. BT and DK acquired and analyzed the data. BT, FR, and ZV interpreted the data. ZV, FR, ZR, and BT obtained funding. BT and DK wrote the first draft of the manuscript. All authors critically revised the manuscript for important intellectual content. All authors gave final approval of the manuscript version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Oxytocin and Opioid Receptor Gene Polymorphisms Associated with Greeting Behavior in Dogs

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Meeting humans is an everyday experience for most companion dogs, and their behavior in these situations and its genetic background is of major interest. Previous research in our laboratory reported that in German shepherd dogs the lack of G allele, and in Border collies the lack of A allele, of the oxytocin receptor gene (OXTR) 19208A/G single nucleotide polymorphism (SNP) was linked to increased friendliness, which suggests that although broad traits are affected by genetic variability, the specific links between alleles and behavioral variables might be breed-specific. In the current study, we found that Siberian huskies with the A allele approached a friendly unfamiliar woman less frequently in a greeting test, which indicates that certain polymorphisms are related to human directed behavior, but that the relationship patterns between polymorphisms and behavioral phenotypes differ between populations. This finding was further supported by our next investigation. According to primate studies, endogenous opioid peptide (e.g., endorphins) receptor genes have also been implicated in social relationships. Therefore, we examined the rs21912990 of the OPRM1 gene. Firstly, we found that the allele frequencies of Siberian huskies and gray wolves were similar, but differed from that of Border collies and German shepherd dogs, which might reflect their genetic relationship. Secondly, we detected significant associations between the OPRM1 SNP and greeting behavior among German shepherd dogs and a trend in Border collies, but we could not detect an association in Siberian huskies. Although our results with OXTR and OPRM1 gene variants should be regarded as preliminary due to the relatively low sample size, they suggest that (1) OXTR and OPRM1 gene variants in dogs affect human-directed social behavior and (2) their effects differ between breeds.

Keywords: dog, wolf, gene-behavior association, OXTR, OPRM1, greeting behavior

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INTRODUCTION

To investigate the comparative biology of human behaviors and uncover the genetic background of behavior disorders, in the past decade several research groups have studied the effects of gene variants on dog behaviors (Hall and Wynne, 2012). Oxytocin and opioid receptor genes are among the candidates for nervous system pathways that regulate canine social behavior toward humans.

Oxytocin is an evolutionarily highly conserved neuropeptide that plays an important role in various complex social behaviors, such as social cognition, trust, attachment, and sociability (Donaldson and Young, 2008). Oxytocin receptor genes have also been investigated in other domestic species; for example, in cats researchers found that the G738A OXTR SNP was associated with the personality trait "Roughness" (irritable, dominant, forceful, and moody behavior), as reported by owner questionnaire (Arahori et al., 2016). However, oxytocin has received the most extensive interest in how it may modulate dogs' behavior toward humans (Buttner, 2016; Jensen et al., 2016; Thielke and Udell, 2017). For example, intranasal administration of oxytocin was found to enhance motivation to approach and affiliate with owners (Romero et al., 2014), and increase looking back at human partners in a situation involving threatening behavior signals by a human (Hernádi et al., 2015).

When examining the genetic background of the oxytocin receptor gene, dog breeds differ from wolves (*Canis lupus*) in the frequency of oxytocin receptor (OXTR) gene variations (Bence et al., 2016), microsatellite markers close to the OXTR gene (Oliva et al., 2016), and OXTR methylation patterns (Banlaki et al., 2017). Methylation levels at certain sites in the OXTR promoter region were found to be different in Border collie females than in males. In males, methylation levels associate with a higher likelihood to approach a threatening unfamiliar person, and a lower likelihood to remain passive or hide behind the owner (Cimarelli et al., 2017).

On a genetically varied sample, Ottenheimer-Carrier et al. (2017) did not find any relationship between two OXTR SNP-s and behavior/personality measures in dogs. The authors suggested that links between OXTR gene SNPs and behavior might be breed-specific. Indeed, on single-breed samples, Kis et al. (2014) reported that three SNPs in the 5′ and 3′ untranslated regions (*UTR*) of the OXTR gene were associated with social behaviors toward humans (namely proximity seeking and friendliness, which were composite traits based on behavioral tests). OXTR gene polymorphisms have also been analyzed in another study conducted on golden retrievers. However, polymorphisms were not linked with separation-related behaviors in this breed (van Rooy et al., 2016).

The oxytocin system interacts with the opioid system that modulates reward, motivation, emotional responses, cognition, nociception, and autonomic functions. In females, opioids inhibit oxytocin release, especially at mu and kappa receptors (Vuong et al., 2010), depending on reproductive state (Evans and Olley, 1988). Opioid-oxytocin interactions probably have anatomical basis (Keverne, 2005). Higham et al. (2011) found that lactating rhesus macaque females possessing the G allele of the mu opioid receptor gene C77G SNP had higher cerebrospinal fluid oxytocin levels (but not different maternal behavior), than homozygous C females.

Several other studies implicate a role for endogenous opioid peptides (e.g., endorphins) in forming stable social relationships, such as pair bonding and attachment [e.g., in prairie voles (Burkett et al., 2011), domestic fowls (Warnick et al., 2005), and zebra finches (Schnelker et al., 2015)].

Morphine was the first chemical discovered to bind to mu opioid receptors. Genetic variations in the mu 1 opioid receptor genes (OPRM1) may primarily explain individual variation in the development of social relations in humans [parent-child interaction (Copeland et al., 2011); rejection by social partners (Way et al., 2009)].

In captive rhesus macaques, functional polymorphisms in the OPRM1 gene have been identified that are associated with social behaviors. Infants carrying the G allele of the C77G SNP exhibited higher levels of attachment behavior and higher distress to separation from their mothers, and they spent more time with their mothers upon reunion than individuals homozygous for the C allele. C/G infants were also less likely to interact with other individuals in the group (Barr et al., 2008). Female rhesus macaques with the G allele held their infants more (Higham et al., 2011).

In dogs, low doses of exogenous opiates have been found to significantly reduce distress vocalization and activity in socially isolated puppies (Panksepp et al., 1978). In a recent study, 34 SNP markers within the 500 kb region around the dog homolog of the OPRM1 gene were analyzed in golden retrievers affected by separation anxiety, and in control dogs, but no significant associations were observed (van Rooy et al., 2016). Two SNPs (C15A and C207T) in the exon 1 of the mu opioid receptor gene have been also studied. According to a preliminary study, dogs carrying the C allele of the C15A SNP have a greater susceptibility for dysphoric state following anesthesia. The authors also concluded that dog breeds closely related to wolves might be predisposed to dysphoria (Hawley and Wetmore, 2010).

In this study, we present data on genetic associations between the oxytocin and mu opioid receptor gene variants and greeting behavior in dogs. Dog breeds and breeding lines differ in several aspects of their social behavior toward humans (for a review see Mehrkam and Wynne, 2014). For example, while herding dog breeds were selected for cooperative work with continuous visual contact with their human partners, Siberian huskies, and other sled dogs were selected for work with no human visual contact (Gácsi et al., 2009). With regards to behavior and gene polymorphism associations, one could expect two different patterns of results. First, it could be hypothesized that all polymorphisms of a gene have the same effect in each breed/breeding lines, and thus in dogs in general. Second, it is also possible that the association patterns differ between populations. Specifically, some polymorphisms may have an effect in one population but not in another, or they have opposite effects in different breeds (due to gene-gene and gene-environment interactions).

Previous findings support the latter, more complex scenario. Hejjas et al. (2007) reported that a variable number of tandem repeats (VNTR) polymorphism in exon 3 of the dopamine D4 receptor gene (DRD4), was associated with activity-impulsivity in police German shepherd dogs (GSD), but not in pet GSDs. The authors suggested that the more homogenous environment in the case of police dogs helped the emergence of the subtle effects of the DRD4 polymorphism. However, it is also possible that the two populations had different ancestries (working line vs. show line); therefore, their genetic background was slightly

different. On the other hand, the lack of association with one DRD4 polymorphism does not not necessarily mean that DRD4 is not involved in the behavior of pet GSDs. In a subsequent study, Hejjas et al. (2009) found that intron 2 VNTR of the same gene (DRD4) was associated with greeting behavior (referred to as "social impulsivity") in pet GSDs (police dogs were not studied in this case). Interestingly, both behaviors has been reported to be linked with DRD4 in racing Siberian huskies (Wan et al., 2013). Although the allele frequency of DRD4 exon 3 is different between GSDs and Siberian huskies, an association with activity behavior score (including activity level during greeting a human) and a marginally significant association with the activity-impulsivity scale has been found in this breed. In sum, we can conclude that despite the genetic isolation of each breed/population and/or the allelic heterogeneity between breeds, the link between DRD4 and activity-impulsivity related measures seems to be generalizable to some breeds of dogs. Similar finding has been reported with regard to the tyrosine-hydroxylase gene (TH) and activity-impulsivity association which has been found to be significant in in pet GSDs (Kubinyi et al., 2012), and marginally significant in racing Siberian huskies (Wan et al., 2013).

The above findings suggest that with some divergence, gene-behavior associations might be similar between breeds. However, the study of Kis et al. (2014) reveals a more complicated picture. The authors found that one OXTR SNP had the same effect in the two herding breeds investigated, but another OXTR SNP had opposite effects in the breeds. Specifically, while carrying the G allele of the −213A/G SNP (formerly[1]: −212A/G) was associated with lower proximity seeking in both German shepherd dogs and Border collies, in the case of the 19208A/G SNP (formerly[2]: 19131AG) the lack of G allele predicted higher friendliness scores in German shepherd dogs, and the lack of A allele was linked to higher friendliness score in Border collies. (Because of linkage disequilibrium, the result was similar in the case of the rs8679684 SNP).

Certainly, the allele frequencies that are typical for a breed could modify the association patterns. Nevertheless, the question remains: how generalizable are gene-behavior associations in dogs?

In this study, we used the Greeting Test behavioral data collected by Kis et al. (2014) on herding dog breeds (Border collie and German shepherd dog) and Wan et al. (2013) on a non-herding dog breed (Siberian husky). The Greeting Test followed the same protocol in the two studies, and both were the first test presented in the test batteries. However, the scoring of the two tests were slightly different, so the raw data of Kis et al. (2014) was modified to be in harmony with the Wan et al. (2013) data (see Methods). We performed association studies between the assessed genetic variants (OXTR −213A/G, 19208A/G, rs8679684, and OPRM1 rs21912990) and the greeting behavior of the dogs. Finding similar or different patterns of gene-behavior associations in herding and non-herding dogs could imply that some OXTR SNPs are linked to social behavior in dogs in general, while others are breed-specific.

We also ran two pilot studies, which are presented in the Supplementary Material. The first is the genotyping of the kappa opioid receptor gene (OPRK1) rs23478162 SNP, and the description of the allele frequencies in three dog breeds and in wolves. The second is a comparative analysis on the expression levels of several opioid receptor genes (OPRM1, OPRD1, and OPRK1) conducted in various brain areas of a male beagle dog (see Supplementary Material).

MATERIALS AND METHODS

Subjects

One hundred and three border collies (1–12-years-old; mean age ± *SD*: 4 ± 3 years; male: 45%) and 104 German shepherd dogs (1–11-years-old; mean age ± *SD*: 4 ± 2 years; male: 57%) were assessed in a Greeting Test by Kis et al. (2014), and 96 Siberian huskies participating in sled dog races (1–14-years-old; mean age ± *SD*: 5 ± 3 years; male: 56%) by Wan et al. (2013).

None of the subjects were closely related, i.e., littermate and parent-offspring relationships were excluded.

For assessing the allele frequencies of the opioid receptor genes, 42 gray wolves (male: 45%) living in Hungarian, Serbian, Austrian, and German zoos and parks were also genotyped but did not participate in the Greeting Test.

Buccal Sample Collection and DNA Isolation and Genotyping

Buccal samples were collected from subjects in a non-invasive way, with cotton swabs from the inner surface of the cheek. Genomic DNA was isolated as described in a previous study (Kotyuk et al., 2013).

We investigated the three OXTR SNPs studied by Kis et al. (2014). The genotyping of −213A/G, 19208A/G (formerly −212A/G and 19131A/G, respectively) and rs8679684 was as described in Kis et al. (2014).

Mu opioid receptor gene (OPRM1) rs21912990 SNP (NCBI, NC_006583.3) was genotyped by allele specific amplification using 5′ATG CAT CTC TAC TAC TAA GG 3′forward and 5′ TTT ACC TCC CTT CTC TTA TC 3′ reverse primers. For genotyping the rs21912990 SNP C allele specific: 5′GGC AGC CCT TCC ATG ATC 3′ and T allele specific: 5′GGC AGC CCT TCC ATG ATT 3′ primers were used. Annealing temperature was 52°C. The PCR products were analyzed by 1.5% agarose gel electrophoresis.

The genotyping of kappa opioid receptor gene (OPRK1) rs23478162 SNP is described in the Supplementary Material.

Behavioral Data

Greeting Test

A female, unfamiliar experimenter greeted the dog, who was kept on leash by the owner (**Figure 1**).

In an open, undisturbed area, the owner stood in place while holding the dog on a 1.5–2 m leash. The dog was allowed to move freely within the range of the stretched leash and was not corrected or rewarded for any behavior. A female experimenter (unfamiliar to the dog) approached the dog in

[1]Position number is determined according to the most recent genome version, this explains the change of nomenclature compared to Kis et al. (2014).
[2]See above.

FIGURE 1 | Sequence of video frames from a Greeting Test. **(A)** Experimenter approaches the non-aggressive dog. **(B)** Experimenter steps sideways, **(C)** Experimenter waits 2–3 s to check whether the dog followed her.

TABLE 1 | Total number of participants in the Greeting Test, greeting score frequencies (%), and means for the three dog populations.

	Total *N*	Score 0 (*N*)	Score 1 (*N*)	Score 2 (*N*)	Mean score (*SD*)
Border collie	103	5.8 (6)	16.5 (17)	77.7 (80)	1.72 (0.57)
German shepherd dog	104	2.9 (3)	11.5 (12)	85.6 (89)	1.83 (0.45)
Siberian husky	96	8.3 (8)	26.0 (25)	65.6 (63)	1.57 (0.64)

a friendly manner (verbally greeted the owner and the dog and smiled). When the dog acted "friendly" (moved toward the experimenter with affiliative behaviors) or showed neutral behavior, the experimenter stepped toward the dog and patted its head, back, and shoulders. Then she stepped 1 m sideways within reach of the leash and waited 2–3 s in order to check whether the dog followed her. If the dog showed aggressive behavior (e.g., barking or growling), the experimenter stayed out of reach of the leash, crouched, and tried to call the dog. If the dog approached the experimenter and was not aggressive, she followed the protocol above. If it was not possible to approach the dog, the test was terminated in 30 s.

In the study of Kis et al. (2014) the Greeting Test was coded with two behavioral variables: "Latency of approaching" and "Latency of following' the experimenter, on a 0–3 scale: 0: 0 s; 1: 1–5 s; 2: 5–15 s; 3: does not approach. For the present analysis, these scores were re-calculated, by applying the coding method of the "Approaches" variable in Wan et al. (2013). This simple coding system enabled Wan et al. (2013) to assess the behavior of numerous dogs in a short time, at the experimental site, which was especially important in the case of the racing Siberian huskies, which were tested around the racing area. Specifically: the "greeting score" in the present study was calculated as follows: we gave 0 points if both "Latency of approaching" and Latency of following' were 3 in the Kis et al. (2014) study (i.e., the dog did not approach the experimenter and could not be patted); 1 point if one of the variables was 0–2 in the Kis et al. (2014) study (i.e., the dog approached the experimenter once in a non-aggressive way); 2 points if both variables were 0–2 in the Kis et al. (2014) study (i.e., the dog approached the experimenter in a non-aggressive way both during their initial encounter and followed her when she stepped away).

Previous studies have shown that the "Latency of approaching" and "Latency of following" variables of the Greeting Test correlated with approaching the experimenter in other contexts (separation from the owner, calling after threatening, Kis et al., 2014). The "Approaches" variable loaded highly on the sociability factor together with contact with the experimenter, body posture and tail wagging variables of the Greeting Test (Wan et al., 2013). Interobserver reliabilities have been reported in the studies of Kis et al. (2014) and Wan et al. (2013).

Statistical Analysis

SNPs deviating from Hardy-Weinberg equilibrium or with minor allele frequency below 0.05 were removed. SPSS 21.0 for Windows was used for all statistical analyses. General Linear Models (GLM) were used to test the effects of age (as covariate), sex, and SNP (with SNK *post-hoc* test) as main effects on behavior (greeting score) with backward elimination on variable selection. We adjusted p values for multiple comparisons with Bonferroni's adjustment: 0.017 for border collies and GSDs (3 GLM-s were run), 0.025 for Siberian huskies (2 GLMs).

RESULTS

OXTR Variation and Behavior

The descriptive statistics of the greeting score are shown in **Table 1**.

Table 2 presents the OXTR SNP genotype frequencies, allele frequencies, and Hardy-Weinberg equilibrium for each breed. In the Siberian husky breed the A allelic variant of the OXTR rs8679684 SNP was infrequent, and the −213A/G genotype frequencies deviated from Hardy-Weinberg equilibrium (chi2 =

TABLE 2 | OXTR SNP genotypes (GT), number of individuals by genotype (*N*), genotype frequencies (%), allele frequencies, chi2 scores, and chi2 test *p*-values in three dog populations.

	–213A/G			rs8679684			19208A/G		
	GT	**N**	**%**	**GT**	**N**	**%**	**GT**	**N**	**%**
Border collie	AA	6	7	AA	1	1	AA	1	1
	AG	28	31	AT	7	7	AG	8	8
	GG	57	63	TT	88	92	GG	94	91
Allele freq	G	0.78		T	0.95		G	0.95	
chi2		0.96			3.25			2.61	
p		0.33			0.07			0.11	
German shepherd dog	AA	12	12	AA	40	40	AA	38	38
	AG	48	49	AT	46	46	AG	49	49
	GG	37	38	TT	15	15	GG	14	14
Allele freq	G	0.63		T	0.38		G	0.38	
chi2		0.35			0.09			0.08	
p		0.55			0.77			0.78	
Siberian husky	AA	17	22	AA	0	0	AA	0	0
	AG	23	30	AT	1	1	AG	12	15
	GG	37	48	TT	74	99	GG	68	85
Allele freq	G	0.63		T	0.99		G	0.93	
chi2		9.94			0.00			0.53	
p		0.00			0.95			0.47	

9.94, $p = 0.002$), therefore these SNP-s were omitted from further analysis. In Border collies and GSDs rs8679684 and 19208A/G are in strong linkage disequilibrium (Kis et al., 2014), therefore the analyses were run only with 19208A/G.

We did not find an age or sex effect in any of the populations. One gene-behavior association was detected in Siberian huskies, and the results of the association analysis are presented in **Figure 2**. Genotype categories only included the G/G and the A/G groups, as the A/A genotype was missing in our relatively small sample. According to the results, the greeting behavior scores of G/G homozygotes for the OXTR 19208A/G SNP was significantly higher than the score of dogs possessing the rare allele (A/G) [$F_{(1, 78)} = 6.786$, original $p = 0.011$, adjusted $p = 0.022$, partial eta squared = 0.08, **Figure 2**], demonstrating the behavioral effect of the rare genetic variant.

OPRM1 Variation and Behavior

The OPRM1 rs21912990 SNP was not in linkage disequilibrium with the OXTR SNP-s in either breed ($p = 0.34$–0.81). The genotype frequencies of Siberian huskies and wolves were similar and C/C genotypes were more frequent than in Border collies and German shepherd dogs (chi2 = 84.533, $p < 0.001$).

The genotype distribution, allele frequencies and Hardy-Weinberg equilibriums are presented in **Table 3**. The measured genotypes of Border collies were not in Hardy-Weinberg equilibrium, but because p value was higher than 0.01 ($p = 0.03$) we performed the association analysis.

Association analysis of behavioral and genetic data on the OPRM1 rs21912990 SNP was conducted in all three dog breeds. There was no association in Siberian huskies [$F_{(1, 85)} = 1.665$, original $p = 0.200$, partial eta squared = 0.019].

Results of the genetic association analysis for the two herding breeds and OPRM1 rs21912990 SNP are shown in **Figure 3**. The genotypes were grouped according to the presence (C/C or C/T) or absence (T/T) of the major allele (C), as the behavioral data of major allele carriers were very similar. Border collies and German shepherd dogs without the major C allele (T/T) had lower greeting scores.

In Border collies OPRM1 rs21912990 T/T homozygotes tended to have lower scores than C/T and C/C dogs [$F_{(2, 78)} = 2.819$, original $p = 0.066$, partial eta squared = 0.067]. C/T and C/C genotypes did not differ. Therefore, the dominant model was applied comparing the genotype groups with the presence (C/C and C/T) or absence (T/T) of the dominant allele. This resulted in a trend toward significance after adjusting p for multiple comparisons [$F_{(1, 79)} = 5.705$, original $p = 0.019$, adjusted $p = 0.057$, partial eta squared = 0.067, **Figure 3A**].

In German shepherd dogs, OPRM1 rs21912990 T/T homozygotes had lower scores than C/T heterozygotes [$F_{(2, 51)} = 4.221$, original $p = 0.020$, partial eta squared = 0.142]. In accordance with the finding among Border collies, the dominant feature of the C allele was also shown among German shepherd dogs. Moreover, applying the dominant model for statistical analysis improved the significance of genetic differences [$F_{(1, 52)} = 8.160$, original $p = 0.006$, adjusted $p = 0.018$, partial eta squared = 0.136, **Figure 3B**].

DISCUSSION

In this study, we assessed the potential association of oxytocin and opioid receptor gene SNPs with greeting an unfamiliar human in three dog populations: pet Border collies, pet German

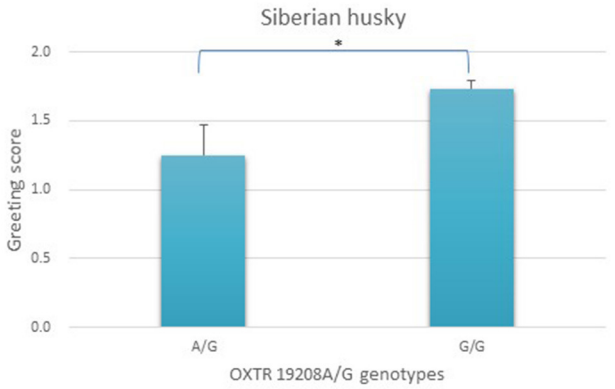

FIGURE 2 | Association between OXTR 19208A/G genotypes and greeting scores (mean + SE) in Siberian huskies ($N = 80$). Genotype categories only included the G/G and the A/G groups, as the A/A genotype was missing the sample. The greeting behavior scores of G/G homozygotes was significantly higher than the score of dogs possessing the minor "A" allele (A/G). *$p < 0.05$.

TABLE 3 | OPRM rs21912990 genotype (GT), number of individuals by genotype (*N*), genotype frequencies (%), allele frequencies, chi2 scores, and chi2 test *p*-values in three dog populations and wolves.

	OPRM1		
	GT	***N***	**%**
Border collie	CC	27	33
	CT	47	58
	TT	7	9
Allele freq	T	0.38	
chi2		4.51	
p		0.03	
German shepherd dog	CC	11	20
	CT	27	50
	TT	16	30
Allele freq	T	0.55	
chi2		0.00	
p		0.95	
Siberian husky	CC	68	78
	CT	18	21
	TT	1	1
Allele freq	T	0.11	
chi2		0.02	
p		0.87	
Wolf	CC	31	74
	CT	11	26
	TT	0	0
Allele freq	T	0.13	
chi2		0.95	
p		0.33	

shepherd dogs, and racing Siberian huskies. The genes were selected as candidates because they have been implicated in the development of complex social behaviors in mammals (e.g., Copeland et al., 2011).

We found that Siberian huskies that lacked the C allele of the OXTR 19208A/G SNP had higher scores in the Greeting test, i.e., they approached a friendly unfamiliar woman more frequently in a non-aggressive way. The same polymorphism was linked to the friendliness behavioral scale in the study of Kis et al. (2014). However, the friendliness scale did not include variables from the greeting test, as it was composed of the variables describing reactions to a threatening and a passive stranger. According to the results of the study by Kis et al. (2014) and the present study, the lack of A allele is associated with increased friendliness in Border collies, and more frequent approach during greeting in Siberian huskies, while the lack of G allele of the 19208A/G is linked to increased friendliness in German shepherd dogs.

In the Kis et al. (2014) study, the variables measured in the Greeting test were part of the proximity seeking scale, which was found to be associated with the −213A/G SNP in both herding breeds. However, this SNP was not in Hardy-Weinberg equilibrium in the Siberian husky population, therefore it was omitted from the investigation.

In conclusion, although the OXTR gene seems to be associated with the broad dimension of human directed social behavior, the precise variables of this dimension may or may not be related to the specific polymorphisms of the gene in different breeds. Thus, the relationship between behavior and genes is complex and breed specific. This is not surprising if we consider that (1) behavior traits are usually associated with many genetic variants, each of which has only a subtle effect on the behavior, thus gene-gene and gene-environment interactions could easily obscure them; (2) the relationship between genetic variations and allele frequencies are different between isolated dog populations with different ancestries; (3) alleles may not directly affect the phenotype, but are correlated with a causative allele located nearby—and the relationship between locations differ between breeds.

In the present study, we investigated not only OXTR, but another gene, which has been implicated in social behavior, the OPRM1. The allele frequencies of the rs21912990 SNP were similar in Siberian huskies and gray wolves, but differed from that of Border collies and German shepherd dogs, which might reflect their genetic relatedness and/or more similar social behavior.

The OPRM1 rs21912990 SNP tended to associate with greeting behavior in Border collies and was also significantly linked to this behavior in German shepherd dogs. T/T homozygotes approached the unfamiliar human less frequently than other dogs. This result is in harmony with findings in rhesus macaques (Barr et al., 2008; Higham et al., 2011), which suggest that individuals with certain OPRM1 allelic variants differ in behavior relating to social affiliation. However, the association was missing in the Siberian husky breed, probably because there was only one individual with the T/T genotype. Once again, the pattern of results suggest that the link between gene polymorphisms and behavior are probably breed specific. Therefore, when considering the applied aspect of opioid receptor studies, it would be important to examine in several

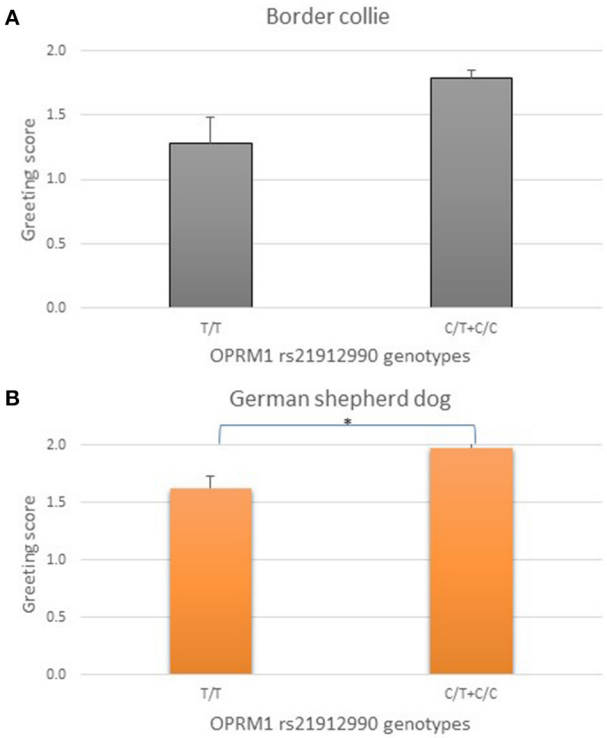

FIGURE 3 | Association between OPRM1 rs21912990 genotypes and greeting scores (mean + SE) in Border collies (**A**, $N = 81$) and in German shepherd dogs (**B**, $N = 54$). T/T homozygotes tended to have lower scores than C/T and/or C/C dogs, therefore, the dominant model was applied comparing the genotype groups with the presence or absence of the dominant C allele. T/T homozygotes had marginally lower scores in Border collies, and significantly lower scores in German shepherd dogs than C/T and C/C individuals. $^*p < 0.05$.

breeds how variations in responses to opioid administration may be influenced by these SNPs (Hawley and Wetmore, 2010).

Although the results should be regarded as preliminary due to the relatively low sample size, our results highlight how the social behavior of dogs toward humans is influenced by the oxytocin and opioid system, but the links between SNP-s and behavioral variables might differ by breeding populations.

ETHICS STATEMENT

Non-invasive studies on dogs are currently allowed to be done without any special permission in Hungary by the University Institutional Animal Care and Use Committee (UIACUC, Eötvös Loránd University, Hungary). The currently operating Hungarian law "*1998. évi XXVIII. Törvény*"—the Animal Protection Act—defines experiments on animals in the 9th point of its 3rd paragraph (3. §/9.). According to the corresponding definition by law, our non-invasive observational study is not considered to be an animal experiment. The owners volunteered to participate and approved of the genetic analyses and behavioral testing of their dogs.

AUTHOR CONTRIBUTIONS

The idea for the paper was conceived by EK and ÁM. The experiments were designed by EK, MB, ZR, and MS. The experiments were performed by EK, MW, MB, DK, and EP. The data were analyzed by EK. The paper was written by EK, MB, and MS.

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SUPPLEMENTARY MATERIAL

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Gaze-Following and Reaction to an Aversive Social Interaction Have Corresponding Associations with Variation in the OXTR Gene in Dogs but Not in Human Infants

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Oláh K, Topál J, Kovács K, Kis A, Koller D, Young Park S and Virányi Z (2017) Gaze-Following and Reaction to an Aversive Social Interaction Have Corresponding Associations with Variation in the OXTR Gene in Dogs but Not in Human Infants. Front. Psychol. 8:2156. doi: 10.3389/fpsyg.2017.02156

It has been suggested that dogs' remarkable capacity to use human communicative signals lies in their comparable social cognitive skills; however, this view has been questioned recently. The present study investigated associations between oxytocin receptor gene (OXTR) polymorphisms and social behavior in human infants and dogs with the aim to unravel potentially differential mechanisms behind their responsiveness to human gaze. Sixteen-month-old human infants ($N = 99$) and adult Border Collie dogs ($N = 71$) participated in two tasks designed to test (1) their use of gaze-direction as a cue to locate a hidden object, and (2) their reactions to an aversive social interaction (using the still face task for children and a threatening approach task for dogs). Moreover, we obtained DNA samples to analyze associations between single nucleotide polymorphisms (SNP) in the OXTR (dogs: −213AG, −94TC, −74CG, rs8679682, children: rs53576, rs1042778, rs2254298) and behavior. We found that OXTR genotype was significantly associated with reactions to an aversive social interaction both in dogs and children, confirming the anxiolytic effect of oxytocin in both species. In dogs, the genotypes linked to less fearful behavior were associated also with a higher willingness to follow gaze whereas in children, OXTR gene polymorphisms did not affect gaze following success. This pattern of gene-behavior associations suggests that for dogs the two situations are more alike (potentially fear-inducing or competitive) than for human children. This raises the possibility that, in contrast to former studies proposing human-like cooperativeness in dogs, dogs may perceive human gaze in an object-choice task in a more antagonistic manner than children.

Keywords: oxytocin, gaze following, social, fear, genotype, dog, human infant

INTRODUCTION

Dogs show various forms of strikingly human-like performance at the behavioral level (for a review see Hare and Tomasello, 2005), and this convergence in behavior is most marked in the social contexts that require dogs to interact with humans (Miklósi and Topál, 2013). More specifically, it has been proposed that dogs possess a sensitivity to human communicative cues that parallels that of human children (for a review see Topál et al., 2014). This similarity is likely to have important functions, as arguably, much of the higher-order cognitive skills of humans rest on the fundamental ability to participate in and make use of communicative interactions in a unique way (e.g., Boyd and Richerson, 1998; Csibra and Gergely, 2011). The special receptivity to communicative signals enables the acquisition of generalizable, culture-specific knowledge, ultimately laying the ground for the accumulation of knowledge over generations. Studies with infants have confirmed that humans already at an early age process information presented in an ostensive context in a specific way: they expect this information to be generalizable and not restricted to the given context (e.g., Topál et al., 2008; Futó et al., 2010). For example, Topál et al. (2008) have shown that children, after repeatedly observing that an object is hidden at one location (A), tend to erroneously search for the hidden object in its initial hiding location even after witnessing that the object has been placed in another location (B). This is the case only if the experimenter has addressed them communicatively before hiding the object. If, however, the original hiding event (A) is not accompanied by ostensive communicative cues, the children commit this search error significantly less often. This, in sum, suggests that they interpret the ostensive (but not the non-ostensive) A trials as a learning situation, and generalize the acquired knowledge to the B trials. Similar results were obtained with dogs using the same paradigm (Topál et al., 2009), suggesting comparable sensitivity to human communication in the two species, at least at the behavioral level.

Such remarkable similarities in performance have initially tempted researchers to assume that they reflect human-like social cognition in dogs (Hare et al., 2002). However, more recent rigorous analyses show that different cognitive mechanisms may be at play. Importantly, Topál et al. (2009) compared the performance of dogs and children in a novel condition where a crucial difference in their behavior emerged: while children continued to search in location A following a communicative demonstration even when there was a new social partner present, the change in social context seemed to provide a clean slate for dogs. Thus, the authors conclude that despite the similarities in superficial behavior, the cognitive processes may be markedly different. While children's behavior can be explained by their bias to interpret information as generalizable across contexts, what dogs may extract from such demonstrations is an instruction to produce a certain action, which retains validity as long as the person giving the instruction is present. Later results have confirmed this interpretation suggesting that dogs tend to pick up information from ostensive communication that is restricted to the "here and now" (Sümegi et al., 2014).

Similarly to the above presented account, it took two decades of research to determine the underlying mechanism of dogs' outstanding success in following human pointing (Lakatos et al., 2009). In contrast to early assumptions that dogs, just as children, interpret pointing as a form of cooperative referential communication that offers them food and information where it can be found (Hare and Tomasello, 2005), recent analyses have showed that dogs take pointing as an imperative that sends them to the highlighted location (Kaminski et al., 2012; Tauzin et al., 2015). Children interpret not only pointing but also directional gaze cues as communicative signals that are supposed to provide them with generalizable knowledge (Senju et al., 2008). Dogs have also been believed to use human gaze similarly to pointing, often ignoring findings that even after a communicative gaze cue, dogs choose one of two food locations randomly (Kaminski et al., 2012). Differences in the two species' reactions to directional cues have been further highlighted by recent studies showing that while children ignore gaze cues in a non-ostensive context (Senju and Csibra, 2008), dogs actually avoid food locations indicated with non-communicative gaze by both a conspecific and a human (Bálint et al., 2015; Duranton et al., 2017). Based on these findings, it has been hypothesized that dogs see an object choice task with non-communicative gaze as food competition and they tend to behave in a way that can help to avoid a potential conflict over food (Duranton et al., 2017). Interestingly, it appears that oxytocin can mitigate this effect: Oliva et al. (2015) found that after intranasal oxytocin administration dogs were more likely to choose the one of two food containers that had been indicated with a human gaze cue.

While oxytocin seems to facilitate social approach and social cognition in general both in dogs and humans (Bartz et al., 2011; for reviews see Kis et al., 2017), one of the best described mechanisms behind these facilitation effects is related to the attenuation of fear responses and anxiety. Most likely contributing to such effects, oxytocin has been shown to inhibit the responsiveness of the hypothalamo-pituitary-adrenal axis (Neumann, 2002) as well as to attenuate the activity of the amygdala in response to both threatening (Huber et al., 2005) and positive stimuli (Domes et al., 2007). Not only oxytocin production but also oxytocin receptor binding is a key component of the oxytocinergic system. In line with this, increasing evidence suggests that genetic polymorphisms of the oxytocin receptor gene (OXTR) also play a role in modulating various behaviors in social interactions, ranging from fearful behaviors through emotion processing to prosociality (see below). A number of studies have looked at associations between human social behavior and different single nucleotide polymorphisms (SNP) in the OXTR, and a few SNPs have emerged as having a prominent role in shaping socio-cognitive skills and social behavior. The OXTR rs53576 polymorphism (in intron 3) is probably the most intensively investigated SNP (for a meta-analysis, see Li et al., 2015), and it has been associated with—among others—stress reactivity (Rodrigues et al., 2009), need for social support (Kim et al., 2010) and emotion processing (Tost et al., 2010). The rs2254298 polymorphisms (in intron 3) in the OXTR have been linked to attachment anxiety (Chen and Johnson, 2012) and depression (Thompson et al., 2011) in

certain populations. A third SNP, rs1042778 (in exon 4 3′ UTR) has also been linked to the regulation of social interactions, in particular by modulating prosocial behavior (Israel et al., 2009). The OXTR SNPs that may account for the variability in the social behavior of dogs are less well known. A few studies have used genetic sequencing to identify loci where significant variations are exhibited between individuals. Kis et al. (2014) have found some SNPs (rs8679682, −212AG, 19131AG) that are associated with proximity seeking and friendliness in dogs. Such variations have been shown to be associated with behavioral differences between dogs and wolves as well, although these loci were not related to within-species behavioral variation (Oliva et al., 2016).

The positive effect of oxytocin on following gaze cues to locate hidden objects may be exerted through at least two mechanisms: either through the reduction of fear (Kirsch et al., 2005; Ring et al., 2006) or through the enhancement of trust (Kirsch, 2015, although see Nave et al., 2015 for the controversy regarding the role of oxytocin in human trust). That is, oxytocin may help to highlight the cooperative aspect of gazing (that is, its perception as an offer of food and information) or it may facilitate approach by reducing fear *despite* the fact that the context remains perceived as competitive. In the present study, following up on recent results described earlier, we set out to test the hypothesis that dogs perceive non-communicative gaze in an object choice task differently to children's interpretation of communicative gaze. More specifically, we aimed to investigate whether OXTR polymorphisms are associated with following human gaze cues in both children and dogs, and if yes whether these associations co-vary with associations the OXTR polymorphisms have with reactions of both species to negative social stimuli. First of all, we hypothesized that the oxytocin system would be related to the modulation of reactions shown in an aversive social context in both species. To test this hypothesis, we used well-established paradigms in both species that have already been shown to evoke distress in participants by violating the expectations of regular adult-infant or human-dog interactions (still face and threatening approach paradigms, respectively). Our second hypothesis was that in dogs, oxytocin would also be related to following of non-ostensive human gaze through the same anxiolytic effect. That is, we predicted that the same OXTR genotypes will be associated with a less fearful reaction to social threat and with higher readiness to follow someone's gaze when searching for food. In contrast, in children, as they do not interpret gaze cues as a threat or competition, we predicted that following gaze will not be associated with OXTR polymorphisms or, if yes, different genotypes will be associated with gaze following and with reactions to a negative social situation. In order to test these hypotheses, we observed (1) the behavior of both infants and dogs in a social context in which their human partner showed negative social behavior unexpectedly, (2) the reaction of infants to communicative gaze, and (3) the reaction of dogs to non-communicative gaze. In addition, buccal samples were obtained from both children and dogs, in order to analyze the associations between behavior and their OXTR polymorphisms located in the intronic as well as the UTR regions of the gene.

METHOD

Participants

Human Participants

Ninety nine toddlers of 15–16 months participated in the study (mean age: 15.73 months; SD: 0.26 months; range: 15.13–16.2 months). Children were selected from a database of families that had previously indicated interest in participating in research studies and were contacted again for this particular study. An additional 19 children were tested, but excluded from the sample due to fussiness (2), missing or insufficient DNA sample (14) or camera failure (3). 48 out of the 99 toddlers successfully completed both tasks; 28 children met the predetermined criteria only for *the gaze following task* and 16 only for the *still face task* (for more details see Procedure). In total, 76 child participants (36 boys/40 girls) were included in the *gaze following* task and 64 (32 boys/32 girls) in the *still face* task (**Table 1**). Experiments with children were conducted at the Institute of Psychology, Hungarian Academy of Sciences, Budapest.

Dog Subjects

Seventy one privately owned adult (older than 10 months) Border Collies (mean age: 4.27 years, SD: 2.88 years, 38 females) were recruited and tested at the Clever Dog Lab, Vienna, Austria. Out of the 71 dogs tested, 22 were castrated or spayed (12 females). An additional 5 dogs were tested, but excluded from analyses due to missing or insufficient DNA sample.

Children and dogs that could not be tested with one of the experimental tasks but provided valid data for the other were only excluded from analyses of the specific task in which they failed to participate. Similarly, if DNA could not be sequenced at a given SNP but there was valid data on the other SNPs, the participant was only excluded from the corresponding analyses (**Table 1** shows the number of dogs and children that were included in each analyses out of the 71 subjects and 99 participants, respectively).

Ethics Statement

The study with child participants was approved by the United Ethical Review Committee for Research in Psychology (Ref No. XIV-I-001/531-4-2012). For dog participants, ethical approval

TABLE 1 | Number of dogs (males/females) and children (boys/girls) included in the different analyses.

CHILDREN			
	Candidate SNP		
	rs1042778	**rs2254298**	**rs53576**
Gaze following task	76 (36/40)	76 (36/40)	76 (36/40)
Still face task	64 (32/32)	64 (32/32)	64 (32/32)

DOGS				
	rs8679682	**−213AG**	**−94CT**	**−74GC**
Gaze following task	56 (27/29)	51(24/27)	56 (27/29)	48 (24/24)
Threatening approach	56 (30/26)	50 (26/24)	56 (30/26)	48 (27/21)

was obtained in accordance with GPS (Good Practice Statement) guidelines and national legislation by the Ethical Committee for the use of animals in experiments at the University of Veterinary Medicine Vienna (Ref No. 04/12/97/2012). Participants' owners (dogs) or caregivers (children) signed informed consent prior to participation.

Procedure

Both children and dogs took part in two tests. *Task 1* was construed to test their sensitivity to a human gaze cue. *Task 2* was construed to assess their reaction to an aversive social interaction with a human experimenter. Testing was conducted by two female experimenters for children and three female experimenters for dogs. In order to standardize their behavior, all experimenters received a detailed experimental protocol and watched the other experimenter(s) conducting the tests. The next sections describe the species-specific testing situations separately.

Task 1: Following a human gaze cue

children

Familiarization trials Prior to the experiment, children engaged in playful activities together with their mothers and the experimenter in order to familiarize them with the environment (10 min).

Test trials Children were seated on their caregivers' lap on a 50 cm high chair. Parents were instructed to hold their children on their laps or were allowed to let children stand on the ground while the parent was holding them at a fixed position. The experimenter kneeled on the floor about 2 meters away from the child and the parent, facing them. She presented two identical opaque boxes to the participant, placing them in front of her 60 cm apart from each other. Once the child's attention was engaged, she opened the two boxes (starting always with the one on her left), revealing that one of the boxes contained a small toy. To make sure children realized the toy in the box, the experimenter lifted the boxes, moved closer to the participant and showed them the content of the boxes close up. During this procedure, she communicated with the child in a natural manner, which included calling the child's name, using attractive facial expressions and engaging in eye-contact repeatedly. After that, she placed the boxes back at their original locations and closed the lids, starting with the one on the left. Then, she switched the location of the boxes three times in view of the child, but with a relatively fast motion in order to confuse children about the location of the baited box. This way, the baited box ended up on the opposite side of the experimenter. The experimenter then looked up at the child in order to initiate eye-contact with them. Once the child was engaged in eye-contact, the experimenter called their name and turned her head toward the baited box and kept looking at it. After 5 s had elapsed, she turned her gaze back toward the child, smiling. At this point, parents (as a priori instructed) let go of their children, and participants were allowed to approach the boxes and look for the toy. If children touched one of the boxes or clearly pointed at one, the test was terminated and the experimenter helped open the box, revealing its content to the child. If children did not make a choice in the first 60 s they were coded as passive and were excluded from analyses.

Dogs

Familiarization trials This phase was included to familiarize the dogs with two small containers (10 cm diameter, 15 cm height). Before the start of the trial, the experimenter placed the two containers on the floor randomly, but about 1.5 m apart from each other, baiting only one of them with food (a small piece of cheese or sausage). The owner then let the dog free to enter the experimental room to search the two containers and eat the food, and waited with the experimenter outside of the room with the door open. If the dog did not start searching within 30 s after being released, the owner entered the room and encouraged the dog to search. A trial ended once the dog ate the food. In total, there were 4 familiarization trials.

Test trial Before the test trial began, the experimenter placed the two containers, in the same way as in the familiarization trials, and a chair at an equal distance of about 2 m from the two containers. The experimenter kneeled between the two containers and waited, keeping her hands behind her back and looking straight ahead. The test began as the owner and the dog entered the room. The owner sat on the chair, keeping the dog on a short leash so that the dog could not approach the containers closer than 1 m. Once the owner sat down on the chair, the experimenter waited to make eye contact with the dog. If the experimenter was not able to do so within 10 s, she tried to get the attention of the dog by calling its name, but minimized other communication. As soon as eye contact was established, the experimenter kept looking into the dogs' eyes with a blank facial expression while staying still and silent. Once the dog broke the eye contact, the experimenter called the dog's name and made another brief eye contact and, with a clear head movement, turned her head to look down at the baited container. After 5 s had elapsed, the owner released the dog to choose a container, while the experimenter was still looking at the baited container. The trial ended when the dog touched one of the containers with its mouth.

Task 2: Reaction to an Aversive Social Interaction

The second task was designed to describe how the participants reacted in a socially aversive situation. As our goal was not to directly compare the behavior of children and dogs but to compare the behavioral associations of the OXTR SNPs across tasks and within species, we chose slightly different paradigms that detect individual variation both in children and dogs (*still face* task, Tronick et al., 1978 and *threatening approach* task, Vas et al., 2005, respectively). Both tasks have been described to evoke distress and frustration in participants through the violation of expectations of regular adult-infant or human-dog interactions. In the *still face* task, this is achieved by the withdrawal of the experimenter's communication and her lack of reactivity. In the *threatening approach* task, the prolongation of her approach and her intense looking evoke this mismatch. Importantly, children and dogs have also been described to show a similar range of reactions to these situations: some try to repair this mismatch

by attempting to engage the partner in friendly interactions, some attempt to leave the unpleasant social situation, whereas others exhibit signs of distress or frustration as a response to the violation of the expected social behavior (Tronick et al., 1978; Vas et al., 2005).

Children: Still face

Children participated in the *still face* task (c.f. Tronick et al., 1978) to test their reactions to the withdrawal of positive social stimulation from the experimenter. The task consisted of two 1-min-long phases. The caregiver was instructed to take a seat on one side of a 1.5 m long blanket and hold their child on their lap. The experimenter sat down at the other end of the blanket, facing the child. In the first phase of the task, the experimenter engaged the child in a session of peek-a-boo game, where she alternated between initiating eye-contact with the child (smiling) and hiding her face behind a veil or her hands. After 1 min had elapsed, a second experimenter signaled the start of the second phase. To ostensibly separate the two phases, upon hearing the signal from the second experimenter, the first experimenter turned her head away from the child and when she looked back, she began the still face phase, during which she was silently looking at the child but did not initiate any further contact and did not respond to the child's attempt to communicate. After 1 min had passed, the test phase ended and the experimenter resolved the possible negative feelings caused by the still face episode by starting the peek-a-boo game again.

Dogs: threatening approach

The test procedure was similar to the procedure described in the experiment of Vas et al. (2005) in which the dog's response to the unexpected threatening behavior of the experimenter was recorded. The dog was on a leash fixed on a wall in the room, while the owner was standing about 30 cm behind the dog. The experimenter, who had previously interacted with the dog and its owner in a friendly manner, entered the room from the side door and stood about 5 m away from the dog. Once the dog looked at her, the experimenter started to approach the dog slowly (one step in every 4 s) with her upper body slightly bent and looking steadily into the eyes of the dog without any verbal communication.

The behavior of the experimenter was determined and standardized across subjects according to the following rules: (1) If the dog kept looking at the experimenter without any other reaction, then she continued to approach the dog until she reached it. (2) If the dog broke the eye contact with her (moving away and/or turning head away), the experimenter stopped and waited motionless for about 4 s and then tried to attract the dogs attention by making some noise (a slight cough or scratching the ground with the foot). If the dog continued to avert his gaze, the experimenter attempted to call the dog's attention two more times (with 2 s in between attempts). Whenever the dog looked at her again, she continued the approach. If, however, the dog did not look at her after the third attempt, the test was terminated. (3) If the dog showed active avoidance, that is, moved behind the owner, the test was immediately terminated. (4) If the dog showed signs of aggression, e.g., barked repeatedly or growled continuously (longer than 4 s) and/or tried to attack the experimenter, the test was terminated. If the subject did not show any form of fear or aggression even when the experimenter reached the dog, she touched the dog's head and gently petted it.

Buccal Sample Collection and SNP Genotyping

Buccal cell samples were collected from each participating dog and child by swabbing the upper gum area with 4 cotton swabs. The cotton swabs were then sealed in a tube and preserved in the freezer until genotyping (Bence et al., 2017). DNA purification was initiated by incubating the buccal samples at 56°C overnight in 0.2 mg/ml Proteinase K cell lysis buffer. It was followed by protein denaturation using saturated NaCl solution. Finally, DNA was precipitated using isopropanol and ethanol by standard procedures and DNA pellet was resuspended in 100 μl 0.5 × TE (1× TE: 10 mM Tris pH = 8, 1 mM EDTA) buffer.

For both species, we genotyped polymorphisms that had been linked to social behavior in former studies. For infants, these were the SNPs rs1042778; rs2254298 and rs53576 (based on Israel et al., 2009; Rodrigues et al., 2009; Chen and Johnson, 2012; for instance). For dogs SNPs −213AG; −74CG; −94TC; and rs8679682 were genotyped (Bence et al., 2017). Note that these SNPs, although all in the OXTR gene, are neither structurally nor functionally equal between dogs and humans.

Dogs

Typical DNA concentration of the dogs' genomic DNA samples isolated from buccal swabs was around 20 ng/μl. The Qiagen Hot-StarTaq polymerase kit was used for PCR amplification. The reaction mixture contained 1 μM of each primer, approximately 5 ng of DNA template, 200 μM dNTP, 0.025 U HotStarTaq DNA polymerase, 1 × buffer, and 1 × Q-solution supplied together with the enzyme. The PCR cycle consisted of an initial denaturation at 95°C for 15 min, 40 cycles of 1-min denaturation at 95°C, 1-min annealing at various temperatures, a 1-min extension at 72°C, and a 10-min final extension at 72°C. The PCR reaction was performed in a total volume of 10 μl. −213AG and the −74CG polymorphisms were genotyped by PCR-RFLP method. PCR amplification was performed as described above using 5′-CCA TTG GAA TCC GCC CCC T-3′ forward and 5′-CAC CAC CAG GTC GGC TAT G-3′ reverse primers. Annealing temperature was 56°C. PCR products were incubated for 3 h at 37°C in a restriction enzyme mixture containing 0.5 U/μl Hpy99I restriction enzyme (NEB) for −213 SNP and 0.5 U/μl BsiEI restriction enzyme (NEB) for −74CG SNP, 1xBSA and 1x NEB4 buffer. Total reaction volume was 16 ml −94TC SNP was genotyped by allelespecific amplification (ASA) using the primers described above. Allele specific primers were 5′-CCG ATC TGC TGG TCC CGG-3′ and 5′-CCG ATC TGC TGG TCC CGA-3′ and the annealing temperature was 60°C. rs8679682 SNP was genotyped by real-time PCR using sequence specific TaqMan probes with minor groove binding (MGB) quencher. Primers were designed by Primer Express 3.0 (forward primer: 59-CTC CTT TAT TTTGGG ATC TTG TGA A-39, reverse primer: 59-CCT GCT CCTTAT TCT GAG CTT AGA A-39, probe specific

for T allele: 59-FAM-AGT GGT AAG TAT AGG ATT G-MGB-39, probe specific for A allel: 59-VIC-AGT GGT AAG TAA AGG ATMGB-39.

The PCR products were analyzed by conventional submarine agarose gel electrophoresis (Biocenter, Szeged, Hungary), using 2.5% agarose gel and visualized by ethidium bromide staining. We investigated frequencies and Hardy–Weinberg Equilibrium analyses of the genotypes. Allele frequencies (**Table 2**) did not deviate significantly from the Hardy-Weinberg equilibrium ($p > 0.05$; Chi-square tests). We also tested whether there were any differences in allele frequencies across dogs tested by Experimenter 1, 2 and 3, and found no significant effects ($p > 0.05$; Chi-square tests; see **Table 3**).

Children

Six PCR amplification was performed as described above using 5′- ACT GGG GCA ACC AAA CAT CT-3′ forward and 5′- ACT CTT CAT GGC CCA GAG TG-3′ reverse (rs53576), 5′- GCT CCA GCC AGA GGA G-3′ forward and 5′-AGT GGG TTC AGG GTG GTA-3′ reverse (rs1042778), 5′- CTG TCT TTG CAC CTT TGC TA-3′ forward and 5′- ATG AAA GCA GAG GTT GTG TG-3′ reverse (rs2254298) primers. Annealing temperatures were 56°C (rs53576 and rs2254298) and 60°C (rs1042778). OXTR rs53576 and rs2254298 SNPs were genotyped by PCR-RFLP method. PCR products were incubated for 3 h at 37°C in a restriction enzyme mixture containing 0.5 U/μl AvaII restriction enzyme (NEB) for rs53576 SNP and 0.5 U/μl DdeI restriction enzyme (NEB) for rs2254298 SNP, 1x BSA and 1x NEB4 buffer. rs1042778 SNP was genotyped by allele specific amplification (ASA) using 5′- AGC CAC CCC AAG GAG T-3′ forward and 5′- AGC CAC CCC AAG GAG G-3′ allele specific primers. The PCR products were analyzed by conventional submarine agarose gel electrophoresis (Biocenter, Szeged, Hungary), using 2.5% agarose gel and visualized by ethidium bromide staining. We investigated frequencies and Hardy–Weinberg Equilibrium analyses of the genotypes. Allele frequencies (**Table 3**) did not deviate significantly from the Hardy-Weinberg equilibrium ($p > 0.05$; Chi-square tests). We also tested whether there were any differences in allele frequencies between children tested by Experimenter 1 and Experimenter 2 and found no significant effects ($p > 0.05$; Chi-square tests; see **Table 4**).

Data Analyses

Behavioral tests were coded offline from the recordings for pre-defined variables. For the *gaze following* task, we coded whether the participants chose the container that had been indicated by the gaze direction of the experimenter. Participants that did not choose a container in the first 90 s were excluded from this part of the analyses. Both for dogs and children, we coded a correct choice if they chose the indicated container.

For the *reaction to an aversive social interaction* task, slightly different measures were used for infants and dogs due to the differences in the procedures. For children, we coded looking times, with a special interest in how much time they spent looking at the experimenter and their caregiver during the still-face period (coding categories: looking at experimenter, looking at caregiver, looking elsewhere). We also coded signs of distress (crying, negative vocalization or negative facial expressions) in the still-face phase. All of the variables were expressed in percentage of time as there could have been slight variations in the total duration times across participants. Infants who left their caregivers' laps during the still face period and spent more than

TABLE 3 | Allele frequencies for all children and the number of children by task and experimenter.

Genotype	rs1042778			rs2254298			rs53576		
	TT	TG	GG	GG	AG	AA	GG	GA	AA
Frequency	0.151	0.353	0.496	0.777	0.222	0	0.374	0.444	0.182
GAZE FOLLOWING									
E1	8	20	25	42	11	0	23	22	8
E2	5	6	12	19	4	0	8	9	6
Σ	13	26	37	61	15	0	31	31	14
STILL FACE									
E1	7	16	21	36	8	0	19	19	6
E2	2	6	12	15	5	0	8	8	4
Σ	9	22	33	51	13	0	27	27	10

TABLE 2 | Allele frequencies for all dogs as well as the number of dogs by task and experimenter.

Genotype	TT	CT	CC	GG	AG	AA	CC	CT	TT	GG	CG	CC
Frequency	0.257	0.60	0.143	0.657	0.20	0.143	0.114	0.571	0.314	0.528	0.257	0.1
GAZE FOLLOWING												
E1	9	13	3	15	6	2	3	12	10	13	5	3
E2	1	11	4	12	3	0	2	7	7	9	3	2
E3	6	8	1	10	2	1	1	11	3	9	3	1
Σ	16	32	8	37	11	3	6	30	20	31	11	6
THREATENING APPROACH												
E1	10	12	3	15	5	2	2	13	10	13	5	3
E2	1	12	4	13	3	0	3	8	6	8	5	2
E3	5	8	1	9	2	1	1	10	3	8	3	1
Σ	16	32	8	37	10	3	6	31	19	29	13	6

TABLE 4 | Summary of the results.

		Human			Dog			
		rs.576	**rs.778**	**rs.298**	**rs.682**	**−213AG**	**−94TC**	**−74GC**
Gaze following	Main effects	–	–	–	–	**G**	**G**	**G**
	Interactions	–	–	–	–	–	–	–
Still face/Threatening approach look at Caregiver/owner	Main effects	–	–	–	–	–	**G;E**	–
	Interactions	–	–	–	–	–	**S×E; S×G; E×G;**	–
Still face/Threatening approach look at Experimenter/Stranger	Main effects	**E**	–	–	–	–		–
	Interactions	–	–	–	**S×E;**	–	S×E	–
Threatening approach first reaction	Main effects	*N/A*	*N/A*	*N/A*	**A;S**	**S;G**	–	**G**
	Interactions	*N/A*	*N/A*	*N/A*	–	–	–	–
Still face signs of distress	Main effects	**G;A**	**A; S**	**A**	*N/A*	*N/A*	*N/A*	*N/A*
	Interactions	**E×G**	**S×E**	–	*N/A*	*N/A*	*N/A*	*N/A*

G, Genotype; A, Age; E, Experimenter; S, Sex Significant effects are bold, while marginally significant effects are indicated by normal font types. N/A, Not applicable.

30% of the time outside of the testing context (that is, were not sitting on the caregiver's lap and were not within a 1 m radius of the experimenter) were excluded from this part of the analyses ($N = 21$). Participants who left the caregiver's lap during the warm-up phase were excluded from all analyses ($N = 14$).

For dogs, we also coded looking times during the *threatening approach* task (looking at the experimenter, the owner or elsewhere). Further on, we coded the dogs' first reaction to the threatening approach of the stranger with the following options: (1), friendly reaction to experimenter (tail wagging while moving toward the experimenter); (2), unfriendly reaction to the experimenter (looking at or approaching experimenter without wagging). Dogs that exhibited extreme stress were excluded from analyses ($N = 14$).

Statistical analyses were performed using SPSS 20.0. Based on the type of the dependent variable (behavioral measures), the associations between genotype and behavior were analyzed using either General Linear Models (Univariate ANCOVA for durations); Binary Logistic Regression (for choice of container and first reaction in the threatening approach task for dogs). We used separate models for each SNPs, and in the ANCOVAs we included age as a covariant, sex (male vs. female), experimenter (2 for children and 3 for dogs) and their two-way interactions both with each other and genotype (3 levels in all cases) in all models. For the regression analyses, we applied a backward elimination method of non-significant effects.

Finally, we also tested whether performance on one test was associated with performance on the other. Thus, we used independent samples *T*-tests to compare behaviors in the *reaction to an aversive social interaction* task between participants that chose correctly vs. incorrectly in the *gaze following* task.

RESULTS

Gaze Following

Children

All 76 children made a choice in this task. Out of the 76 children, 30 chose the baited container (thus, used the gaze direction of the experimenter as a cue to find the hidden object). This does not differ significantly from choosing randomly (though shows a marginal below chance effect) (binomial: $p = 0.085$).

The SNP rs1042778 did not have a significant effect on children's choices and none of the control variables (age, sex and experimenter) did so either (all $p > 0.283$ at removal) (**Figure 1**). Similarly, we did not find any significant main or interaction effects in the analyses on SNP rs2254298 (all $p > 0.283$ at removal) and SNP rs53576 (all $p > 0.283$ at removal).

Dogs

Altogether 57 dogs were included in the sample that both made a choice and had at least one identifiable SNP. Out of the 57 dogs, 38 chose the baited container (thus were successful in using gaze direction as a cue), which does not significantly differ from chance (binomial: $p = 0.111$)

The rs8679682 polymorphism did not have a significant main effect on dogs' choices of container [$\chi^2_{(2)} = 0.754$, $p = 0.449$], and the analyses did not yield any significant effects of the control variables or interaction effects either (all $p > 0.195$ at removal) (**Figure 2**).

The −94TC polymorphism, however, had a significant effect on dogs' choices of container [$\chi^2_{(2)} = 8.267$; $p = 0.016$], showing that while dogs with the homozygous C and the heterozygous genotypes made their choices at random, dogs with the homozygous T genotype chose the baited container more often. All other effects were not significant (all $p > 0.222$ at removal).

We also found a marginally significant effect on dogs' choices by the −213AG polymorphism [$\chi^2_{(2)} = 5.948$; $p = 0.051$]. Dogs with the homozygous G genotype were more likely to follow the correct, baited container; however, this was not true either of the homozygous A or the heterozygous genotypes. All other effects were not significant (all $p > 0.066$ at removal).

SNP −74GC also had a significant effect on dogs' behavior in the task [$\chi^2_{(2)} = 13.21$; $p = 0.001$], showing that dogs with the homozygous G genotype were most likely to choose the baited

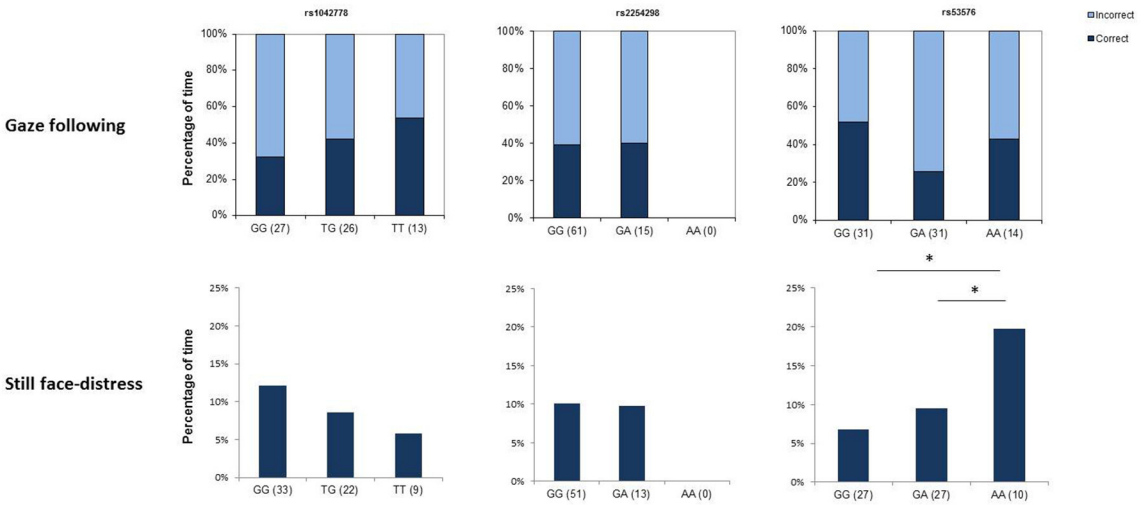

FIGURE 1 | Genotype × behavior associations in children. The upper row shows associations between genotype at the three selected SNPs and success in using communicative gaze as a cue to locate a hidden object, while the lower row depicts associations with the amount of time spent with showing distress signals in an unpleasant social situation (*still face* task). Asterisks mark significant gene × behavior correspondences.

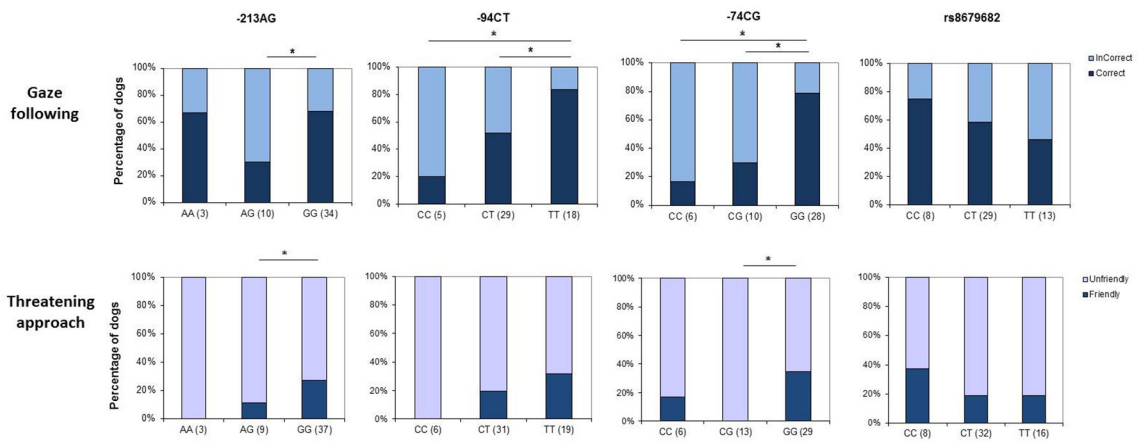

FIGURE 2 | Genotype × behavior associations in dogs. The upper row shows associations between genotype at the four selected SNPs and success in using non-communicative gaze as a cue to locate a hidden object, while the lower row depicts associations with reactions to social threat (*threatening approach* task). Asterisks mark significant gene × behavior correspondences.

container compared to the homozygous A or the heterozygous genotypes. All other effects were not significant (all $p > 0.109$ at removal).

Reaction to an Aversive Social Interaction

Children—looking at the Experimenter

Allele variations at rs1042778 had no significant effect on the amount of time children spent looking at the experimenter during the still face phase [$F_{(2)} = 0.39$; $p = 0.679$], nor did the analyses yield any significant interaction effects (all $p > 0.319$). Similarly, no main [$F_{(2)} = 0.065$; $p = 0.8$] or interaction effects were found involving SNP rs2254298 (all $p > 0.15$). The model including the SNP rs53576 yielded a significant effect of experimenter [$F_{(1)} = 4.057$; $p = 0.049$], but no main effect of allele variations [$F_{(2)} = 1.478$; $p = 0.238$] and no interaction effects (all $p > 0.231$). Children spent more time looking at Experimenter 2 than at Experimenter 1 [M(E1) = 25.4; M(E2) = 34.24].

Dogs—looking at the experimenter

SNP rs8679682 had no significant main effect on the amount of time dogs spent looking at the threateningly approaching experimenter [$F_{(2)} = 0.607$; $p = 0.55$]. However, there was a significant two-way interaction between sex and experimenter [$F_{(1, 54)} = 4.578$; $p = 0.017$], showing that whereas females reacted differently to the two experimenters, males did not. Allele variations at SNP−213AG had no main effect on the time dogs spent looking at the experimenter [$F_{(2)} = 1.048$; $p = 0.362$], and

we did not find any significant main effect of the control variables, nor any interaction effects (all $p > 0.301$).

Analyzing the effects of variations at SNP−94TC, we found a marginal effect of allele variation[$F_{(2)} = 2.647$; $p = 0.084$] and a marginal interaction between sex and experimenter [$F_{(1,54)} = 2.599$; $p = 0.087$]. Results indicate that males differentiated more between experimenters than females, and dogs with the homozygous T genotype spent less time looking at the experimenter than the other two genotypes [M(TT) = 81.06%; M(CT) = 93.11%; M(CC) = 91.39%].

Analyses of the SNP −74GC yielded no main effect of allele variation [$F_{(2)} = 0.783$; $p = 0.466$] and no other effects (all $p > 0.364$).

Children—looking at the caregiver

The SNP rs1042778 did not have a significant effect on the amount of time children spent looking at their caregivers [$F_{(2)} = 0.2$; $p = 0.146$]. The interactions involving rs1042778 were not significant either (all $p > 0.151$). Similarly, variations at SNP rs2254298 did not significantly modulate gazing at the caregiver [$F_{(2)} = 0.002$; $p = 0.965$] and the interaction effects were not significant either (all $p > 0.139$). The same was true for SNP rs53576 [main effect: $F_{(2)} = 0.165$; $p = 0.849$; interaction effects: all $p > 0.296$].

Dogs—looking at the owner

Rs8679682 had no main effect on the amount of time dogs spent looking back at their owners [$F_{(2)} = 0.11$; $p = 0.896$] and there were no significant main effects of the control variables and no significant interactions either (all $p > 0.154$). Similarly, no effects were found analyzing either SNP −213AG [main effect: $F_{(2)} = 0.034$; $p = 0.967$; other effects: all $p > 0.377$] and SNP −74GC [main effect: $F_{(2)} = 1.396$; $p = 0.263$; other effects: all $p > 0.523$].

However, allele variations at SNP −94TC had a significant effect on dogs' looking times at their owners [$F_{(2)} = 3.446$; $p = 0.042$] and the analyses also yielded a main effect of experimenter [$F_{(2)} = 6.014$; $p = 0.005$], showing that the amount of time spent looking at the owner differed as a function of who was administering the test. These effects were qualified by significant two-way interactions between sex and experiment [$F_{(2,54)} = 4.675$; $p = 0.015$]; sex and allele variations [$F_{(2,54)} = 3.673$; $p = 0.035$]; experimenter and allele variations [$F_{(4,54)} = 4.913$; $p = 0.003$] and a three-way interaction between sex, experimenter and allele variations [$F_{(2,54)} = 6.355$; $p = 0.004$]. Results show greater variability in the case of males than females. Specifically, looking times increased when Experimenter 2 was administering the test for male dogs with the homozygous C genotype compared to all other cases ($M = 19.55\%$, all other Ms<7%.)

Children—signs of distress

The analyses on the effects of SNP rs1042778 yielded no main effect of genotype [$F_{(2)} = 1.579$; $p = 0.216$] and no interaction effects involving rs1042778 (all $p > 0.102$) (**Figure 1**). However, age and sex had marginal effects on the amount of time children exhibited signs of distress [sex: $F_{(1)} = 3.781$; $p = 0.057$; age: $F_{(1)} = 3.722$; $p = 0.059$] and the interaction between sex and experimenter was significant [$F_{(1,63)} = 4.555$; $p = 0.038$]. The results indicate that younger children exhibited more signs of distress than did older children and boys exhibited more distress than girls [M(girls) = 8.48% of the total duration of the phase; M(boys) = 11.47%]. The interaction shows that there was not a considerable difference in the amount of distress signals in the case of girls [M(E1) = 8.069%; M(E2) = 7.934%]; however boys showed more signs of distress when the test was administered by Experimenter 2 [M(E1) = 8.147%; M(E2) = 21.51%].

In the analyses involving SNP rs2254298, we replicated the connection between age and distress signals [$F_{(1)} = 5.208$; $p = 0.026$], but we found no main effect of genotype [$F_{(2)} = 0.477$; $p = 0.493$] and no interaction effects involving rs2254298 (all $p > 0.352$).

The rs53576 polymorphism had a significant effect on the amount of distress signals children produced in the still phase period [$F_{(2)} = 5.796$; $p = 0.005$], showing that children with the homozygous AA genotype exhibited more distress [M(AA) = 21.52%] than children with the other two genotypes [M(GG) = 7.69%; M(GA) = 7.56%]. There was also a significant interaction effect between experimenter and genotype [$F_{(1,63)} = 5.601$; $p = 0.006$] showing that this difference was mainly attributable to tests administered by Experimenter 2. When Experimenter 1 administered the test, the amount of distress signals produced showed less variation across genotypes and in general, distress signals were scarcer [M(GG) = 6.88%; M(GA) = 10.59%; M(AA) = 9.55%]. The analyses also replicated the effect of age [$F_{(1)} = 6.067$; $p = 0.017$].

Dogs—first reactions to the threatening experimenter

Allele variations at rs8679682 did not have a significant effect on dogs' first reactions to the experimenter [$\chi^2_{(2)} = 1.144$; $p = 0.564$] (**Figure 2**). However, sex [$\chi^2_{(2)} = 4.511$; $p = 0.034$] had a significant modulatory effect, showing that while dogs were more likely to react with looking at or approaching the experimenter without tail wagging than to produce a friendly reaction, this was stronger in the case of males. All other effects were non-significant ($p > 0.236$ at removal)

The analyses on the effects of SNP −213AG yielded a significant main effect of allele variations [$\chi^2_{(2)} = 8.383$; $p = 0.015$], showing that while dogs with the homozygous A ($N = 3$) or the heterozygous genotype ($N = 10$) all reacted with looking at the experimenter without tail wagging, the behavior of the homozygous GG genotype was more diverse with 11 out of 37 dogs reacting in a friendly way. All other effects were not significant ($p > 0.086$ at removal).

Similarly, SNP−74GC significantly modulated dogs' behavior [$\chi^2_{(2)} = 10.861$; $p = 0.004$]. While dogs with the heterozygous genotype all ($N = 13$) reacted with looking at the experimenter without tail wagging, participants with the homozygous G genotype also produced friendly reactions (10 out of 29). −94TC polymorphism did not have a significant effect on dogs' first reactions [$\chi^2_{(2)} = 3.356$; $p = 0.187$].

Correspondence between Tasks

Children that chose correctly in the first task spent less time (mean: 24.92 s) looking at the experimenter in the *still face*

situation than those that could not find the reward [mean: 34.71 s; $t_{(46)} = 2.37$; $p = 0.022$]. The same was true for dogs: those that chose the baited container spent significantly less time looking at the experimenter in the *threatening approach task* [mean: 93.29 vs. 85.39 s; $d_{(49)} = 2.482$; $p = 0.017$]. We found no other associations between performance in the *gaze following task* and the variables coded for the *reaction to an aversive social interaction task*.

DISCUSSION

The present study explored associations between variation in the OXTR and reaction to an aversive social interaction as well as use of a gaze cue to locate hidden food in dogs and humans. Results seem to support our hypotheses that the oxytocinergic system may play a similar role in shaping dogs' and human infants' reactions to their partner's unexpected negative (distressing) behaviors but not to her gaze cue in a search task. We suggest that this is because the latter is potentially a competitive (and thus distressing) context for dogs, while it would be a cooperative context for human infants.

Our results show that SNP in the gene coding for oxytocin receptor binding are indeed associated with both dogs' and children's reactions to a violation of normal social interactions. We found that dogs' first reactions (either friendly or neutral/fearful) were significantly modulated by two of the four polymorphisms analyzed (−213AG, −74GC). One of these polymorphisms (−213AG) had already been shown to be associated with proximity seeking, a composite measure that included latency to approach the experimenter after the threatening approach task (Kis et al., 2014). Also, it has been shown that intranasal administration of oxytocin influences dogs' reaction in the threatening approach task (Hernádi et al., 2015). In the corresponding analyses with children, we found that the amount of distress signals produced after the withdrawal of positive social stimulation was significantly modulated by one of the three polymorphisms analyzed (rs53576). These results confirm that variation in the oxytocinergic system influences how dogs as well as humans respond to social threat or a socially ambiguous situation (Huber et al., 2005; Hernádi et al., 2015; Kovács et al., 2016)

Analyzing participants' behavior in the *gaze following* task, we found that three out of the four identified polymorphisms (−213AG, −95TC, and −74GC) were connected to whether dogs approached a food location the human experimenter had looked at beforehand. Importantly, two of these three polymorphisms (−213AG, −74GC) were linked to the dogs' friendliness in the *threatening approach* task as well. For example, dogs with the homozygous G genotype at SNP −74GC were not only more likely to search for food using the gaze direction of a human, but were also less threatened by the experimenter in the subsequent task. The same was true for dogs with the homozygous G genotype at −213AG. Although the present study does not allow us to assign specific functions to specific polymorphisms, these consistencies suggest that similar mechanisms regulate dogs' reaction to a clear social threat and to non-ostensive gaze in a food searching context.

In contrast to this, we found no such associations in the case of the toddlers: none of the candidate polymorphisms affected the children's use of communicative human gaze to locate the hidden toy. However, in the same group of infants, we detected a significant association between the subjects' OXTR genotype and the amount of distress the infants displayed in the *still face* task. This suggests different mechanisms underlying dogs' use of non-ostensive and children's use of ostensive gaze. Research in developmental psychology suggests that even younger infants are prepared to follow the gaze of an interactional partner while they ignore similar gaze cues if those are not addressed to them (e.g., Senju and Csibra, 2008). While it has been shown that infants develop an expectation that the direction of ostensive gaze is referential and it delivers generalizable knowledge (Senju et al., 2008), much less research addressed how humans interpret non-ostensive gaze. In contrast to the infants' performance, a number of studies found that without training and extended experimental pre-experiences, dogs follow communicative human gaze only with their gaze but do not approach a food location indicated in this way (Kaminski et al., 2012; Téglás et al., 2012; Duranton et al., 2017). Furthermore, dogs do not only ignore non-ostensive gaze but in fact tend to avoid a food location that another dog or a human has looked at in this way beforehand (Bálint et al., 2015; Duranton et al., 2017). Confirming these results, our findings suggest that dogs perceive such scenarios as competition over food and do not interpret non-ostensive gaze as a cooperative communicative signal that offers food to them. Dogs seem to respond to the context with markedly more social anxiety than children while at the same time it is still possible that they both interpret the non-ostensive gaze cue itself as an intentional cue that indicates the experimenter's interest in this location (Duranton et al., 2017). Further research will have to investigate this latter question.

Nevertheless, in this study we did not find that dogs as a group would avoid a food location indicated with non-ostensive gaze. On the contrary, our results suggest a surprising strong effect of oxytocin on how dogs perceive such a situation. The percentage of dogs following non-ostensive gaze varied very strongly with genotype of the OXTR, with only 20% of the dogs carrying two C alleles on the −94CT choosing the indicated container in contrast to the 80% of the TT dogs doing so.

Although in dogs, both OXTR polymorphisms that were associated with the dogs' reaction to a social threat were also linked to following a gaze cue to a food location, in human infants, we found only one OXTR polymorphism that was associated with reaction to an aversive social situation. Therefore, further studies should look at additional OXTR SNPs to investigate whether they have corresponding associations in the two tasks used here. There certainly are more human SNPs that merit investigation. For example, an association has been found between the rs4686302 SNP and social cognition deficits (e.g., facial emotion recognition) in children with ADHD (Kalyoncu et al., 2017). Especially interesting for dog-human comparisons, this SNP might be functionally similar to the canine rs851376227 located in the last exon of OXTR. Another study

(Isgett et al., 2016) revealed that the rs1042778 SNP in the human OXTR gene is associated with gaining positive emotions from loving-kindness training. This particular SNP is located in the 3' UTR that is a region where canine SNPs have also been found.

Interestingly, although we used different gaze cues in children and dogs and we found that only the dogs' gaze following was linked to how they reacted to an aversive social interaction, we found similar associations in dogs and children between their other behaviors in the two tasks. In particular, we found that those participants—both dogs and children—that followed the experimenter's gaze to a hiding location tended to spend less time looking at the experimenter threatening them (in dogs) or looking at them with a still face (in children). At a first sight this seems to suggest that dogs' and children's behavior are guided by similar mechanisms. This might be even correct at the level that participants that are more skilled at utilizing gaze cues may generally be more adept in social situations and, as such, faster to process negative social stimuli as well. Alternatively, it is also possible that gazing during social threat reflects different motivations in dogs and in children and thus, the consistency is only manifested at the behavior level but is not present in the underlying mechanisms. Analyses on the looking times (both in the case of children and dogs) focused on the attention participants paid to the two potential partners in the situation. The caregiver or the owner represented a secure base for participants; therefore looks directed at them can be interpreted as security or information seeking in a negative or ambivalent social situation. Gaze directed at the experimenter can either show fear or curiosity. However, looking at the experimenter in the *still face* task may not only reflect how fearful they perceived the situation, but how much they were interested in re-engaging her in play.

Note that we found that the identity of the experimenter affected the participants' behavior in both species. Despite of the training all experimenters received, this is understandable considering the social nature of the tasks. Most importantly, the genotype of the subjects was not confounded with identity of the experimenter for either SNP, thus the associations between genotype and behavior found in the study cannot be accounted for by an experimenter effect.

Finally, an interesting puzzle in our data concerns the children's generally low success in using gaze direction to locate the hidden object. As children at this age are typically good in following human communicative gaze, we suspect that the procedure we used explains their low success in this study. One could argue that the fact that the experimenter looked back at the children after her gaze cue made it more difficult for the children to remember which container they should choose. If so, we would expect random choices, in contrast to which we found that children had a tendency to choose the empty container. Therefore, we suggest that children's difficulty in locating the toy stems from their immaturity of inhibitory control. Instead of the training trials that we used for the dogs, we wanted to make sure that the toddlers also understood what they would be searching for by allowing them to see the toy inside the box at the non-cued location before the trial (see in procedure). As such, children's execution of action may be strongly biased by the last seen location of the object which may prevent other cognitive abilities (i.e., gaze following) from being exhibited. A similar dissociation between performance in overt behavior and cognitive processing has been documented in other areas of cognitive development as well (e.g., Onishi and Baillargeon, 2005). Importantly, problems with inhibition may also make genotype × behavior associations unobservable. Thus, we cannot discard the hypothesis that similarly to dogs, children's use of communicative cues is affected by OXTR polymorphisms. Further studies using another experimental procedure will have to address this question. However, even if an OXTR genotype × gaze following association is found, our prediction is that this will not be the same association as we found between OXTR genotype and reaction to still face.

In sum, these results support the idea that similarities observed in the overt behavior of dogs and human children may result from different mechanisms. While variations in the OXTR receptor gene affected both species behavior in a negative social situation, we could find corresponding associations in a gaze following task only in dogs. This raises the possibility that for dogs, the two situations are more alike (potentially fear-inducing or competitive) than for human children. Although the aversive social interaction tasks differed between species, the genotype × behavior associations we found were related to the distressing nature of these tasks both in dogs and children. However, while the same polymorphisms modulated the dogs' behavior in the *gaze following* task as well, we found no such consistencies across tasks in the children. We suggest that this is because young children interpret others' object-directed behavior as a learning opportunity (Csibra and Gergely, 2006) and as mostly cooperative, while dogs may view a social partner in a food searching task in a more antagonistic manner. If so, the oxytocin system can facilitate the success of dogs in participating in fundamentally cooperative, communicative interactions by fostering social approach through the reduction of fear responses in social interactions (Huber et al., 2005).

AUTHOR CONTRIBUTIONS

JT and ZV designed the study; all authors prepared the study material and data acquisition; KO, KK, SY, and DK entered the data and prepared it for statistical analyses; KO and JT analyzed the data; KO, JT, and ZV interpreted the data; ZV and JT obtained funding; KO wrote the first draft of the manuscript; KO, JT, ZV, and AK critically revised the manuscript for important intellectual content. All authors gave final approval of the manuscript version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Nasally-Administered Oxytocin Has Limited Effects on Owner-Directed Attachment Behavior in Pet Dogs *(Canis lupus familiaris)*

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The present study explored the effects of intranasal oxytocin, a naturally occurring hormone, on the behavior of pet dogs during an attachment test. Each dog participated in two testing sessions. On one visit saline was administered nasally, and on another, oxytocin was administered nasally. For half of the dogs ($n = 20$), solutions were administered with a Mucosal Atomization Device (MAD) and for half of the dogs ($n = 20$), solutions were administered using a nasal spray bottle. Condition order was counterbalanced and a double-blind methodology was employed. Following a 30-min wait period after administration of solutions, dog-owner pairs participated in the Secure Base Test, a short attachment test consisting of three 2-min phases: (1) Baseline- the owner was present, dogs were able to freely explore the testing room (2) Alone- dogs were left alone in the testing room (3) Return- owners re-entered the room and were reunited with their dog. In each phase the dog was evaluated for contact seeking, exploration, and avoidance behaviors. Although, oxytocin administration was expected to increase owner-directed proximity and contact seeking behavior, this effect was not observed. In fact, in the baseline phase, dogs spent significantly more time seeking the proximity of their owners when they received saline than when they received OT ($p < 0.05$). Sex differences were also assessed for the behavioral variables of interest in the Secure Base Test, and results indicated that OT did not affect dogs' behavior in the alone phase, but when saline was administered, females spent significantly more time in contact with the door than males in the alone phase ($p < 0.05$). Overall, the effects of nasally administered oxytocin on attachment related behavior appeared to be limited or inconsistent for this pet dog population.

Keywords: oxytocin, secure base, dogs, social behavior, human-animal interactions, attachment style

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INTRODUCTION

Oxytocin is a hormone involved in the formation and maintenance of social bonds between a variety of mammalian species (for a review, see Carter et al., 2008), including dogs and humans (for a review, see Beetz et al., 2012). Dogs have been found to display increased OT levels within a variety of social contexts involving exposure to a familiar person after a separation including when the human engages in verbal praise and physical contact with the dog, verbal praise alone,

or even when the dog simply has visual access to the person (Rehn et al., 2014). Humans may also experience elevated OT after interacting with their dogs, however results have been mixed or gender dependent. For example, Miller et al. (2009) demonstrated that OT levels increase in women after interaction with a bonded dog, but this effect was not seen in men. Evidence of a correlation in OT levels between dogs and their female owners has also been found (Handlin et al., 2011, 2012). Furthermore, there is evidence that owners who report having positive relationships with their dogs have higher urinary OT concentrations after interacting with their dogs, and in turn, their dogs gaze at them for longer durations in comparison to owners who report having less positive relationships with their dogs (Nagasawa et al., 2009).

Given OT's important role in relationships between dogs and humans, a growing area of research has explored the effects of nasally administered OT on dogs' behavior in a variety of contexts. In one study, dogs displayed more affiliative behaviors toward familiar humans and conspecifics when given OT nasally, compared to when they received saline (Romero et al., 2014). Another study demonstrated that female dogs gazed at their owners more after OT was administered intranasally, and urinary OT concentrations of their owners increased as a result, but these effects were not seen in male dogs and their owners (Nagasawa et al., 2015). Other studies have shown that administering OT nasally to dogs influences performance on object-choice tasks (Oliva et al., 2015), influences play behavior between familiar conspecifics (Romero et al., 2015), induces positive expectancy biases (Kis et al., 2015), and modulates reactions to owners and strangers in a threatening approach test (Hernádi et al., 2015). In addition, OT has recently been shown to have an impact on dogs' motion perception in a study comparing dogs' responses to a two-dimensional projection of a moving human (a "biological stimulus") and to the same video when scrambled and inverted, after OT or saline administration (Kovács et al., 2016). Dogs that received OT spent less time looking at the biological stimulus than dogs receiving saline indicating that dogs may have a natural preference for the motion of biologically-relevant stimuli, and OT administration decreases this preference.

While oxytocin is often associated with bond formation, to date, no studies have directly investigated whether increased OT levels might influence the secure base effect, or other behaviors indicative of "secure" attachments of pet dogs toward their owners. One of the first investigations into dog-human attachment utilized a seven episode version of the Strange Situation Test, originally designed to evaluate a human infant's attachment to their mother (Topál et al., 1998). Since then a variety of approaches to the study of dog-human attachment behavior- including the use of methodologies with additional controls and counterbalanced conditions- have been used successfully (Palmer and Custance, 2008; Rehn et al., 2013). The majority of these studies would suggest that dogs are capable of forming attachment bonds to their human caregivers, which includes proximity seeking behavior and preference for their owner. However, another important component of attachment is the secure base effect. Beyond the basic attachment bond, the secure base effect requires that a bonded individual be able to use their attachment figure as a source of comfort when challenged or stressed (Bowlby, 1969). Currently it is unknown whether OT might (1) exclusively promote increased proximity seeking and gaze behavior toward a human attachment figure (Romero et al., 2014; Nagasawa et al., 2015) independent of attachment security or (2) whether OT administration also facilitates feelings of security (the secure base effect). This distinction would have important implications for understanding the role of OT in bond formation and maintenance, and could also have important applied implications for the treatment of social anxiety disorders (see Thielke and Udell, 2015 for a review).

There is also a great need for additional double blind replications of studies measuring the behavioral outcomes of intranasal oxytocin administration in dogs in general. Relatively few studies have been conducted that directly evaluate how this procedure influences the behavior of dogs toward their owners, and of those that do exist, the effects have often been relatively small (Hernádi et al., 2015; Oliva et al., 2015; Romero et al., 2015).

Therefore, the current study asked how administration of intranasal oxytocin would affect the attachment behavior, and attachment style, of dogs in both the presence and absence of their owners. Given that we were specifically interested in the secure base effect, we utilized a modified version of the test originally developed by Harlow (1958) designed to study the secure base effect in infant rhesus macaques *(Macaca mulatta)*. This Secure Base Test consisted of three episodes, each 2 min in length: Baseline- dogs experienced an unfamiliar testing room in the presence of their owners, Alone- a phase in which owners left dogs in the room alone, and Reunion- in which the owner returns. As in expanded version of this test, including the SST, the return phase is critical for assessing greeting and proximity seeking behavior, as well as assessing the style of attachment to the owner (Waters, 1978; Rehn et al., 2013). Importantly a double-blind methodology was used to prevent expectations of the OT administrator, experimenter, owners or coders from influencing the behavior of the dog. If OT administration had a significant influence on the attachment behavior of dogs toward humans, we predicted that it might function in one of two ways:

(1) If OT increases affiliative behavior in dogs, time spent in contact with, and in proximity to, owners should increase when dogs receive OT vs. saline. In the absence of a secure attachment, increased motivation to seek the proximity of the owner could result in increased rates of search and anxiety related behaviors when the dog is left alone.
(2) If OT increases feelings of security in attachment relationships, then dogs should seek the proximity of their owner but also (a) display fewer stress-related behaviors when left alone after receiving OT and (b) be more likely to exhibit behaviors associated with a secure attachment after OT administration (reunion behavior, contact-exploration balance) when compared to saline administration.

Additionally, we thought it was possible, that if OT increases affiliative behavior in dogs, OT could also increase attachment security or result in changes in attachment style. Although it may take several interactions between individuals for an attachment relationship to form, dogs living in shelters have been shown to form this relationships relatively quickly-after

just a few interactions with an experimenter (Gàcsi et al., 2001). Furthermore, research with humans has shown that a significant proportion of men who were classified as insecurely attached prior to a single dose of OT administration displayed increased attachment security on an attachment task (Buchheim et al., 2009).

A secondary aim of this study was to test two administration methods-a nasal spray bottle and a Mucosal Atomization Device (MAD) to determine if the methods differ in the amount of stress-related behaviors that dogs display upon administration. One study used a nasal spray bottle (Romero et al., 2014) while another study used MADs for administration (Oliva et al., 2015). Thus, we aimed to evaluate whether stress responses during administration differed depending on the type of administration device used, as differences in stress during administration could impact results of behavioral tests.

MATERIALS AND METHODS

Subjects

A total of 40 pet dogs were volunteered by their owners. All dogs were required to be over 10 months of age, in good health, not have a history of separation anxiety and not be pregnant or lactating, due to oxytocin's known role in inducing labor and lactation. As breed was not a variable under evaluation, a variety of breeds and mixes were enrolled in the study. There were 18 male and 22 female subjects. **Table 1** lists each dog's breed and age.

Ethics Approval Statement

This study was conducted under ethical approval from the Institutional Animal Care and Use Committee (IACUC) of Oregon State University (ACUP number 4664). Consent was provided by dog owners via a consent form approved by Oregon State University's IACUC committee.

Experimental Protocol

The order of treatments in this study (saline vs. OT) was counterbalanced. Fifty microgram (24 IU) of OT (Extreme Peptide, United States) were diluted in 0.5 ml of a 0.65% saline solution (Ocean Saline Nasal Spray, Bridgewater, NJ). Dogs receiving the saline solution received 0.5 ml of the 0.65% saline solution. All solutions were prepared within 48 h of each testing session and given a code associated with the testing session by an assistant who did not participate in testing. This kept the experimenter blind to the solution being administered during the test. OT or saline was administered intranasally in an unfamiliar room by an experimenter.

Nasal administration methods have varied across scientific studies. Therefore, in the current study we utilized and compared two previously cited administration methods: A nasal spray bottle (Romero et al., 2014) and a MAD (Oliva et al., 2015). Half of the subjects ($n = 20$) experienced the nasal spray bottle (Sinox Pharma, China) administration method and the other half experienced the MAD (Live Action Safety, Eugene, Oregon) administration method for both their OT and saline administrations. For each type of administration, food was placed in a container so that the dogs could smell it but not access it in order to ensure that they were sniffing while the administration occurred. Each dog participated twice on 2 different days (visits were spaced at least 5 days apart) receiving either saline or OT prior to the attachment test. Administration was filmed and videos were coded for stress-related behaviors (**Table 2**). Thirty percent of the videos were coded by a second independent observer, blind to the treatment each dog received on each day, to assess inter-rater reliability. Total duration of administration was also measured. Thirty minutes after OT or saline administration, dogs and owners participated in a modified attachment test, the Secure Base Test (modified from Harlow, 1958). This time period was chosen as previous work has shown that effects of OT can be seen after this time period (Woolley et al., 2014).

The Secure Base Test occurred in a second unfamiliar room ∼3.6 m by 4.2 m in size. The room contained a chair with a semi-circle 1 m in radius taped around the chair. Three dog toys were placed on the testing room floor during each testing session (**Figure 1**). Testing consisted of three phases, each lasting 2 min. **Table 3** summarizes instructions that owners received for each phase of the attachment test. Each phase lasted for 2 min and immediately followed the phase preceding it, and the test began immediately after the dog and owner entered the testing room. In phase one (Baseline), the owner received instructions to sit neutrally in a chair in an unfamiliar testing room. Owners were able to reciprocate affection if the dog approached and entered the circle by petting the dog twice without restraining it by the collar if the dog approached (i.e., placed at least two paws or half of a body length inside the circle) or initiated contact. Dogs were able to freely explore the room. In phase two (Alone), the owner exited the testing room so that the dog was left alone. In phase three (Return), the owner re-entered the testing room. Owners were asked to sit neutrally, as in baseline, during this phase and were able to reciprocate affection by petting the dog twice without restraining it by the collar if the dog approached or initiated contact. Videos of the Alone phase were coded for search and separation anxiety behaviors (**Table 4**; McCrave, 1991; Overall et al., 2001; Storengen et al., 2014). Videos of the Baseline and Return phases were coded for attachment related behaviors (**Table 5**). An independent observer, blind to the treatment each dog received on each day, coded all videos. A second independent observer, also blind to condition, recoded 30% of the videos to ensure inter-rater-reliability.

While duration-based measures are commonly used to evaluate the effects of OT administration on behavior, they may not be the most sensitive or reliable method for detecting the secure base effect or attachment style (Schöberl et al., 2016). Therefore, we also coded the videos using a holistic scoring approach specifically designed to assess attachment style categories (Schöberl et al., 2016) using the definitions in **Table 6**. For this method, all videos were double coded by two attachment style experts to ensure reliability. Both coders independently watched the Return phase of each video for each dog, and categorized the dogs according to the definitions in **Table 6**, allowing for an inter-rater reliability score. The two coders were then asked to come to a consensus as described by Schöberl et al. (2016) on all videos by jointly viewing videos where initial

TABLE 1 | Breed, age, treatment order, and attachment style categorizations for each subject.

Dog's Name	Age (years)	Breed	Treatment Order	OT Attachment Style	Saline Attachment Style
Annie	5	American Pit Bull Terrier Mix	OT-Saline	Secure	Secure
Annie	10	Border Collie	OT-Saline	Secure	Unclassifible
Blue	2	Dachshund	Saline-OT	Secure	Secure
Bohdie	3	American Pit Bull Terrier Mix	Saline-OT	Secure	Secure
Boss	7.5	German Shepherd	OT-Saline	Insecure avoidant	Insecure disorganized
Bree	4.7	Collie	Saline-OT	Insecure disorganized	Unclassifible
Bruno	5.3	Poodle/Border Collie/Papillion Mix	Saline-OT	Secure	Secure
Carmella	9.6	Golden Retriever	OT-Saline	Insecure ambivalent	Insecure ambivalent
Ducky	3.5	Corgi	OT-Saline	Insecure avoidant	Secure
Ellie	3.9	Cane Corso	Saline-OT	secure	Secure
Ember	3	Border Collie	Saline-OT	Insecure ambivalent	Insecure ambivalent
Grace	10	Golden Retriever	Saline-OT	Secure	Secure
Gryphon	8	Black Russian Terrier	OT-Saline	Insecure avoidant	Insecure avoidant
Guinness	4	Standard Poodle	OT-Saline	Secure	Secure
Hampton	6.7	Rottweiler/American Pit Bull Terrier Mix	OT-Saline	Secure	Secure
Honey	2	Labrador Retriever Mix	Saline-OT	Secure	Secure
Ian	8	Border Collie	OT-Saline	Secure	Secure
Jac	1.5	Brittany Spaniel	Saline-OT	Secure	Secure
Jade	1.75	Black Russian Terrier	OT-Saline	Secure	Secure
Kenny	3.2	Golden Retriever	Saline-OT	Secure	Secure
Kobe	7	Akita/American Pit Bull Terrier Mix	Saline-OT	Secure	Secure
Lily	5.3	Border Collie Mix	Saline-OT	Secure	Secure
Lizzie	8	Australian Shepherd Mix	Saline-OT	Insecure ambivalent	Insecure avoidant
Loke	3	Alaskan Malamute	Saline-OT	Secure	Secure
Louie	2.6	Labrador Retriever Mix	OT-Saline	Secure	Secure
Molly	2.2	Shepherd/Husky/Labrador Retriever/American Pit Bull Terrier Mix	Saline-OT	Secure	Secure
Pumpkin	3	Australian Cattle Dog Mix	Saline-OT	Secure	Secure
Raven	1	Dachshund	OT-Saline	Secure	Secure
Riley	8	Golden Retriever	OT-Saline	Secure	Secure
Ripley	1	Border Collie	OT-Saline	Secure	Secure
Rowan	10 months	Australian Shepherd	Saline-OT	Secure	Secure
Shelby	11	Golden Retriever	Saline-OT	Secure	Secure
Tahoma	3	Labrador Retriever	OT-Saline	Secure	Secure
Tara	11	American Pit Bull Terrier Mix	OT-Saline	Secure	Secure
Teddy	13.5	Shetland Sheepdog	OT-Saline	Insecure avoidant	Unclassifible
Tenaya	12.9	Collie	OT-Saline	Secure	Secure
Willow	12.5	Collie	OT-Saline	Secure	Secure
Wrigley	7	Labrador Retriever/Akita Mix	Saline-OT	Secure	Secure
Zoey	4	Australian Shepherd/McNab Shepherd/Border Collie Mix	OT-Saline	Secure	Secure
Zum	11	American Pit Bull Terrier Mix	Saline-OT	Secure	Secure

disagreement occurred and mutually deciding on an attachment style classification. Dogs where consensus could not be reached were labeled as unclassifiable (Schöberl et al., 2016).

Statistical Methods

All statistical analyses were conducted using RStudio. The frequency of each of the following stress-related behaviors was recorded during OT and saline administration for each testing session: lip licking, head shaking, shivering, whining, and yawning. The total amount of time that administration took was also recorded for each session. No instances of yawning were observed during administration for any dog in this study, so yawning was excluded from the analysis. The stress-related behaviors were coded as being mutually exclusive, and were summed to create an overall score of stress during administration. For analysis, data from each dog's first testing

TABLE 2 | Stress-related behaviors (Adapted from Deldalle and Gaunet, 2014).

Behavior	Description
Lip licking (frequency)	Dog licks lips
Yawning (frequency)	Dog opens mouth and yawns
Shivering (frequency)	Dog trembles
Whining (frequency)	Dog makes high pitched noise
Head shaking (frequency)	Dog moves head from side to side

session was used so that for half of the dogs ($n = 20$), administration was performed with an MAD, and for half of the dogs ($n = 20$), administration was performed using a nasal spray bottle. Order of solution presentation (OT or saline) was counterbalanced equally between groups as well. Inter-rater reliability for duration of administration was 79.2 and 75% for the overall stress-related behavior score. The distribution of data for duration scores was heavily right skewed, therefore a log transformation of duration was used in the analysis which aided in the normalization of the data for analysis. Following this transformation the average duration of administration using the MAD vs. the nasal spray bottle method was compared using a *t*-test, as was the average number of stress-related behaviors for each administration type. Fishers Exact tests were used to compare the number of dogs with secure and insecure attachment styles for the MAD vs. the nasal spray bottle method for both saline and OT conditions separately. A power analysis was conducted during the procedural design phase of this study as recommended in the statistics literature (Das et al., 2016). It was determined that 20 subjects would give us 90% power to detect a large effect size, with a two-tailed alpha of 0.05, therefore a sample size of 20 subjects per administration type was utilized.

The variables measured for the baseline and return phases of testing include proportion of time spent engaging in the following activities: avoiding, exploring, inside the circle (a measure of proximity seeking), playing and contact with the owner, as well as the latency to enter the circle and the latency to make contact with the owner, and the number of times per session each dog made contact with its owner. Inter-rater reliability was high for all behaviors coded in the baseline and return phases: Baseline phase: mean 91% agreement, range 79–96% agreement across individual behaviors; Return phase: mean 86% agreement, range 75–92% agreement across individual behaviors. Histograms were used to assess normality. A Shapiro-Wilk test was used to assess normality of residuals for both treatment type (the within-subjects variable) and sex (the between-subjects variable) for each behavioral measure of interest within the Return phase. The assumption of normality was violated for the proportion of time dogs spent avoiding, exploring, in proximity to their owners, playing, in contact with their owners, the latency to make contact with their owners and the latency to seek proximity to their owners. The normality assumption was not violated for the frequency at which dogs made contact with their owners. A paired *t*-test was used to compare proportion of time spent inside the circle when dogs were given OT vs. when they were given saline.

For the Alone phase, we were interested in the relative frequency of behaviors associated with the presence or absence of separation distress as identified in canine attachment (Schöberl et al., 2016) and separation anxiety (McCrave, 1991; Overall et al., 2001; Storengen et al., 2014) literature. The proportion of time the dog spent engaging in the following activities was recorded: being out of sight, looking at the door, playing, and touching the door. The number of times each dog engaged in hypersalivation, elimination, repetitive movement, vocalizing, and destruction, touching the door, and looking at the door during the alone phase was measured as well. No instances of hypersalivation, elimination, repetitive movement, or destruction were observed during the alone phase of the attachment test, so these behaviors were excluded from all analyses of the alone phase. While duration of looking at the door (38% agreement), frequency of looking at the door (63% agreement) and number of vocalizations (38% agreement) produced during the alone phase had moderate reliability scores, all other reliability measures indicated strong agreement: mean 93%, range 75–100% agreement). Histograms were used to assess normality. The Shapiro-Wilk test was used to assess normality of residuals for both treatment type (the within-subjects variable) and sex (the between-subjects variable) for each behavioral measure of interest within the Alone phase. The assumption of normality was violated for the proportion of time dogs spent out of sight, exploring, playing, and touching the door, the frequency at which dogs touched the door and the frequency at which dogs vocalized for both treatment and sex. However, the mixed design ANOVA is generally considered robust to violations of normality, and therefore was chosen to allow for the targeted evaluation of interaction effects. The normality assumption was not violated for the frequency at which dogs looked at the door or the proportion of time dogs spent looking at the door for either treatment type or sex. Within-subject comparisons were analyzed using a paired *t*-test to determine whether any differences were present when dogs were given OT vs. when they were given saline with respect to the variables measured during the alone phase of the attachment test.

To investigate the interaction between treatment and subject sex in the alone and return phases, we also compared the behavior of males and females when given OT or saline with a 2×2 Mixed Design ANOVA. This is an important consideration as studies have demonstrated that male and female dogs/humans can show different behavioral trends after OT administration (Nagasawa et al., 2015; Oliva et al., 2015).

Inter-rater reliability for holistic coding, when two independent coders categorized dogs according to the definitions outlined in **Table 6**, was 77.5%. In order to ensure that all dogs were reliably categorized according to the appropriate attachment styles, the same two coders re-watched the videos for the dogs for which they did not independently agree and mutually decided on an attachment style (Waters, 1978; Schöberl et al., 2016). Videos for three dogs were scored as unclassifiable and were dropped from analysis (resulting in six total testing sessions being dropped from this portion of the analysis). A Fishers Exact test to compare the number of dogs categorized as having a secure attachment (demonstrating the secure base effect) after administration of OT vs. saline.

TABLE 3 | Owner instructions for attachment test.

Phase	Description
Phase 1 (Baseline) 2 min	Owners were instructed to sit in chair in testing room and pet the dog twice each time it entered the circle.
Phase 2 (Alone) 2 min	Owner and experimenter exited the room and the dog was left alone.
Phase 3 (Return) 2 min	Owner and experimenter quietly re-entered the room without greeting the dog. Owners were instructed to sit in chair in testing room and pet the dog twice each time it entered the circle. (Identical to baseline.)

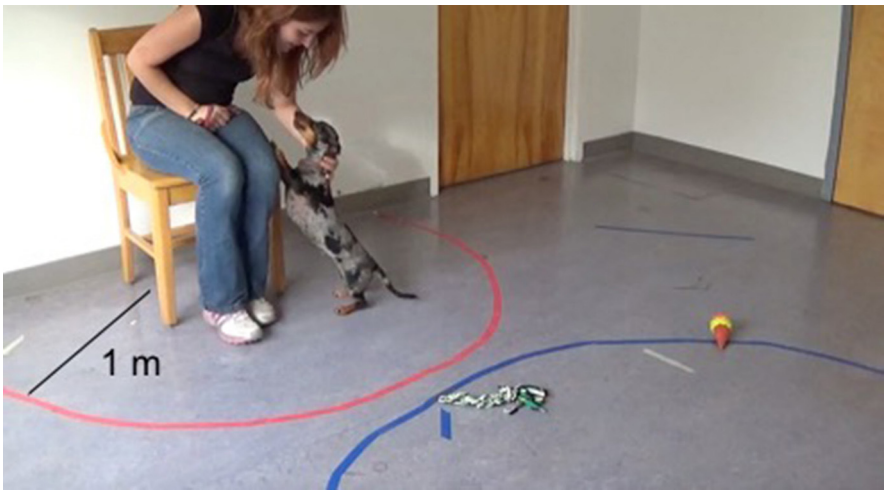

FIGURE 1 | Layou of testing room for SBT. Written consent was obtained from this participant for the inclusion of this image in the manuscript.

All tests were two tailed and had an alpha level of 0.05 unless otherwise specified. *Post-hoc* comparisons were made using *t*-tests with a Tukey-Kramer adjustment for all pairwise comparisons.

RESULTS

Administration

Results indicated that administration in the MAD group (Mean duration = 18.25 s) was shorter compared to the nasal spray bottle group (Mean duration = 32.75 s), this difference was not statistically significant [$t_{(38)} = -1.8019$, $p = 0.08$]. No significant differences were observed with respect to overall stress-related behavior between administration types [$t_{(27.679)} = -1.2303$, $p = 0.23$]. No significant differences were found with respect to attachment style based on administration type when either the saline ($p = 1.00$) or the OT solutions ($p = 1.00$) were administered. As no significant differences were found on these measures based on administration type, data from both administrative methods was pooled for the remaining analyses. However, since sample size is relatively small ($n = 20$ per group) when grouped according to administration type, we have also included analyses (using a Mixed Design ANOVA) for MAD and nasal spray bottle groups separately.

Duration and Frequency Based Measures: Baseline Phase

Dogs that received saline spent significantly more time inside the circle compared to dogs that received OT [$t_{(39)} = 2.11$, $p = 0.04$]. On average, dogs spent 6.5% more time in proximity to their owners when saline was administered compared to when OT was administered (see **Table 7**). No other measures in the Baseline phase differed significantly when dogs received OT compared to when they received saline. While we predicted that the proportion of time dogs spent in contact with their owners would significantly differ when they were given OT vs. saline-based on the proximity and contact seeking effects previously reported (Romero et al., 2014)—this was not observed in the current study [$t_{(39)} = 0.35$, $p = 0.73$]. Additionally, a binomial test was used to compare the number of dogs that showed an increase in proximity seeking when given OT compared to saline. A total of 17 dogs out of 40 showed an increase in proximity seeking after OT administration when compared to saline ($p = 0.43$). Of these dogs, 6 dogs belonged to the MAD group and 11 dogs belonged to the nasal spray bottle group.

For comparisons between administration type, a significant interaction was found between method of administration and treatment for avoidance behavior [$F_{(1, 1)} = 4.67$, $p = 0.04$]. A trend was found with respect to saline administration, as, on average, dogs in the nasal spray bottle group spent 8.3% more time exhibiting avoidance behavior than dogs in the MAD group [$t_{(31.95)} = -1.90$, $p = 0.07$], see **Table 8**. Within-subjects comparisons for the MAD group revealed that there was a trend of dogs spending an average of 6.1% more time exhibiting avoidance behavior when OT was administered compared to when saline was administered [$t_{(19)} = 1.93$, $p = 0.07$]. For a summary, see **Table 9**. Averages for time spent displaying avoidance behavior for dogs in the MAD group

TABLE 4 | Alone phase focal behaviors.

Behavior	Description
Vocalizing (frequency)	Whining or barking
Touching or scratching at testing room door (frequency and duration)	Using any part of body to make contact with door
Elimination (frequency)	Urinating or defecating
Destruction (duration)	Destroying/chewing non-toy objects in testing room
Excessive motor activity (duration)	Pacing or other repetitive movements
Hypersalivation (frequency)	Excessive drooling or salivation
Exploring (duration)	Walking around room
Looking at door (frequency and duration)	Gazing in direction of door without making contact with door
Playing (duration)	Picking up/making contact with toys

TABLE 5 | Attachment behaviors.

Behavior	Description
Inside circle (proximity seeking) (duration)	Laying, sitting or standing inside of the circle taped around the owner's chair
Outside circle (duration)	Laying, sitting or standing outside of the circle taped around the owner's chair
Exploring (duration)	Moving around the room or walking in a non-repetitive manner (i.e., not pacing)
Contact with owner (frequency and duration)	Physical contact with owner (or owner making contact with dog) with any part of body
Playing (duration)	Picking up/making contact with toys
Avoiding (duration)	Sitting, standing or laying out of reach outside circle

include 9.62% when OT was administered and 3.62% when saline was administered. Dogs in the nasal spray bottle group spent an average of 8.20% of the session displaying avoidance behavior when OT was administered and 11.92% of the session when saline was administered. *Post-hoc* comparisons did not reveal any other trends or significant differences. With respect to proximity seeking, no significant interactions between method of administration and treatment were found. On average, dogs in the MAD group spent 36.98% of the session in proximity to their owners when OT was administered and 46.09% of the session in proximity to their owners when saline was administered. Dogs in the nasal spray bottle group, on average, spent 35.60% of time in proximity to their owners when OT was administered and 38.54% of the session in proximity to their owners when saline was administered. No significant interactions between method of administration and treatment were found for any other behaviors measured in baseline.

Duration and Frequency Based Measures: Alone Phase

No statistically significant differences were observed with respect to any of the variables of interest. However, there was a trend of dogs vocalizing more during the alone condition when OT was administered compared to when saline was administered [$t_{(39)} = -1.87$, $p = 0.07$]. On average, dogs vocalized 5.4 times more when OT was administered than when saline was administered. Overall, no significant differences or trends were found according to type of administration device used for any of the behaviors coded in the alone phase. Dogs assigned to the MAD administration group vocalized an average of 31.2 times during the alone phase when OT was administered and an average of 26.15 times when saline was administered. For the nasal spray bottle group, dogs vocalized an average of 33.7 times during the alone phase when OT was administered and an average of 28.05 times when saline was administered.

Only moderate effects of treatment, sex or treatment by sex interaction were found with respect to the behavioral variables of interest. A trend was seen with respect to treatment on the frequency at which dogs vocalized [$F_{(1, 1)} = 3.40, p = 0.07$]. There was a trend of an interaction between treatment and sex for the frequency at which dogs looked at the door [$F_{(1, 1)} = 3.7$, $p = 0.06$]. When saline was administered, there was a trend in which males tended to look at the door with a greater frequency than did females, $t_{(30.13)} = -2.00, p = 0.05$, with males looking at the door 2.11 more times than females. In the saline condition, females spent significantly more time touching the door than did males [$t_{(21.26)} = 2.28$, $p = 0.03$]. On average, females spent 9.5% more time in contact with the door than did males (see **Table 10**).

Duration and Frequency Based Measures: Return Phase

No significant differences were found with respect to the variables of interest in the return condition when within-subject comparisons were made. No significant differences or trends were found for any of the behaviors observed in the return phase with respect to type of administration device used. Of special interest was the fact that there was not a statistically significant difference with respect to the proportion of time dogs spent in proximity to their owners when treated with OT vs. saline [paired t-test, $t_{(39)} = 0.63, p = 0.53$]. On average, dogs in the MAD group spent 57.02% of the return session seeking proximity when OT was administered, and 59.0% of time seeking proximity when saline was administered. Dogs in the nasal spray bottle group spent an average of 52.36% of the session seeking proximity when OT was administered and 56.35% of the session seeking proximity when saline was administered. There was also not a significant difference in the proportion of time dogs spent in contact with their owners when treated with OT vs. saline [paired t-test, $t_{(39)} = 0.59$, $p = 0.55$]. Dogs assigned to the MAD group spent an average of 19.36% of the session in contact with owners when OT was administered and an average of 19.05% of time in contact with their owners when saline was administered. Dogs assigned to receive administration via a nasal spray bottle spent an average of 16.83% of time in contact with their owners after OT administration and an average of 20.86% of the session in contact with owners after saline administration.

To investigate a possible interaction between treatment and sex, we compared the behavior of males and females when given OT or saline with a 2 × 2 Mixed Design ANOVA. While no

TABLE 6 | Attachment style definitions (adapted directly from Schöberl et al., 2016).

Attachment Style	Definition
Secure	Dog approaches owner promptly at reunion and follows, makes physical contact or signals for contact, seeks and is comfortable with contact. Little or no gaze aversion or proximity avoidance. Little or no resistance to contact or interaction.
Insecure avoidant	Dog shows little tendency to approach, to seek contact, or to follow. Dog turns or looks away during reunion. Dog shows lack of response to invitations to approach or interact for 30 s or more. Dog explores the room and objects during pre-separation and post-separation. There is little active search for owner.
Insecure ambivalent	On reunion, they mixed persistent distress with efforts to maintain physical contact and/or physically intrusive behavior directed toward the owner. These dyads were characterized by a degree of conflict regarding physical contact or play activities. For example, the dog wished to maintain contact and was uncooperative with the owner's attempt to encourage play or exploration, or the owner maintained firm physical contact which the dog merely passively tolerated. (Dogs who the judges agreed seemed essentially secure but with ambivalent tendencies, were included in the secure group).
Insecure Disorganized	Evidence of strong approach avoidance conflict or fear on reunion, for example, circling owner, hiding from sight, rapidly dashing away on reunion, "aimless" wandering around the room, shying away from contact, or proximity. "Dissociation" may be observed, that is, staring into space without apparent cause; still or frozen posture for at least 20 s (in the non-resting, non-sleeping dog).
Unclassifiable	Dogs showed ambiguous evidence of disorganization or other disturbance, for example, "depressed"-a marked lack of enthusiasm in a dog that otherwise seemed secure or showed other behavior suggesting a neurologic or compulsive disorder. Classifiers were unable to reach consensus on group placement for dogs from this classification category. Unclassifiable dogs were excluded from further analysis on dog attachment.

Only descriptions pertaining to the return phase were used.

TABLE 7 | Effect sizes for behaviors of interest for overall comparisons with pooled data.

	OT		Saline				95% CI		
Variable	***M***	***SD***	***M***	***SD***	$t_{(39)}$	***p***	***LL***	***UL***	**Cohen's *d***
Baseline-Inside circle (proximity seeking, duration)	0.36	0.26	0.42	0.25	2.11	0.04	0.002	0.13	0.33
Alone- Vocalizing (frequency)	32.45	27.81	27.10	25.65	−1.87	0.07	−11.15	0.45	−0.30

TABLE 8 | Effect sizes for behaviors of interest for dogs by administration type (i.e., with separate analyses for MAD and nasal spray bottle administration types) in the saline condition.

	MAD		Nasal spray bottle				95% CI		
Variable	***M***	***SD***	***M***	***SD***	$t_{(31.95)}$	***p***	***LL***	***UL***	**Cohen's *d***
Baseline-Avoiding (duration)	0.04	0.10	0.12	0.17	−1.90	0.07	−0.17	0.006	−0.57

TABLE 9 | Effect sizes for behaviors of interest for dogs by solution type in the MAD condition only.

	OT		Saline				95% CI		
Variable	***M***	***SD***	***M***	***SD***	$t_{(19)}$	***p***	***LL***	***UL***	**Cohen's *d***
Baseline-Avoiding (duration)	0.096	0.19	0.04	0.10	1.93	0.07	−0.005	0.13	0.43

TABLE 10 | Effect sizes for behaviors of interest for comparisons of males and females with pooled data.

	Females		Males				95% CI		
Variable	***M***	***SD***	***M***	***SD***	***t*(df)**	***p***	***LL***	***UL***	**Cohen's *d***
Alone- Looking at door (duration)-saline condition	7.27	2.71	9.39	3.76	−2.00	0.05	−4.28	0.04	−0.65
Alone- Contact with door (duration)-saline condition	0.10	0.19	0.007	0.14	2.28	0.03	0.008	0.18	0.56
Return- Contact with owner (latency)-saline condition	2.86	3.01	16.67	37.44	1.75	0.09	−29.99	2.37	0.52
Return-Playing (duration)-OT condition	0.15	0.21	0.31	0.35	−1.75	0.09	−0.35	0.03	−0.55

statistically significant differences were found, there was a trend of an effect of sex on latency to make contact with their owners [$F_{(1, 1)} = 2.90$, $p = 0.09$]. A trend was present in which females had a shorter latency to engage in contact with their owners than males in saline condition [$t_{(38)} = 1.75$, $p = 0.09$], with females making contact with their owners an average of 13.8 s faster than males. A trend was seen with respect to playing, as there was a tendency for males to spend a greater proportion of time engaging in play than females after OT administration [$t_{(26.28)} = -1.75$, $p = 0.09$]. On average, males spent 16.3% more time engaging in play compared to females after OT administration. See **Table 10** for a summary of effect sizes for results of interactions between treatment and sex.

Evaluation of the Secure Base Effect and Attachment-Style

Attachment style categorizations for each dog for both OT and saline conditions can be found in **Table 1**. For the OT condition, 31 dogs were scored as secure, 3 dogs were scored as insecure avoidant, and three dogs were scored as insecure ambivalent. For the saline condition, 32 dogs were scored as secure, two dogs were scored as insecure avoidant, two dogs were scored as insecure ambivalent, and one dog was scored as insecure disorganized. The Fisher's exact test comparing the number of dogs with secure and insecure attachments for both the OT and saline conditions was not significant ($p = 1.00$), indicating that OT did not impact the attachment styles of dogs in the present study. Only one dog was scored as insecurely attached in the OT condition but securely attached in the saline condition. The remaining dogs who were scored as insecure, were scored as being insecurely attached in both phases (although the type of insecure attachment did vary for a few individuals). Overall, attachment style category changes were only observed for three dogs: one dog was classified as insecure avoidant when OT was administered, but was categorized as insecure disorganized when saline was administered, one dog was classified as insecure avoidant when OT was administered but scored as secure when saline was administered, one dog was classified as insecure ambivalent when OT was administered, but was classified as insecure avoidant when saline was administered.

DISCUSSION

The results of the baseline phase were unexpected, as previous literature has shown that OT results in increased affiliative behavior and proximity seeking (Romero et al., 2014; Nagasawa et al., 2015). In contrast, we found that dogs sought proximity for longer durations after the saline administration, not OT administration. One possible explanation for this result may have to do with the relationship between the effects of OT and sex. For example, some research has suggested that female dogs may be more sensitive to the prosocial effects of OT and males may exhibit increased vigilance after administration of OT, particularly if OT binds to receptors for vasopressin, a structurally similar molecule (Nagasawa et al., 2015). As **Figure 2** shows, male dogs tended to spend less time in proximity to their owner (within the 1 m circle) when given OT, while females do not differ with respect to the proportion of time spent in owner proximity when given OT or saline. Thus, it is possible that OT resulted in increased vigilance in males that led them to spend less time in proximity to their owners during baseline driving this effect. Evidence from studies with both humans and prairie voles indicates that vasopressin has sexually dimorphic effects, and it is associated with defensive behaviors (for a review, see Carter, 2014). Thus, if OT binds to vasopressin receptors, it could lead to an increase in these behaviors, particularly for males. In humans, vasopressin has also been shown to increase defensive behaviors in an adaptive manner (Heinrichs and Domes, 2008) and plays a role in bonding and aggressive behavior by increasing encoding of positive and negative social cues (Guastella et al., 2010).

The effects of OT on the behavior of dogs during an Alone condition where their owner leaves them alone in an unfamiliar room, had never previously been evaluated. This condition has important implications for the potential mechanisms underlying behavioral responses associated with OT. We hypothesized that if administration of OT resulted in greater attachment security, that when given OT, dogs would engage in fewer stress-related behaviors during a brief separation period from their owners compared to the saline control condition. The results did not support this hypothesis. Instead, there was a trend of dogs vocalizing more frequently when they had received OT compared to when they received saline. As vocalizing may indicate stress, it is possible that administration of OT may increase stress when dogs experience a short separation period from their owners. Based on the findings of prior studies demonstrating proximity seeking behavior after OT administration (Romero et al., 2014), increased vocalizations or stress when left alone could be due to the disruption of proximity seeking behavior at a time when motivation to engage in this behavior is especially high. However, it should be noted that this effect was minimal, at least among the pet dogs tested in this study, and that in the current study we did not find a significant increase in proximity seeking behavior by dogs toward their owners during baseline. There was also a trend of males spending a greater proportion of time looking at the door of the testing room in their owner's absence in the saline condition, but this effect was not seen with OT. This may suggest that there are sex differences that predict owner-directed search-related behaviors in the absence of their owners, and OT may decrease these differences.

As two different methods of administration were tested in this study, we conducted additional analyses without pooling data for both groups. Overall, the only instance an interaction effect between type of administration and behavior was for avoidance behavior in the baseline phase. However, it should be noted that only 19 of 40 dogs exhibited any avoidance behavior in baseline when OT was administered, and only 19 of 40 dogs displayed avoidance behavior when saline was administered. In addition, it should be noted that although differences in duration of administration and overall stress experienced during administration were not statistically significant, sample size was relatively small ($n = 20$ per administration type). Thus, it is possible that different methods of nasal administration could

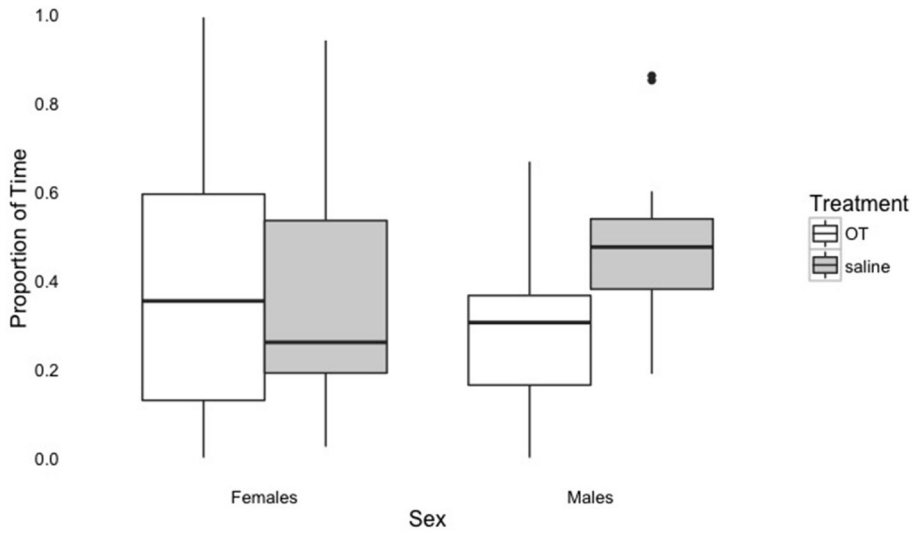

FIGURE 2 | Proportion of time spent within 1 m of the owner in the Baseline phase by treatment and sex. The dark line indicates the median, the box indicates the interquartile range, or the middle portion of the data, the upper bar indicates scores above the middle 50%, the lower bar indicates scores below the middle 50%, and outliers are greater than the upper bar by more than 1.5 times the interquartile range.

lead to different outcomes in behavioral tests, although limited evidence of this was found in the present study.

Additionally, we hypothesized that OT administration would result in an increase in proximity seeking and/or behaviors associated with secure attachment when dogs were reunited with their owners after a short separation period. No such effect was found. A trend toward a greater proportion of time spent engaging in play was identified for males, but not females, when OT was administered. Play is often thought to be an indicator of welfare (Held and Špinka, 2011) and is sometimes used as a measure of the secure base effect during the reunion phase of attachment tests (Schöberl et al., 2016), so a trend toward an increase in time spent engaging in play could suggest that OT may have some impacts on welfare. However, the relationship between the increase in play behavior and OT administration for males was not statistically significant and other methods, such as direct human interaction and petting (Mehrkam et al., 2014), have been found to have a more robust and immediate effect on rate of play.

Overall, while some significant differences were found between OT and saline conditions, and between male and female responses to OT administration, such differences are often modest in both this and other studies. For example, several studies have found fairly small effect sizes of nasally administered OT in dogs including in relation to dogs' ability to use pointing gestures to find hidden food in an object-choice task (Oliva et al., 2015) responses to the threatening approach of owners (Hernádi et al., 2015), and relatively small changes in affiliation rate with familiar humans and conspecifics (Romero et al., 2014). While the temptation might be to increase sample size, this raises an important issue for future research. Studies evaluating intranasal administration of OT are often concerned with the effect of treatment on the behavior of individual animals, especially in cases where OT administration might be recommended as a behavior modification tool or aid (Thielke and Udell, 2015). Therefore, increased sample size for studies of this type might actually be problematic, as a larger body of averaged data could mask the relative weakness of behavior change that might be expected for a single dog. In contrast, other treatments have shown a greater behavioral effect with similar sample sizes. For instance, one study comparing the efficacy of a dog appeasing pheromone to clomipramine (an antidepressant medication) for the treatment of separation anxiety in 57 dogs measured improvement on several different behaviors before and after either treatment intervention (Gaultier et al., 2005). The smallest improvement was seen in 65% of dogs vocalized less or did not vocalize at all in the absence of their owners after treatment. Therefore, an improvement in separation anxiety symptoms was seen for the majority of dogs in the study, regardless of treatment type. Therefore, future research should attempt to further explore predictive variables that could explain the different degrees of behavioral change reported across studies investigating the effects of OT administration, at both the individual and group level, such as sex differences, OT dosage or administration methods, and not simply increase sample size. Conversely, some researchers and clinicians may find moderate effect sizes informative for some applications, therefore providing a diversity of information on the effects of OT administration with different sample sizes and effect sizes will likely be important for future directions.

The current study was also conducted with pet dogs without known anxiety disorders, and although some effects of OT have been found on the social behavior of this population in the past, more substantial effects may still be found in specific social populations, for example in dogs with separation anxiety. Furthermore, many pet dogs in this and other (e.g., Schöberl et al., 2016) attachment studies have demonstrated a secure

attachment to their owners. For this reason, pro-social changes associated with OT administration may be less detectable in the general population, but might be more salient for dogs who initially display insecure attachments to their owners. It should also be noted that the present study did not include measuring dogs' plasma OT levels after OT administration, therefore we cannot rule out the possibility that OT levels decreased during the waiting period for the SBT. While a waiting period between OT administration and behavioral testing is considered standard practice the duration of the waiting period itself varies across studies. Although Romero et al. (2014) suggests an optimal waiting period of about 15 min, one study used 40 min waiting periods (Hernádi et al., 2015), while another employed a 45 min waiting period (Oliva et al., 2015). Variation in waiting period is also found in studies with other species, including humans, for example one study used a 30 min waiting period (Woolley et al., 2014), while another used a waiting period that ranged from 45 to 90 min (Guastella et al., 2009). More research is needed in both of these areas to determine if specific populations or methods may lead to greater or more consistent affects of OT on behavior than others.

In addition, while some studies involving nasal OT administration in dogs have also used double-blind methodologies (Hernádi et al., 2015; Kis et al., 2015; Oliva et al., 2015, 2016; Kovács et al., 2016) as was the case in the current study, other studies have used a single-blind procedure in which either the owner or coders were not aware of which treatment was given at each session or to each subject, but where the experimenters conducting the study may have known which treatment was administered at each session (Romero et al., 2014; Nagasawa et al., 2015). Future research should consider to what degree experimenter knowledge or bias could influence the behavior of dogs or their owners in studies of this type.

Finally, it is worth noting that these findings are similar to findings from research with human subjects, where OT has been shown to have nuanced effects-increasing prosocial behavior in some contexts, while yielding negative results or leading to antisocial behavior in other contexts. For a review, see (Bartz, 2016). As a result, applications of OT in applied contexts, may be limited or minimally require further investigation targeting subpopulations experiencing specific behavior problems or disorders, as the effect may be larger in these populations compared to the general population.

AUTHOR CONTRIBUTIONS

LT, GR, SS, and MU designed the study. LT collected the data. LT and MU performed data analyses and interpreted the data. LT and MU wrote the first draft of the manuscript. LT, GR, SS, and MU revised the manuscript.

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Nasal Oxytocin Treatment Biases Dogs' Visual Attention and Emotional Response toward Positive Human Facial Expressions

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The neuropeptide oxytocin plays a critical role in social behavior and emotion regulation in mammals. The aim of this study was to explore how nasal oxytocin administration affects gazing behavior during emotional perception in domestic dogs. Looking patterns of dogs, as a measure of voluntary attention, were recorded during the viewing of human facial expression photographs. The pupil diameters of dogs were also measured as a physiological index of emotional arousal. In a placebo-controlled within-subjects experimental design, 43 dogs, after having received either oxytocin or placebo (saline) nasal spray treatment, were presented with pictures of unfamiliar male human faces displaying either a happy or an angry expression. We found that, depending on the facial expression, the dogs' gaze patterns were affected selectively by oxytocin treatment. After receiving oxytocin, dogs fixated less often on the eye regions of angry faces and revisited (glanced back at) more often the eye regions of smiling (happy) faces than after the placebo treatment. Furthermore, following the oxytocin treatment dogs fixated and revisited the eyes of happy faces significantly more often than the eyes of angry faces. The analysis of dogs' pupil diameters during viewing of human facial expressions indicated that oxytocin may also have a modulatory effect on dogs' emotional arousal. While subjects' pupil sizes were significantly larger when viewing angry faces than happy faces in the control (placebo treatment) condition, oxytocin treatment not only eliminated this effect but caused an opposite pupil response. Overall, these findings suggest that nasal oxytocin administration selectively changes the allocation of attention and emotional arousal in domestic dogs. Oxytocin has the potential to decrease vigilance toward threatening social stimuli and increase the salience of positive social stimuli thus making eye gaze of friendly human faces more salient for dogs. Our study provides further support for the role of the oxytocinergic system in the social perception abilities of domestic dogs. We propose that oxytocin modulates fundamental emotional processing in dogs through a mechanism that may facilitate communication between humans and dogs.

Keywords: domestic dog, nasal oxytocin, facial expressions, eye movements, pupil diameter, emotional arousal

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INTRODUCTION

The hypothalamic neuropeptide oxytocin plays a significant role in the regulation of a variety of social behaviors in both humans and other mammals. Oxytocin is known especially as an "affection hormone" facilitating parental care and pair-bonding, but it can also affect other social behaviors, such as social approach, trust, cooperation and empathy (reviewed in Campbell, 2010; Bartz et al., 2011; Romero et al., 2016; Shamay-Tsoory and Abu-Akel, 2016). The influence of oxytocin on complex social behavior is likely based on its modulating effects on rudimentary social processing, as the perception of social cues and encoding emotional information (Bartz et al., 2011; Ellenbogen et al., 2012). It has been proposed that this neuropeptide enhances the salience of cues important for social interaction and can additionally reduce withdrawal behaviors in the presence of socially aversive cues (Bartz et al., 2011; Shamay-Tsoory and Abu-Akel, 2016). To understand more broadly how the oxytocinergic system mediates pro-social behavior and emotions, it is necessary to investigate the effects of oxytocin on basic social perception in different species (Parr et al., 2013; Kovács et al., 2016a).

Domestic dogs (*Canis familiaris*) may be ideal subjects for studying the behavioral phenomena related to oxytocin because they resemble humans more than any other animal with regard to those aspects of social-cognitive functioning (e.g., attachment to their human partners – Topál et al., 2009), which have been linked to the central release of oxytocin. For example, endogenous oxytocin levels rise both in humans and dogs after positive human–dog interactions (Odendaal and Meintjes, 2003; Miller et al., 2009; Nagasawa et al., 2009, 2015; Handlin et al., 2011, 2012; Mitsui et al., 2011; Rehn et al., 2014) and correlate with the quality of dog–owner relationship (Handlin et al., 2012). Interestingly, polymorphisms in the oxytocin receptor gene are related to contact seeking and amicability toward humans (Kis et al., 2014) suggesting that oxytocin may contribute to the genetic background of socio-cognitive behavior in dogs (Bence et al., 2013). The oxytocin-facilitated communication may have supported the development of interspecies relations between humans and dogs through the domestication of dogs (MacLean and Hare, 2015; Nagasawa et al., 2015).

Increasing evidence suggests that the intranasal administration of oxytocin underpins a valid approach to study mechanisms underlying social behavior and cognition in dogs (Kis et al., 2017). The inhaled oxytocin is able to cross the blood–brain barrier and thus has the potential to modulate the behavior of dogs by directly affecting the brain (Romero et al., 2014). Importantly, however, oxytocin probably exerts its effects through multiple mechanisms, such as enhancing social motivation, increasing the salience of social cues and reducing social anxiety (Bartz et al., 2011). Intranasal oxytocin treatment has been found to promote dogs' affiliation toward both humans and conspecifics (Romero et al., 2014), to facilitate playful interactions between dogs (Romero et al., 2015), to increase eye contact in dog–human interactions (Nagasawa et al., 2015; Oliva et al., 2015; Kovács et al., 2016b) and to improve the reading of human body language (Oliva et al., 2015). Oxytocin biases dogs' behavior toward positive expectations (Kis et al., 2015) and moderates dogs' behavior both in affiliative (Romero et al., 2014; Nagasawa et al., 2015) and threatening interactions (Hernádi et al., 2015; Kovács et al., 2016b). The investigations, so far, have focused mainly on how oxytocin affects the dog's behavior. However, it is likely that oxytocin regulates dogs' socio-cognitive functions already at the level of visual perception (Kovács et al., 2016a).

In primates, oxytocin alters the interconnection patterns of those brain regions involved in attention, perception and emotion regulation (Kirsch et al., 2005; Gamer et al., 2010). Intranasal oxytocin administration improves the recognition and memorization of facial emotional expressions (e.g., Savaskan et al., 2008; Lischke et al., 2012, for a review see Shahrestani et al., 2013) and increases gazing at faces and eyes (in humans: Guastella et al., 2008a; Domes et al., 2012; in monkeys: Ebitz et al., 2013; Dal Monte et al., 2014). Typically, intranasal oxytocin suppresses vigilance to threatening social cues (in humans: Kirsch et al., 2005; Di Simplicio et al., 2009; Kim et al., 2014; in monkeys: Ebitz et al., 2013; Parr et al., 2013) but also improves visual processing of socially rewarding stimuli such as smiling faces (Guastella et al., 2008b; Domes et al., 2012, 2013). Dogs, to some extent, view and process faces like primates (Guo et al., 2009; Törnqvist et al., 2013; Somppi et al., 2014, 2016; Dilks et al., 2015; Cuaya et al., 2016). Dogs can distinguish human facial expressions from pictures and modulate their reactions in accordance with the emotional information in them (Nagasawa et al., 2011; Racca et al., 2012; Albuquerque et al., 2016; Barber et al., 2016; Somppi et al., 2016). However, there are no studies to date explicitly focusing on the effects of intranasal oxytocin on face perception in dogs.

Recent evidence suggests that during visual inspection of emotionally arousing events, pupils dilate in response to sympathetic nervous system activation (e.g., Bradley et al., 2008). Hence, pupil diameter can be used as a physiological index of emotional arousal (see Laeng et al., 2012 for a review). Oxytocin can promote social information gathering by affecting subjective emotional states and modulating pupil dilation (Ebitz et al., 2013; Leknes et al., 2013; Prehn et al., 2013) because the oxytocinergic system interacts with brain mechanisms regulating motivation, emotional arousal and attentional processes (Shamay-Tsoory and Abu-Akel, 2016). Examination of pupil dilation, therefore, may give a deeper perspective for the interpretation of gazing behavior.

It is increasingly accepted that oxytocin selectively amplifies social approach and inhibits social vigilance in both humans and non-human animals (e.g., Guastella et al., 2008a; Domes et al., 2012; Ebitz et al., 2013; Theodoridou et al., 2013; Romero et al., 2016), and in this study, we aimed to assess the validity of this assumption in the case of domestic dogs. In a placebo-controlled, within-subjects design, dogs after having received either oxytocin or a placebo (saline) treatment were presented with pictures of unfamiliar male human faces displaying either positive or negative emotional expressions. The voluntary attention (eye gaze patterns) and the indicator of sympathetic arousal state (changes in pupil dilation) were recorded by an eye tracking device. We hypothesized that

intranasal oxytocin would affect the rudimentary attentional and affective processes in dogs which manifests itself in: (i) increased gaze toward the eye region of human faces, especially during the viewing of positive (smiling) faces (ii) decreased vigilance when presented with threatening facial expressions and, (iii) changes in emotional arousal as reflected in changes in pupil diameter.

MATERIALS AND METHODS

The experiments were conducted at the Veterinary Faculty of the University of Helsinki from August to October 2012. The oxytocin treatment was licensed by the Finnish Medicines Agency, Fimea (vetkl-nro05/2012). The procedures were approved by the Ethical Committee for the Use of Animals in experiments at the University of Helsinki (minutes 9/2012) and all dog owners completed an informed written consent to participate in the study.

Animals and Pre-training

A total of 46 dogs were recruited for the study, but three dogs were not able to complete the final tests (because of illness, $n = 1$; nervousness, $n = 1$; unsuccessful calibration, $n = 1$). The final experimental group included eight 6-years-old purpose bred beagles housed in a group kennel (6 castrated males, 2 sterilized females) and 35 privately owned 1- to 10-years-old pet dogs (mean ± *SD*: 6.5 ± 2.2 years; 23 intact females, 7 sterilized females, 3 intact males and 2 castrated males). Pet dogs represented 16 different breeds and mongrels (**Table 1**). The average weight of the dogs was 20.5 kg (*SD* = 9.1, range: 3.5–40 kg; **Table 1**). None of the dogs were on medication during the study and the female dogs were in the anestrus phase. Throughout the whole study period, the daily routines of the dogs were kept similar to that in their regular life. Pet dogs were fed one to two times and taken outdoors three to five times daily. Kennel dogs lived in the kennel facilities at the University of Helsinki. They were fed twice a day, and once a day they were released into an exercise enclosure for 2 h.

Prior to conducting the experiments, the dogs were clicker trained to lie still and lean their chins on a specially designed rack, as described in Somppi et al. (2012). The criterion for passing the training period was that the dogs took the pre-trained position without being commanded to do so and remained in that position for at least 30 s while their owners and the experimenters were positioned behind an opaque barrier. During the training, the dogs were not encouraged to fix their eyes on a monitor or images and they were not restrained or forced to perform the task. Pet dogs were trained by their owners and kennel dogs were trained by the first and second authors. The owners with their dogs visited the experimental room approximately 2–3 (range 1–10) times prior to the actual experiment until they were fully familiar with the experimental environment and instrumentation setup. Most of the dogs visited the experimental room 2–3 times, but some dogs needed more visits because they had difficulties concentrating on the task.

Eye Tracking System

The binocular eye movements of the dogs were recorded with an infrared-based contact free eye tracking system (iView X^{TM} RED250, SensoMotoric Instruments GmbH, Germany) which was integrated below a 22" LCD-monitor (1680 pixels × 1050 pixels) placed at 0.50–0.75 m (mean ± *SD*: 0.69 ± 0.05 m) distance from the dogs' eyes.

During the tests, the chin rest, the monitor and the eye tracker were placed in a cabin constructed of plywood and cardboard. The cabin had one open wall, three solid walls, and a roof ($h = 1.5$ m, $w = 0.9$ m, $d = 0.9$ m). Two additional fluorescent lamps were placed in front and above the monitor. The average illumination intensity measured on the sides of the dogs' heads was 11000 (*SD* = 2300 lx: range 4200–13400 lx). These differences in the photometric values (luminous intensity) were due to the fact that different sized dogs were placed at different distances from the monitor and light sources.

The eye tracker was calibrated and the calibration accuracy was checked twice using a five-point procedure (Somppi et al., 2012, 2014). The calibrated area was a visual field of 40.5° × 24.4° from the average distance of 0.70 m (equal to the size of the monitor). The criterion for an adequate calibration was achieved if the dogs' eye fixations hit within a 1° radius off the central calibration point and at least three of four distal points. The fixation was encoded if the minimum fixation duration was 75 ms and the maximum dispersion value $D = 250$ pixels (Somppi et al., 2012). On average, five calibration trials were required for each dog to achieve an adequate calibration (*SD*: 4.2, range: 1–27). For the final calibrations, the average accuracy was 96% (*SD*: 8%, range: 70–100%), calculated as a proportion of fixated points out of five calibration points over two calibration checks of all dogs. To maintain vigilance and motivation of dogs, the calibration and image viewing were carried out on separate days. According to our previous findings, the stored calibration can be used repeatedly during separate days (Somppi et al., 2012). Illumination and the position of the chin rest, monitor, and eye tracker were kept the same during the calibration and the image viewing. The accuracy of the central point fixations was re-assessed visually immediately before the image viewing.

Stimuli

A total of eight digital color facial photographs from the Radboud Faces Database (RaFD, Langner et al., 2010) were used (see an example in **Figure 1**). We selected four male faces with happy (smiling, teeth visible) and angry (teeth not visible) expressions. The size of the image area was an average 800 pixels × 580 pixels (height 800 pixels, width range 500–620 pixels depending on the width of the faces). The stimuli were presented on a black background using Experiment Center 3.0^{TM} software (SensoMotoric Instruments GmbH, Berlin, Germany).

During one testing session, one happy face and one angry face ('a stimulus set') of the same male person, unfamiliar for the participating dogs, were presented consecutively on the left or on the right side of the screen in altering orders, 7 s per image. During the face presentation, a 3-s-long neutral sound is played. Before each face presentation a 4-s-long 'attractor,' a swinging pendulum and tick-tock sound, was presented to the

dogs in order to get the dogs' attention. The stimulus sets were pseudo-randomized between the dogs.

Experimental Procedure

In a balanced within-subject experimental setup the dogs were given oxytocin nasal spray (OXT; Syntocinon® 40 IU/ml, Novartis, Australia) in one testing session and a placebo saline nasal spray (PLB; Naso NaCl 0.9%, Ratiopharm, Germany) in other testing session, 7–25 days (mean interval ± *SD*: 9.7 ± 4 days) between them. Different stimulus sets were presented in the first and second testing sessions.

The experimental setup consisted of four phases. First, the dogs were brought to the test room for warm-up trials, in which they viewed a series of images of landscapes and wild animals while the experimenter rewarded them randomly after one to five images. The warm-up phase lasted for 5–10 min, depending on the dog's behavior. After the warm-up trials, the dogs were intranasally administered 12 IU PLB or

TABLE 1 | Breeds, ages, sex and weights of the subjects.

Breed	Age (years)	Sex	Weight (kg)	Group
Australian kelpie	6	Sterilized female	14	Pet dog
Beauce Shepherd	3.5	Female	38	Pet dog
Beauce Shepherd	4.5	Sterilized female	30	Pet dog
Beauce Shepherd	5	Female	36	Pet dog
Border collie	1	Female	15	Pet dog
Border collie	1.5	Male	20	Pet dog
Border collie	2.5	Female	19	Pet dog
Border collie	4	Female	13	Pet dog
Border collie	6.5	Female	15	Pet dog
Border collie	8	Castrated male	18	Pet dog
Border collie	10	Sterilized female	18.5	Pet dog
Bouvier	6.5	Female	30	Pet dog
Boxer	1.5	Female	27	Pet dog
Boxer	5.5	Female	26	Pet dog
German Shepherd	3	Female	27	Pet dog
German Shepherd	5	Female	30	Pet dog
German Shepherd	6	Female	27	Pet dog
Hovawart	3.5	Female	30	Pet dog
Hovawart	4.5	Sterilized female	27.5	Pet dog
Hovawart	7	Sterilized female	30	Pet dog
Lagotto Romagnolo	5	Male	15	Pet dog
Miniature schnauzer	3.5	Sterilized female	7	Pet dog
Mixed breed (half rottweiler)	2.5	Female	30	Pet dog
Mixed breed (half Labrador)	3.5	Female	32	Pet dog
Mixed breed (unknown breeds)	8.5	Sterilized female	20	Pet dog
Rottweiler	2.5	Female	40	Pet dog
Rough Collie	3.5	Female	21	Pet dog
Rough Collie	4	Female	20	Pet dog
Smooth collie	8	Male	24.5	Pet dog
Swedish Vallhund	6.5	Female	11	Pet dog
Toy poodle	5.5	Female	3.5	Pet dog
Toy poodle	2	Female	4.5	Pet dog
Welsh corgi cardigan	5	Female	16	Pet dog
Welsh corgi cardigan	6.5	Female	15	Pet dog
Welsh corgi cardigan	8	Castrated male	18	Pet dog
Beagle	6	Castrated male	12	Kennel dog
Beagle	6	Castrated male	11.5	Kennel dog
Beagle	6	Castrated male	16.5	Kennel dog
Beagle	6	Castrated male	12	kennel dog
Beagle	6	Castrated male	11.5	Kennel dog
Beagle	6	Castrated male	14.5	Kennel dog
Beagle	6	Sterilized female	12	Kennel dog
Beagle	6	Sterilized female	16	Kennel dog

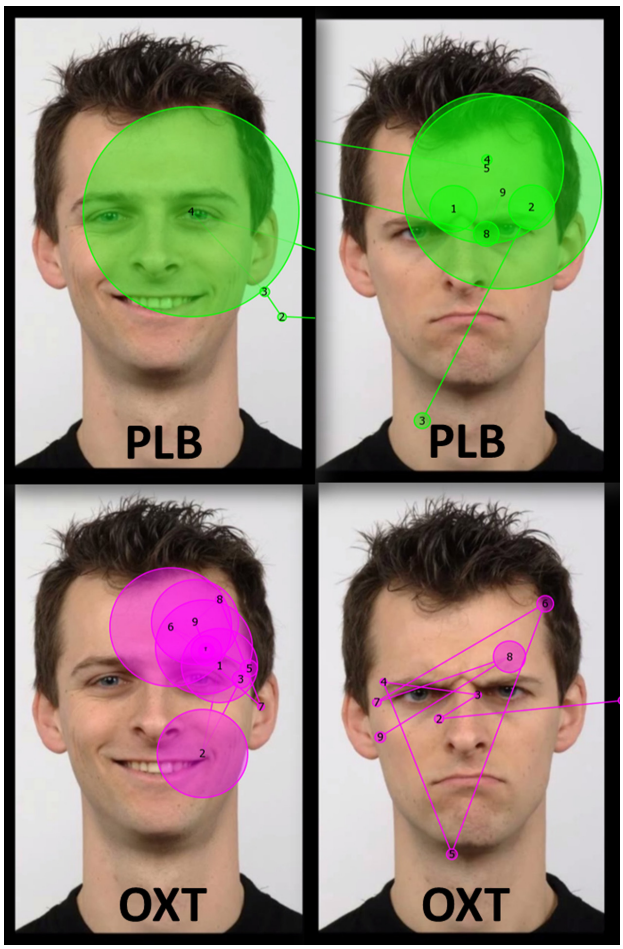

FIGURE 1 | Examples of the stimuli images (from Radboud faces database; Langner et al., 2010) and scan paths of one dog during 7000 ms viewing after the placebo (PLB) and the oxytocin treatments (OXT). The dotted white rectangular area in the top left image represents the analyzed area of interest. Circles represent the gaze fixations of one dog and the lines trace the path that the gaze traveled across the image. Circle size is proportional to the fixation time. The numbers in the circles represent the order of the fixations.

OXT in a different room (3 puffs in alternating nostrils; the sequence for half of the dogs was right-left-right while for the others left-right-left). Only two puffs were given (one in each nostril) for dogs that weighed less than 5 kg. After the treatment, subjects waited in their owners' car or in a separate room without social stimulation. Dog owners were instructed to avoid social contact with their dogs and to avoid getting the dog excited (i.e., no stroking, playing or training during the waiting time). Finally, the dog was brought back into the testing room. It settled down in the pre-trained position, and eye tracking data recording was started after the eye tracker had detected the dog's eye properly. The dog was rewarded after the stimulus presentation regardless of its behavior. If the dog changed its predetermined position during the stimulus presentation (i.e., lifted its head from the chin rest), it was not re-positioned by the experimenter/owner. The average time that elapsed between the nasal spray administration and the presentation of stimulus set was 46 min (*SD*: 2 min, range 44–61 min).

The data from original testing session of six dogs were lost due to eye tracker software crashes ($n = 2$) or the dogs lifting their heads from the chin rest ($n = 4$). These testing sessions were redone 23–33 days later with the same treatment as in the original test (2 dogs PLB, 4 dogs OXT), but with a different stimulus set.

Data Analysis

Eye movement data were obtained from a total of 169 images (PLB Happy $n = 43$; PLB Angry $n = 40$; OXT Angry $n = 43$; OXT Happy $n = 43$). Data from three dogs in the PLB/Angry image condition were insufficient for analysis because of technical problems or due to dogs' head movements.

Different aspects of dogs' gazing behavior toward a rectangular eye region (area of interest – see **Figure 1**), which was one-third the size of the entire face region, were recorded. Two gaze variables derived from eye movement data were considered for analyses: fixation count (the sum of all fixations that hit on the eye region during the entire stimulus presentation time) and revisits (how many times the dog glances back at the eye region). In addition, the average pupil size (pupil diameter in mm; recorded during the fixations targeted at the monitor) was calculated using the manufacturer's built-in algorithm. (BeGaze 3.0^{TM} software, SensoMotoric Instruments GmbH, Berlin, Germany).

The effects of the treatment (OXT or PLB) on the gazing behavior (number of fixations, revisits) and pupil diameter were analyzed with generalized linear mixed models (GENLINMIXED, SPSS 24.0, IBM, New York, NY, United States) using normal distribution and identity link function with first-order autoregressive covariance structure (AR1). The model fitting was based on the evaluation of Akaike Information Criteria and Pearson residual observed-by-predicted plots. The fixed factors included in the final model were treatments (OXT or PLB), facial expression (Happy or Angry) and the interaction between the facial expression and the treatment. The subject (i.e., tested dog) and interaction between the treatment and the testing day were included as random effects, latter to take into account the cross-over design of the experiment. The testing day and sequence of the image were included as repeated measures. In the analysis for the pupil diameter, total number of fixations was used as a covariate to take into account the possible dependency between gaze variables and pupil dilation (Aspinall et al., 2014; Ebitz et al., 2014). The sex and dog population (pet or kennel dog) were tested both as fixed and random effects, but discarded from the final analysis because they were statistically significant ($p > 0.05$) as fixed effects, and redundant for the models as random effects. The *post hoc* tests with sequential Bonferroni adjustment were included in the GENLINMIXED procedure. The gazing variables were log-transformed for analyzes to acquire better model fitting, and they are reported as log-transformed values. All results are reported as estimated means with their standard errors (SE) using the significance level $p < 0.05$.

RESULTS

Number of Fixations

The overall mean number of fixations on the eye region of the human faces did not differ significantly between oxytocin and placebo treatments ($F = 0.499$, $df = 165$, $p = 0.481$) or between happy and angry facial expressions ($F = 0.555$, $df = 165$, $p = 0.457$). However, the interaction between the treatment (OXT/PLB) and facial expressions (Happy/Angry) was statistically significant ($F = 10.182$, $df = 165$, $p = 0.002$). *Post hoc* tests revealed that dogs fixated at the eye region of angry faces less frequently after oxytocin than after the placebo treatment ($t = -2.721$, $df = 165$, $p = 0.007$, **Figure 2A**) and tend to fixate at the eye region of happy faces more after oxytocin than after the placebo treatment, although this was not significant ($t = 1.767$, $df = 165$, $p = 0.079$, **Figure 2A**). After the placebo treatment dogs tended to fixate at the eyes of the angry faces more than eyes of the happy faces, although this was not significant ($t = 1.711$, $df = 165$, $p = 0.089$, **Figure 2A**). Oxytocin treatment instead induced more fixations toward the eye region of happy faces compared to the corresponding region of angry faces ($t = 2.813$, $df = 165$, $p = 0.006$, **Figure 2A**).

Revisits

The overall mean number of revisits made to the eye regions of the human faces did not differ significantly between oxytocin and placebo treatments ($F = 0.657$, $df = 116$, $p = 0.419$) or between happy and angry expressions ($F = 1.560$, $df = 116$, $p = 0.214$). However, the interaction between the treatment (OXT/PLB) and facial expressions (Happy/Angry) was statistically significant ($F = 8.908$, $df = 116$, $p = 0.003$). *Post hoc* tests revealed that dogs revisited on the eye regions of happy faces more frequently (i.e., glanced back to eyes) after oxytocin treatment than after placebo treatment ($t = 2.854$, $df = 116$, $p = 0.005$). In addition, after the intranasal administration of oxytocin, the eye regions of happy faces were revisited more often than those of the angry faces ($t = 2.909$, $df = 116$, $p = 0.004$, **Figure 2B**).

Pupil Diameter

Similarly, to 'Number of fixations' and 'Revisits', the overall mean pupil diameter of dogs while viewing human emotional faces did not differ significantly between oxytocin and placebo treatments ($F = 0.107$, $df = 163$, $P = 0.744$) or between happy and angry expressions ($F = 0.142$, $df = 163$, $p = 0.706$). However, the interaction between treatment (OXT/PLB) and facial expressions (Happy/Angry) was statistically significant ($F = 9.998$, $df = 163$, $p = 0.002$). *Post hoc* pairwise comparisons revealed that after the placebo treatment dogs had larger pupils while viewing angry as compared to happy faces ($t = 2.488$, $df = 163$, $p = 0.014$), but oxytocin treatment caused the opposite pupil response ($t = -2.95$, $df = 163$, $p = 0.038$, **Figure 2C**).

DISCUSSION

Intranasal administration of oxytocin modulates different aspects of social cognitive functioning in social mammals, including humans (Campbell, 2010; Romero et al., 2016). The behavioral effects of oxytocin are also increasingly evidenced in domestic dogs (Romero et al., 2014; Hernádi et al., 2015; Kis et al., 2015; Nagasawa et al., 2015; Oliva et al., 2015), an important model animal for studying the evolutionary emergence of human social cognition (Topál et al., 2009). Based on these results, the purpose of the present study was twofold: (i) we explored how oxytocin influences the allocation of visual attention in dogs when viewing emotionally expressive human faces and, (ii) we also investigated, for the first time in domestic dogs, the effects of oxytocin treatment on pupil size modulations related to attention allocation.

The results show that intranasal oxytocin treatment has a significant effect on both attentional focus and emotional arousal confirming that neuropeptide oxytocin mediates social perception and emotional states in dogs. Intranasal administration of oxytocin selectively attenuated dogs' attention to the eye region of angry faces and reduced emotional arousal, reflected in their pupil sizes, when viewing negative facial expressions. In contrast oxytocin increased both emotional arousal and the allocation of attention toward the eye region of emotionally positive (smiling) faces. Thus, oxytocin treated dogs made fewer fixations on the eyes of angry faces and more revisits to the eyes of happy faces compared with the placebo treatment. Moreover, after oxytocin treatment dogs fixated and revisited more frequently the eye region of happy than angry faces. However, oxytocin did not increase the dogs' gazing toward human eyes at general level, which is in line with the results of human and monkey studies showing that oxytocin administration does not generally enhance approaching social stimuli of any kind, but specifically increases the salience of particular social stimuli (Domes et al., 2012; Theodoridou et al., 2013; Dal Monte et al., 2014).

It seems that oxytocin treatment has the potential to bias dogs' attention away from threat and toward smiling faces due the social anxiety relieving and pro-social behavior promoting properties of the neurohormone. This is in line with earlier reports suggesting that oxytocin promotes social interactions via focusing attention on social signals of potentially approaching friendly encounters and reducing social contact with potentially threatening encounters (Domes et al., 2012). Dogs, like many other animals, may consider direct eye contact threatening, even in artificial contexts (Somppi et al., 2016). Threatening stimuli typically evokes prolonged attention, which is the consequence of delayed disengagement of attention from threat (Fox et al., 2002; Belopolsky et al., 2011). The fear-attenuating effect of oxytocin, however, may reduce this response and allows more flexible processing of positive stimuli (Kirsch et al., 2005; Guastella et al., 2008a,b; Bartz et al., 2011; Ebitz et al., 2013; Parr et al., 2013; Theodoridou et al., 2013; Kim et al., 2014). Furthermore, oxytocin increases trustworthy and approach behavior (Bartz et al., 2011; Romero et al., 2014, 2016; Shamay-Tsoory and Abu-Akel, 2016), which may have increased the dogs' willingness to look at the smiling eyes as well.

Previous studies have shown that oxytocin increases attention to the eye region of conspecific faces (Guastella et al., 2008a; Andari et al., 2010; Ebitz et al., 2013; Dal Monte et al., 2014), in

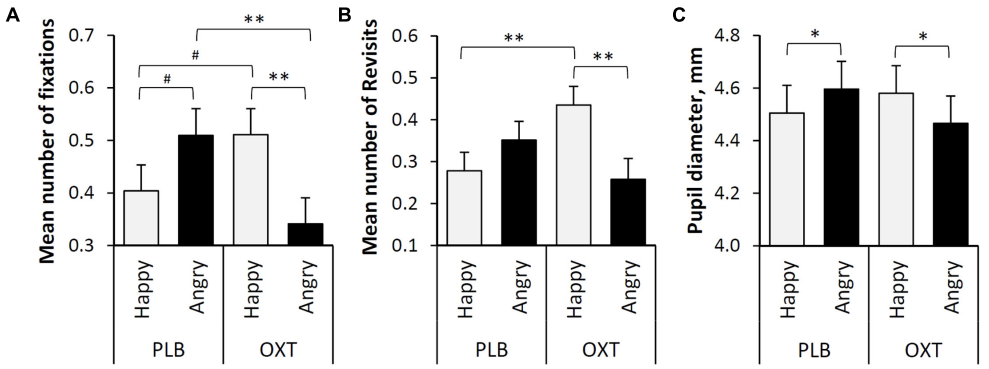

FIGURE 2 | (A) The mean number of fixations targeted to the eye regions of happy and angry faces (Lg10 transformed values). **(B)** The mean number of revisits targeted to the eye regions of happy and angry faces (Lg10 transformed values). **(C)** The mean pupil diameter (mm) during the presentation of two facial expressions (Happy and Angry) and two treatments (PLB and OXT). Error bars represent *SE*. Asterisks indicate significant differences (**$p < 0.01$, *$p < 0.05$) and a statistical trend ($^{\#}p < 0.09$).

domestic dogs, however, this phenomenon seems to go beyond the boundaries between species. The present study provides the first evidence that oxytocin can affect the visual processing of heterospecific emotional facial expressions, which is consistent with the observations showing that exogenous oxytocin promotes dogs' social behaviors toward human partners. Oxytocin facilitates interspecies attachment by enhancing dogs' motivation to approach and affiliate with humans (Romero et al., 2014; Nagasawa et al., 2015). Recently, Nagasawa et al. (2015) have found that after intranasal oxytocin administration dogs gazed more toward their owners' faces, which consequently facilitated owners' affiliative behavior toward their dogs, suggesting that the mutual oxytocin-mediated gaze between dog and owner promotes human–dog-bonding. Oxytocin also improves dogs' ability to interpret the social cues of humans other than their owners, probably because oxytocin helps dogs to tolerate shifting their gaze to the human eyes (Oliva et al., 2015; Kovács et al., 2016b). Oxytocin increased looking back at the eyes of friendly faces also in our artificial setup suggesting that oxytocin has a role in maintaining prolonged eye contact at a very rudimentary level.

The enhanced eye gazing during viewing of emotional faces is likely related to dogs' emotional state. As an indicator of emotional arousal (Bradley et al., 2008; Machado et al., 2011), we measured the pupil diameters of the dogs and found that oxytocin-treated dogs had larger pupils while viewing happy faces while placebo-treated dogs had larger pupils while viewing angry faces. In humans, greater pupil dilation is associated with both visual attention and increased emotional arousal (Bradley et al., 2008; Gredebäck et al., 2012; Kret et al., 2013). It is noteworthy that oxytocin can both increase positive arousal and attenuate negative arousal related to fear reactions (Bradley et al., 2008; Kret et al., 2013; Ellenbogen et al., 2014) and pupils can dilate due to either pleasant or unpleasant emotional states (Bradley et al., 2008; van Steenbergen et al., 2011), as we found in dogs.

Altogether the link between gazing patterns and pupil dilation in dogs provides further support for the notion that fluctuations in pupil diameter may reflect allocation of attentional resources (Leknes et al., 2013). Oxytocin can directly affect the effectiveness of social information gathering by selectively modulating pupil dilation (Leknes et al., 2013; Prehn et al., 2013; Ebitz et al., 2014). Pupil dilation-linked arousal may adjust the balance of processing resources in those situations in which both goal-relevant stimuli and conflicting but biologically important objects compete for recruiting the subject's attention (Ebitz et al., 2014). Attention is typically focused on stimuli of great biological and social significance, such as threatening stimuli if a subject is in a negative arousal state. Oxytocin may control pupil dilation through attenuation of negative arousal thus biasing attention toward other relevant targets (Prehn et al., 2013; Ebitz et al., 2014). Although the happy and angry faces in our study were not actually competing for the dogs' attention (i.e., stimuli were presented consecutively), the changes in the dogs' focus of attention and pupil dilation were probably based on the aforementioned mechanism. According to a recent theoretical framework, oxytocin has a major role in regulation of social attention through its interaction with the dopaminergic system and amygdala, both brain regions responsible for emotional arousal, rewarding system and detection of socially relevant stimuli (Shamay-Tsoory and Abu-Akel, 2016). We suggest that after receiving placebo treatment angry faces were more relevant and salient for dogs because seeing the directly staring threatening face induced negative emotional arousal in them. Conversely, oxytocin treatment reduced this social anxiety response and probably also facilitated the positive arousal evoked by the viewing of smiling faces. It should be noted that one factor which may have affected the results is the general emotional state of dogs, which was positive as dogs were highly motivated to participate in the task due to reward based positive operant conditioning. Importantly, however, eye movements and pupil data alone are insufficient to draw firm conclusions about dog's emotional state or oxytocin's role in attention and emotion regulation in dogs.

Further studies are needed to determine whether the results we report are typical only for domestic dogs due their human-tunedness in their communicative skills, or, whether a similar phenomenon exists in other mammalian species.

Moreover, in our study dogs were presented with unfamiliar human faces, thus, at present we do not know whether familiarity would modulate the observed effect of oxytocin on emotional face processing. Recent findings highlight the potential role of familiarity: familiar faces attract dogs' attention more than unfamiliar ones (Somppi et al., 2014) and the effect of oxytocin may be more pronounced toward socially more relevant partners (Hernádi et al., 2015). In further studies, therefore, both stimuli of familiar/unfamiliar conspecifics, hetero-specifics as well as inanimate object should also be tested to clarify the effects of oxytocin on dogs' gazing behavior. We may assume that oxytocin regulates the viewing of own-species faces differently, because the effects of oxytocin are highly context dependent (Shamay-Tsoory and Abu-Akel, 2016). Besides, the emotional signals of other-species and own-species may elicit differential viewing strategies in dogs (Somppi et al., 2016).

In future studies, the effects of the dogs' breed, sex and personality traits should also be taken into account, as the impact of oxytocin are not uniform across all individuals (Kovács et al., 2016b; Shamay-Tsoory and Abu-Akel, 2016). Previous studies had also reported both sex- and breed-specific effects of intranasal oxytocin: e.g., female dogs and dog breeds selected for enhanced cooperative abilities were found to be more susceptible to the effects of intranasal oxytocin (Kis et al., 2014, see also Nagasawa et al., 2015; Kovács et al., 2016a,b). The subjects of our eye tracking experiment, however, were not appropriate for investigating sex and breed effects: the vast majority of the dogs were females (32/43) and most of the participant dogs represent herding dog breeds (22/43),other breeds selected for cooperative work (11/43, including two mixed breed dogs with known pedigree). We should note, however, that a potential confound to the effect of oxytocin in this (and many previous) research is that breed and sex as well as age, reproductive viability, size, pre-training success and time between testing sessions varied among subjects. Further studies are needed to examine whether these differences have a real impact on the oxytocin-mediated effects of dogs' facial emotion processing or not.

CONCLUSION

Both gazing patterns and pupillary data underpin the links between oxytocinergic, attentional and emotional circuits in domestic dogs. Oxytocin administration selectively biases the emotional arousal and attention allocation in domestic dogs by suppressing vigilance toward threatening social stimuli and increasing the arousal inducing effect of smiling human faces and making the eye gaze of friendly humans more salient for dogs. Taken together, our study support the hypothesis that oxytocin modulates fundamental emotional processing in dogs through a mechanism that facilitates communication between humans and dogs.

AUTHOR CONTRIBUTIONS

Conceptualization: JT, SS, and HT. Methodology: SS, HT, JT, AK, and CK. Data collection: AK, SS, and HT. Data analyses: SS, LH, and HT. Contributed in writing: SS. Contributed in review and editing: LH, HT, OV, AK, CK, and JT.

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The Way Dogs (*Canis familiaris*) Look at Human Emotional Faces Is Modulated by Oxytocin. An Eye-Tracking Study

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Dogs have been shown to excel in reading human social cues, including facial cues. In the present study we used eye-tracking technology to further study dogs' face processing abilities. It was found that dogs discriminated between human facial regions in their spontaneous viewing pattern and looked most to the eye region independently of facial expression. Furthermore dogs played most attention to the first two images presented, afterwards their attention dramatically decreases; a finding that has methodological implications. Increasing evidence indicates that the oxytocin system is involved in dogs' human-directed social competence, thus as a next step we investigated the effects of oxytocin on processing of human facial emotions. It was found that oxytocin decreases dogs' looking to the human faces expressing angry emotional expression. More interestingly, however, after oxytocin pre-treatment dogs' preferential gaze toward the eye region when processing happy human facial expressions disappears. These results provide the first evidence that oxytocin is involved in the regulation of human face processing in dogs. The present study is one of the few empirical investigations that explore eye gaze patterns in naïve and untrained pet dogs using a non-invasive eye-tracking technique and thus offers unique but largely untapped method for studying social cognition in dogs.

Keywords: dog, eye-tracking, oxytocin, emotion, face processing

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INTRODUCTION

In human visual communication the face has a unique function, because it is the most reliable source of one's emotional or mental states and intentions (Todorov et al., 2008). The ability to recognize behavioral indicators of emotions in others plays a key role in the social organization of group-living species as it might help to predict others' subsequent behavior. The development of such skills can also be highly beneficial for those sociable domestic animals that live in mixed-species social systems and are commonly kept as companions (Nagasawa et al., 2011; Racca et al., 2012).

Dogs have long coexisted with humans, and have developed a uniquely human-tuned social competence, which, among others, make it possible for dogs to efficiently communicate with humans (for a review see Miklósi and Topál, 2013). Dogs are not only able to detect and recognize the human face (Racca et al., 2010), but also to connect facial expressions with probable outcomes (Nagasawa et al., 2011). Furthermore faces play an important role in how dogs recognize

their owners (Adachi et al., 2007; Marinelli et al., 2009). Dogs, similarly to adult humans, show left gaze bias only towards upright positioned human faces but not towards monkey or dog faces or objects (Guo et al., 2009) and they can also learn to discriminate between neutral and happy facial emotional expressions (Deputte and Doll, 2011; Nagasawa et al., 2011). Although this does not necessarily reflect emotion recognition ability in dogs, the finding that they look longer at their owners' happy vs. sad faces may indicate that dogs are sensitive to human emotional states (Morisaki et al., 2009). Importantly, however, the neuromodulatory mechanisms involved in dogs' social-emotional receptivity are still largely unexplored.

Several studies have revealed that human socio-cognitive processing is influenced by the neurochemical state of the central nervous system (Kirsch et al., 2005). One of the most prominent neuromodulators is oxytocin, a nine aminoacid long oligopeptide that is produced in the hypothalamus (Lee et al., 2009). Ample evidence suggests that oxytocin influences different aspects of human social behavior (Kosfeld et al., 2005; Buchheim et al., 2009; Heinrichs et al., 2009; Scheele et al., 2012) and it has also been shown to regulate social behavior in many nonhuman species (Lee et al., 2009). According to Guastella et al. (2008) after a single dose of intranasally administered oxytocin people look more to the eye region of human faces. Guastella et al. (2009) also suggest that oxytocin enhances the connection of facial expressions to emotional states. This notion is further confirmed by studies showing that intranasal oxytocin administration selectively increases the recognition ability of certain emotions in humans, although the results are contradictory. While some studies have found an effect regardless of the valence of emotional faces (Domes et al., 2007; Rimmele et al., 2009), in other cases oxytocin only had an effect regarding negative facial emotions such as fear (Fischer-Shofty et al., 2010), anger (Savaskan et al., 2008) and both anger or fear (Kis et al., 2013). The idea that oxytocin differentially modulates human visual attention towards positive or negative facial emotional expressions has been corroborated by an eye-tracking study (Domes et al., 2013) which found that intranasal oxytocin treatment increased gaze to the eye region in case of neutral and happy, but not angry dynamic faces.

The effects of oxytocin on dogs' social behavior are increasingly explored, and most of the findings support a role of the oxytocin system in dogs' human-like social skills (for recent reviews, see: Buttner, 2016; Kis et al., 2017). There are some general concerns about peripheral oxytocin measurements (McCullough et al., 2013), and some claims about dog-human co-evolution based on peripheral oxytocin measurements have been widely criticized (Kekecs et al., 2016). This is an ongoing debate, as some authors think that the role of oxytocin in the co-evolution of humans and domestic animals is clear (Herbeck et al., 2016), while others have a more critical attitude towards oxytocin research in dogs (Rault et al., 2017). The literature on the effect of intranasal oxytocin administration to dogs is less controversial, although not only "positive", e.g., increased ability to follow human pointing, (Oliva et al., 2015; Macchitella et al., 2017), social sensitivity (Kovács et al., 2016b), cognitive bias (Kis et al., 2015), but also "negative", e.g., less friendly reaction to a threatening owner (Hernádi et al., 2015) effects have been found. This is, however, consistent with human literature suggesting that oxytocin is not a magical "trust elixir" (Mikolajczak et al., 2010), and that despite increasing prosocial behaviors, it does not make people blind to negative social stimuli, but on the contrary in some cases it even increases the salience of negative social stimuli (Theodoridou et al., 2013).

Furthermore recent studies have proved that applying the eye tracking method to dogs is viable (Williams et al., 2011), and it might provide new insights into dogs' face processing and social-communication skills. It has been found Somppi et al. (2012) that dogs, without any task-specific pre-training, focus their attention on the informative regions of facial images, which support the notion that eye tracking technology offers promising possibilities for studying the effects of oxytocin on visual processing of human emotional expressions in the dog. Using the eye-tracking method it was also proven that dogs follow human gaze if it is preceded by communicative signals directed to them (Téglás et al., 2012). These three research groups that have so far conducted eye-tracking studies on dogs have used different methodological solutions (e.g., head-mounted vs. contact-free eye-tracking, family dogs vs. laboratory dogs, trained vs. untrained dogs), which all come with different advantages. Téglás et al. (2012) was able to collect data from a representative sample of untrained family dogs, Somppi et al. (2012) could achieve sustained attention and long fixation times with purpose-trained laboratory dogs, Williams et al. (2011) developed a method that promises application to real-life situations (as opposed to computer-screen images).

In the present study we capitalized on the eye tracking technology, and set out to address the question whether dogs' face processing, as measured by subjects' looking pattern, changes due to the oxytocin treatment and if these changes are specific to certain facial emotion expressions. In order to do so, first we assessed the most adequate presentation method in terms of number of stimuli, to allow dogs to maintain a focused attention. We assessed (Study I) the maximum number of stimuli that could be presented without risking that an order effect would overwrite any other effects of interest. Human faces were presented from both genders and with different emotional expressions in order to determine if these factors have a major effect on dogs' viewing patterns. Then we used eye-tracking to investigate the effects of a single dose of intranasal oxytocin on pet dogs' viewing patterns of emotional faces (Study II). We hypothesized that: (1) most looking times will be focused on informative regions (e.g., eyes and mouth) as in previous studies (Somppi et al., 2012); that (2) after oxytocin treatment angry faces will be more salient for dogs (Theodoridou et al., 2013) making them avert gaze from these images; and that (3) oxytocin will increase looking time to the eye region (as in humans, Guastella et al., 2008). Dogs' age, sex, training level and head shape were also considered as confounding variables.

STUDY I

Background

Previous studies investigating visual processing in dogs presented a very limited number of stimuli both in eye-tracking test (Somppi et al., 2012; Téglás et al., 2012) and in preferential looking (image projection) paradigms (Faragó et al., 2010; Racca et al., 2012; Péter et al., 2013), which raises concerns of pseudoreplication (Lazic, 2010), e.g., the effect found might be specific to those images only and might not generalize to other stimuli. In our first study we aimed to investigate dogs' visual attention span in a sequential image presentation task in order to determine the maximum number of stimuli that could be presented without a serious order effect (that would potentially mask other effects of interest). In order to do this we presented a sequence of six images of male and female faces expressing happy, angry and fearful emotions.

Methods

Subjects

Fifty-eight adult pet dogs (females/males: 30/28; mean age ± SD: 4.26 ± 3.07; from 25 different breeds and 16 mongrels) were recruited from the Family Dog Project Database built and maintained by Department of Ethology, Eötvös University. In order to be selected for this study the subject had to be naïve to the task, and older than a year. 27 dogs had to be excluded due to subjects' inattentiveness and/or their head shape (too long nose, lateral position of the eyes) that made the eye-tracker calibration impossible. The final sample consisted of 31 dogs (male/female: 15/16; mean age ± SD = 4.18 ± 2.76; from 15 different breeds and 8 mongrels).

Experimental Procedure

The experiments took place in a laboratory room (4 m × 4 m). The eye gaze data was collected with a Tobii X50 Eye Tracker (Stockholm, Sweden) at 50 Hz, that was the same temporal resolution used by a previous dog eye tracker study (Téglás et al., 2012). The eye tracker had 0.5–0.7 degree accuracy 30 × 16 × 20 cm freedom of head movement. The stimuli were presented on a 17-inch LCD monitor positioned behind the eye tracker.

When the owner and the dog arrived at the laboratory dogs were allowed to freely explore the room and to interact with the experimenter for approximately 5 min. During this time owners were informed in detail about the experimental procedure. Then we checked whether the dog's eyes could be captured by the Track Status viewer to determine if a subject had the potential to successfully pass the calibration. The experimenter placed a treat on top of the eye tracker and encouraged the dog to take the treat from there. Once the dog became familiar with the equipment the owner was asked to sit the dog in front of the eye tracker and hold the dog by placing both hands on its chest (**Figure 1**). Depending on the size of the dog the distance of the equipment from the dog varied (approx. 50–80 cm) and the angle was adjusted until the eye-tracker could register both of the dogs' eyes. During the calibration and stimulus presentation phase the owner did not interfere with the dog nor did he/she force it to watch the screen.

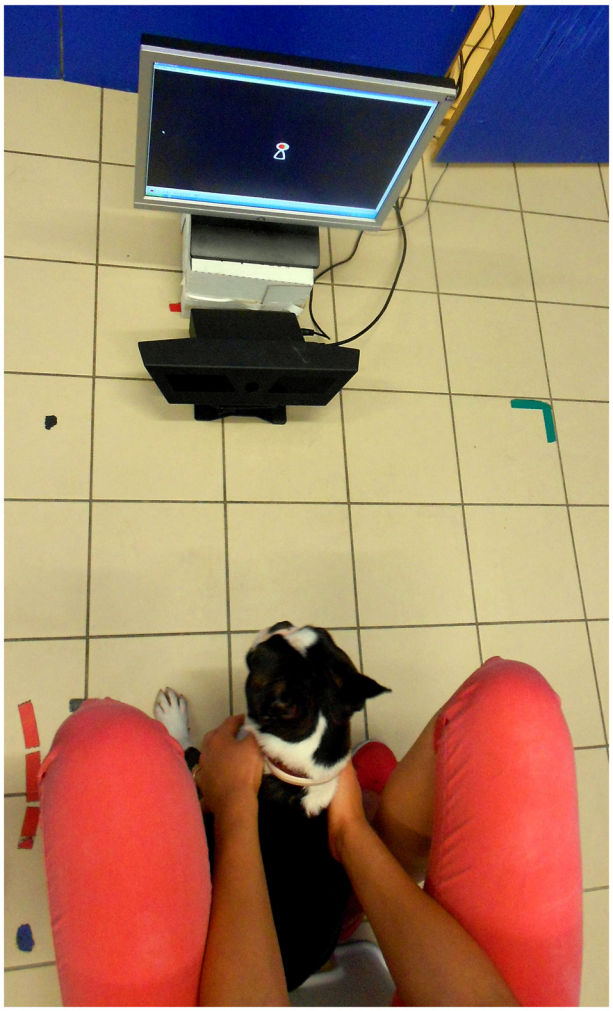

FIGURE 1 | The dog's position during stimuli presentation.

Calibration

The eye gaze recording was preceded by a five-point calibration phase. This was run using the ClearView 2.5.1 software package and the procedure was identical to that reported by Téglás et al. (2012). The calibration was considered successful if both of the dog's eyes were registered on at least four of the five points.

Stimulus Presentation

After successful calibration the experimenter left the room and the test trial followed during which Clearview 2.5.1 software presented six images of three different male and three different female faces from the Radboud Faces Database (Langner et al., 2010) showing happy, angry or fearful emotional expressions. The stimulus presentation started with an introductory phase during which an attention getter stimulus (a rattling and moving

toy) was presented in the middle of the screen for 4 s. It was followed by the presentation of a face stimulus for 5 s in the middle of the screen. The attention-getter reappeared on the screen between each facial stimuli to redirect the dogs' attention. The presentation order of the first two facial stimuli was counterbalanced between subjects (first stimulus: angry female, second: happy male, $N = 12$ dogs; first stimulus: happy male, second: angry female, $N = 19$ dogs), while the order of the other stimuli was fixed (third: fearful female, fourth: angry male, fifth: happy female, sixth: fearful male). During the presentation of emotional facial expressions a neutral beep sound was played.

Data Analysis

Gaze duration was calculated as the time subjects spent looking at the screen during the presentation of the face stimuli. Gaze duration data of the first presented stimuli (mean looking time at the first presented stimuli) was used to test the effects of age (Pearson correlation), training experience (trained vs. untrained dogs; independent samples *t*-test), head shape (short vs. long nose dogs; independent sample *t*-test) as well as the potential differences between male and female subjects (independent samples *t*-test).

Linear Mixed Model (LMM) was used to determine how the presentation order (from first to sixth; within subjects covariate), as well as the emotional expression (happy, angry, fearful; within subject factor) and the gender (male or female; within subject factor) of the stimuli faces influenced the total gaze duration towards the screen.

Based on the results of the first model (see later) data of the first two faces (angry female and happy male, in a counterbalanced order across subjects) was entered in another model (LMM) in order to test the effects of order (first/second, within-subjects factor), angry female/happy male (within-subjects factor) and their interaction. As a strong order effect was found across the six images, with this second model we aimed to see if restricting the analysis to two images only would yield different results.

Data of the first image was used to test how long dogs look into the different regions of the face. Each stimulus face was divided into four *AOIs*: eyes, mouth, forehead and neck regions. The size of AOIs for the eye, mouth and forehead were the same for all faces, the neck AOI was 33% smaller. Gaze durations were calculated for each of the AOIs. Then *gaze preference scores* were calculated for each dog based on the gaze duration data: we ranked the four facial AOI according to their efficiency in attracting a subject's attention by assigning rank 1 to the lowest value, and assigning the mean of ranks to ties. In order to correct for the fact that the neck region was 33% smaller, data from this region was multiplied by 1.5 before the rank transformation. Friedman test was used to test if dogs preferred to look at one region over another (we first tested for the data pooled together for all subjects, and then tested separately the looking pattern for the two different images).

Results

Gaze duration toward the first stimuli was not affected by the dog's sex ($t_{(29)} = 0.65$; $p = 0.52$), age (Pearson $r_{(29)} = -0.22$; $p = 0.26$), training experience ($t_{(15,2)} = 1.48$; $p = 0.16$) and head shape ($t_{(5,29)} = 1.23$; $p = 0.27$).

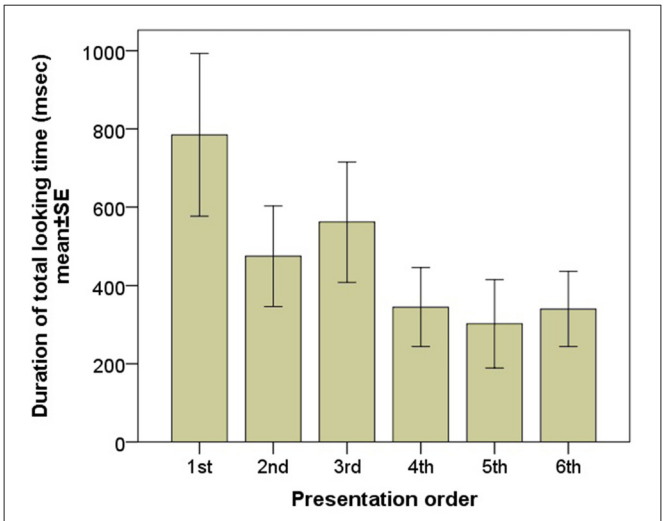

FIGURE 2 | Gaze duration of the subjects during the six consecutive image presentations.

According to the LMM there was a significant main effect of the sequence of presentation on mean gaze duration towards the screen indicating a strong decrease in viewing duration ($F_{(1)} = 8.743$, $p = 0.004$; **Figure 2**). No effect of emotional expression (happy vs. angry vs. fearful; $F_{(2)} = 1.287$, $p = 0.287$) and gender ($F_{(1)} = 0.869$, $p = 0.3521$) was found.

When only data of the first two images entered in the LMM the order effect also disappears (first/second: $F_{(1)} = 1.329$, $p = 0.254$; angry female/happy male $F_{(1)} = 0.286$, $p = 0.595$; order × image interaction: $F_{(1)} = 0.449$, $p = 0.505$).

Analysis of gaze preference scores for the first image (**Figure 3**) showed that dogs differentiate between the facial regions in their looking pattern ($\chi^2 = 24.260$, $p < 0.001$). They

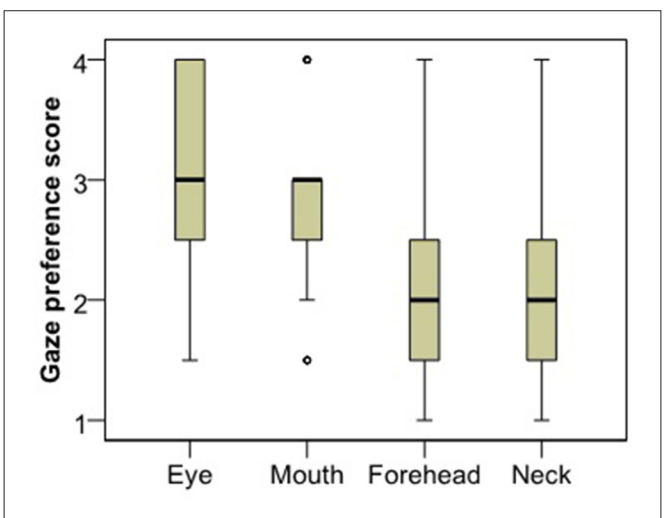

FIGURE 3 | Viewing preference of the different face regions as expressed in the rank of viewing times.

look more to the eye region compared to both the neck (Dunn *post hoc*, $p < 0.001$) and the forehead region ($p = 0.003$); and they look more to the mouth compared to the neck ($p < 0.001$), although not the forehead ($p = 0.084$). There was no difference between the eye and the mouth ($p = 0.240$) or the neck and the forehead ($p = 0.098$) regions. The same result remained both for subjects that viewed the angry female image ($\chi^2 = 12.108$, $p = 0.007$) and those who viewed the happy male image ($\chi^2 = 12.770$, $p = 0.005$).

Discussion

This pilot study investigated how certain properties of human faces e.g., gender, emotional expression and sequence of the presented faces influence the dogs' looking behavior. We found that the presentation order had an important effect on the dogs gaze duration toward the screen, that confirms the study design of previous studies using both eye-tracking (Somppi et al., 2012; Téglás et al., 2012) and projected images (Faragó et al., 2010; Racca et al., 2012) and suggests that due to the limited attention span of dogs fewer stimuli should be used. We found no influence of the model's gender and there was no difference in the gaze duration toward faces expressing different emotional expressions either. This is somewhat in contrast with previous studies suggesting that dogs recognize the gender of humans (Wells and Hepper, 1999; Deputte and Doll, 2011) as well as the different emotions (Morisaki et al., 2009; Deputte and Doll, 2011; Nagasawa et al., 2011). This difference might be due to special circumstances that dogs face whilst participating in an eye tracking experiment (e.g., watching a computer screen without a task might not be a natural behavior for a dog). Note also that while previous studies coded the dogs' behavior/head movement (Morisaki et al., 2009; Deputte and Doll, 2011) or used touch screen technique (Nagasawa et al., 2011), here we measured gaze durations, a more specific indicator of attentional engagement. It is also possible that the strong order effect that we found masked other more subtle effects, although the fact that we found no effect in the model that analyzed the first two images (angry female vs. happy male) makes this explanation somewhat less likely. Our results are also in line with the notion (Somppi et al., 2012) that dogs show a greater visual preference for emotionally meaningful face areas (e.g., the eyes as opposed to the neck and the forehead).

STUDY II

Background

Based on the results obtained in Study 1, we designed the second study that aimed to test the effect of intranasal oxytocin treatment on dogs' human face and emotion processing. As no effect of image gender was found, we decided to restrict our stimuli to one gender only. In order to minimize the confound arising from order effects only two stimulus images were used for longer presentation duration (7000 ms). Although no effect of emotion was found in Study I, we decided to use both happy and angry facial expressions as stimuli, due to the extended human literature showing an emotion-specific effect of oxytocin on face processing (Domes et al., 2007; Savaskan et al., 2008; Guastella et al., 2009; Marshall-Pescini et al., 2009; Fischer-Shofty et al., 2010; Kis et al., 2013).

Methods

Subjects

A total of 125 family dogs naïve to the experimental setting were recruited on a voluntary basis from the Family Dog Project (Abdai and Miklósi, 2015) database. Of these 48 dogs were excluded as their eyes could not be captured by the eye tracking device due to subjects' inattentiveness and/or their head shape (too long nose, lateral position of the eyes). The remaining 77 dogs received either placebo (PL group, $N = 32$ dogs) or oxytocin (OT group, $N = 45$ dogs) pretreatment. However, further 31 dogs (8 in the PL and 23 in the OT groups) had to be excluded because they did not provide eye gaze data for both of the stimuli pictures. Surprisingly, oxytocin pre-treated dogs had to be excluded in a much higher ratio than was the case for both previous studies and placebo treated dogs in the present study. One possible explanation is that as oxytocin has an effect on pupil dilatation (especially when viewing emotional stimuli; e.g., Leknes et al., 2013), this might underlie the high drop-out rate we experienced (e.g., changes in dog's pupil size caused that the eye-tracker did not record valid gaze data in some cases).

The final sample consisted of 46 subjects from 20 different breeds and 10 mongrels; $N = 24$ in the Placebo (mean age $\pm$ SD: 4.52 ± 2.23; females/males: 10/14) and $N = 22$ in the Oxytocin (mean age $\pm$ SD: 4.31 ± 2.5; females/males: 8/14) groups.

Pre-Treatment

If the eye tracker was able to detect both eyes of the dog a single intranasal dose of oxytocin (Syntocinon-Spray, Novartis) or placebo (isotonic natriumchlorid 0.9% solution) was administered. The amount of solution sprayed into nostrils depended on the dogs' body size: large and medium sized dogs (over 18 kg) received 12 IU (1 and 2 puffs per nostril), small dogs (under 18 kg) received 8 IU (1-1 puff per nostril). Treatment was followed by a waiting period of 40 min (similarly to human experiments; e.g., MacDonald et al., 2011) presumed to be necessary for intranasally administered neuropeptides to develop their effect on the central nervous system (Born et al., 2002). This pre-treatment procedure has been validated for dogs by showing that oxytocin as compared to placebo decreases heart rate and increases heart rate variability (Kis et al., 2014) and was used in several studies that yielded behavioral differences between oxytocin vs. placebo pre-treated dogs (Hernádi et al., 2015; Kis et al., 2015; Kovács et al., 2016a,b).

Calibration

After the waiting period the dog-owner dyad entered the laboratory again and the owner was asked to set her dog into the testing position. The eye gaze recording was preceded by the same five-point calibration process used in Study I (section "Study I: Methods: Calibration").

Stimulus Presentation

After the successful calibration the experimenter left the room and the test trial followed during which Clearview 2.5.1 software presented two images of male faces expressing two different emotions (happy and angry). Stimulus presentation started with an introductory phase during which an attention getter (a rattling and moving toy) was present on the screen for 4 s—in order to direct the dogs' attention to the center of the screen. It was followed by the presentation of a happy or angry face for 7 s displayed on either the left or the right side of the screen (We presented images to the left and right side in order to avoid that the fixating to the attention getter, presented to the middle immediately preceding the stimuli, causes fixations to relevant target regions). Then the whole presentation procedure was repeated (attention-getter stimulus for 4 s and facial image for 7 s) in this case the location (left or right) and the emotional expression (angry or happy) were reversed.

The stimulus material included facial photographs of four male individuals from the Radboud Faces Database (Langner et al., 2010). Images were randomly selected from the 20 Caucasian adult males the database contained. Models wore black t-shirts, had no hair on the face and wore no glasses, makeup or jewellery. The chosen images all showed the emotional expression with eyes directed straight ahead and from a 90° camera angle. Photos had been corrected for white-balance, and spatially aligned according to facial landmarks. We did not make any modification to the images obtained from the database. All images were of an original size of 1024 × 681 pixels, and in our monitor were presented in the size of 26 cm × 17.3 cm. The images were randomly assigned to the dogs during the test with the restriction that each dog would see the different emotional expressions of the same person. The type (happy or angry) and the location (left or right side) of the firstly presented images were counterbalanced between subjects in both OT and PL groups. During the presentation of emotional facial expression neutral beep sound was played.

Data Analysis

Due to a generally low duration that dogs spent looking at the stimulus (see "Results" section), only gaze duration data could be analyzed, but not the number of fixations. The 200 ms criteria commonly used for human infant eye-tracking (Gredebäck et al., 2009) would result in zero fixations for a considerable proportion of dogs. While some studies address this problem by lowering the fixation threshold for dogs to 0 or 75 ms we decided to use only the gaze durations instead, as it is hard to argue that a fixation of 0 ms is meaningful.

Gaze duration was calculated as the time subjects spent looking at the screen during the presentation of the stimuli. Each stimulus face was divided into four *AOIs*: eyes, mouth, forehead and neck regions. The size of AOIs for the eye, mouth and forehead were the same for all faces, the neck AOI was 33% smaller. We summed up these AOIs to get a whole face region as well. Gaze durations were calculated for each of the AOIs. The *relative gaze durations* toward eye, mouth, forehead, neck and whole face regions were calculated by dividing the means of the gazing time toward these regions by the means of the total gazing time at the screen. Then *gaze preference scores* were calculated for each dog based on the gaze duration data: we ranked the four facial AOI according to their efficiency in attracting a subject's attention by assigning rank 1 to the lowest value, and assigning the mean of ranks to ties. In order to correct for the fact that the neck region was 33% smaller, data from this region was multiplied by 1.5 before the rank transformation.

Gaze duration data (mean looking time at the two presented stimuli) was used to test the effects of age (Pearson correlation), training experience (trained vs. untrained dogs; independent samples *t*-test), head shape (short vs. long nose; independent samples *t*-test) as well as the potential differences between male and female subjects (independent samples *t*-test). LMM was used to determine how the treatment (OT or PL; between subjects factor), as well as the emotional expression (happy or angry; within subject factor) and the presentation order (first or second; within subject factor) of the stimuli influenced the relative gaze durations towards the different AOIs.

Gaze preference scores were used to test if dogs in the OT and PL groups have any preference for a designated facial region of the happy/angry faces (Friedman test, Dunn *post hoc* test). For the statistical analysis the SPSS 18.0 statistical package and InStat software were used.

Results

There was no difference in age ($t_{(117)} = 0.39$; $p = 0.69$), gender ($\chi^2_{(1)} = 1.23$; $p = 0.27$) and training experiences ($\chi^2_{(1)} = 0.27$; $p = 0.604$) when comparing dogs who successfully passed to those who failed to pass the calibration. Dogs in the final sample looked at the screen on average 19.7% (2759.54 ms) of the total (2 × 7000 ms) time (ranged between: 80–10,508 ms) when the facial images were presented. Gaze duration toward the screen was not affected by the dog's gender ($t_{(44)} = 0.15$; $p = 0.88$), age (Pearson $r = 0.009$; $p = 0.95$), head shape ($t_{(44)} = 1.33$; $p = 0.19$) and training experience ($t_{(44)} = 0.29$; $p = 0.77$).

There was a significant interaction (LMM; for full models see Supplementary Materials) between emotional expression and sequence of presentation in case of relative gaze to the eye ($F_{(1,84)} = 7.37$; $p = 0.008$) and mouth ($F_{(1,84)} = 7.54$; $p = 0.007$) region. In case of the first stimulus, dogs looked more to the happy face's eyes than to the angry face's eyes and more to the angry face's mouth than to the happy face's mouth. In contrast in case of the second stimulus, dogs looked more to the angry face's eyes than to the happy face's eyes and more to the happy face's mouth than to the angry face's mouth. Relative gaze duration to the forehead region was also affected by the sequence of presentation ($F_{(1,84)} = 3.94$; $p = 0.05$). Subjects looked more to the forehead region at the first presented faces than at second one.

Relative gaze duration toward the whole face indicates a significant interaction between emotional expression and pretreatment type ($F_{(1,84)} = 4.67$; $p = 0.03$). After having received intranasal administration of oxytocin, dogs gazed less toward

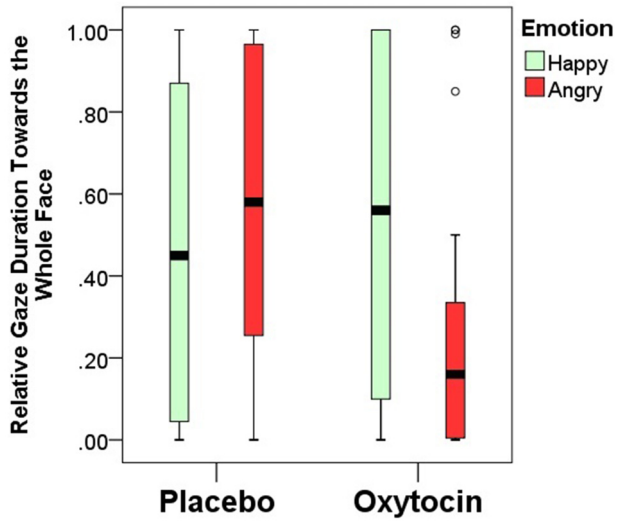

FIGURE 4 | Relative gaze duration (mean ± SE) towards the whole face expressing happy/angry emotion in the placebo and oxytocin groups. Median, quartiles, whiskers, outliers.

the human face expressing negative, but not positive emotion (**Figure 4**). Relative gaze durations towards the other face regions (eye, mouth, neck and forehead) were not influenced by the pretreatment or emotional expression and no interaction between pretreatment and emotional expression was found either (all $p > 0.05$; for full models see Supplementary Materials).

Based on the distribution of gaze durations toward the different parts of angry and happy faces, the facial regions were ranked and the gaze preference scores for the different AOIs of happy and angry faces in both OT and PL groups were analyzed (**Figure 5**). In the placebo-treated group, we found significant differences in terms of dogs' looking patterns for both the happy ($\chi^2_{(3)} = 19.705$; $p < 0.001$) and the angry ($\chi^2_{(3)} = 19.123$; $p < 0.001$) facial images. Replicating our results in study 1 dogs preferred to look to the eye region compared to the forehead (Dunn *post hoc* test; $p < 0.05$) and neck region (Dunn *post hoc* test; $p < 0.01$) of both happy and angry faces. A similar attentional bias was found in the oxytocin-treated group for the angry faces ($\chi^2_{(3)} = 9.333$; $p = 0.025$), although the *post hoc* test did not reach significance. This differential looking pattern, however, was not found in case of the happy ($\chi^2_{(3)} = 6.706$; $p = 0.082$) faces for the oxytocin-treated group. Directly compared, the oxytocin and the placebo groups did not differ in their rank scores for any of the facial regions (all $p > 0.05$ for both happy and angry faces), see supplementary material.

GENERAL DISCUSSION

In the present study, we investigated visual processing of human faces in dogs and demonstrated differential effects of oxytocin on the eye gaze patterns towards faces expressing positive and negative emotions. Dogs in the control groups (i.e., all subjects receiving no pre-treatment in study 1 and those subjects in the main study that received placebo treatment) displayed a general preference towards the eye region of the human face regardless of valence of the emotional expression.

Our results are also important from the methodological point of view, as they add to the handful of experiments that have so far employed eye-tracking in order to measure gaze patterns in non-human animals (e.g., chimpanzee: Kano and Tomonaga, 2009; Hattori et al., 2010; dog: Williams et al., 2011; Somppi et al., 2012). We confirmed previous claims that eye-tracking can be applied to study task- naïve pet dogs (Téglás et al., 2012). However the large number of subjects that had to be excluded raise some concerns about the representativeness of the subjects participating in these studies and also pose considerable practical difficulties for future research. Despite some general "rules of thumb" (e.g., dogs should have no hair in the eyes) we did not find any factor that would predict successful eye-tracker calibration as no effect of head shape or training experience was found. A possible solution to this methodological problem is to train the dogs to lie still for the purpose of an eye-tracking study. For example, in a recent study in which dogs were specifically trained to meet the requirements of eye-tracking (Somppi et al., 2017) 43 of the 46 recruited subjects successfully completed the experiment. However training dogs for such a task might heavily influence their looking pattern as well as their cognitive processes during image viewing, as training has been shown to modulate attention in general (Vas et al., 2007). Specific trainings (Marshall-Pescini et al., 2009) as well as general training level (Marshall-Pescini et al., 2008) have also been found to influence performance and certain aspects of behavior in social and cognitive tasks The future combination of two approaches would be ideal. It is also important to mention that our study suggests that eye-tracking can only be used with short stimuli presentation as the looking time of dogs quickly decreases over time.

Our findings fit well with the widely held notion that the eye region of another is a strong attention getter for group members in many social species (Emery, 2000). However after oxytocin pre-treatment this preference only remained for the angry but not the happy faces, contrary to human findings where oxytocin increased gaze to the eye region (Guastella et al., 2009). This difference between dogs and humans might be attributed to a difference in the meaning of gaze cues. In humans, staring eyes (establishing eye contact) have two distinct functions as they can signal either competitive (threatening—Wieser et al., 2009) or collaborative (information sharing—Senju and Csibra, 2008) attitudes toward the partner. Although direct gaze in face–to–face situations is commonly used to indicate a positive, information sharing attitude in humans from very early on Csibra (2010), the predominant role of this signal between non-human subjects is evoking fear or aggression and has little (if any) collaborative property. Even among dogs direct gaze is mainly used for signaling dominance and as a form of ritualized aggression (Schenkel, 1967).

Further studies could follow up on the finding that dogs use certain relevant regions of the face to assess

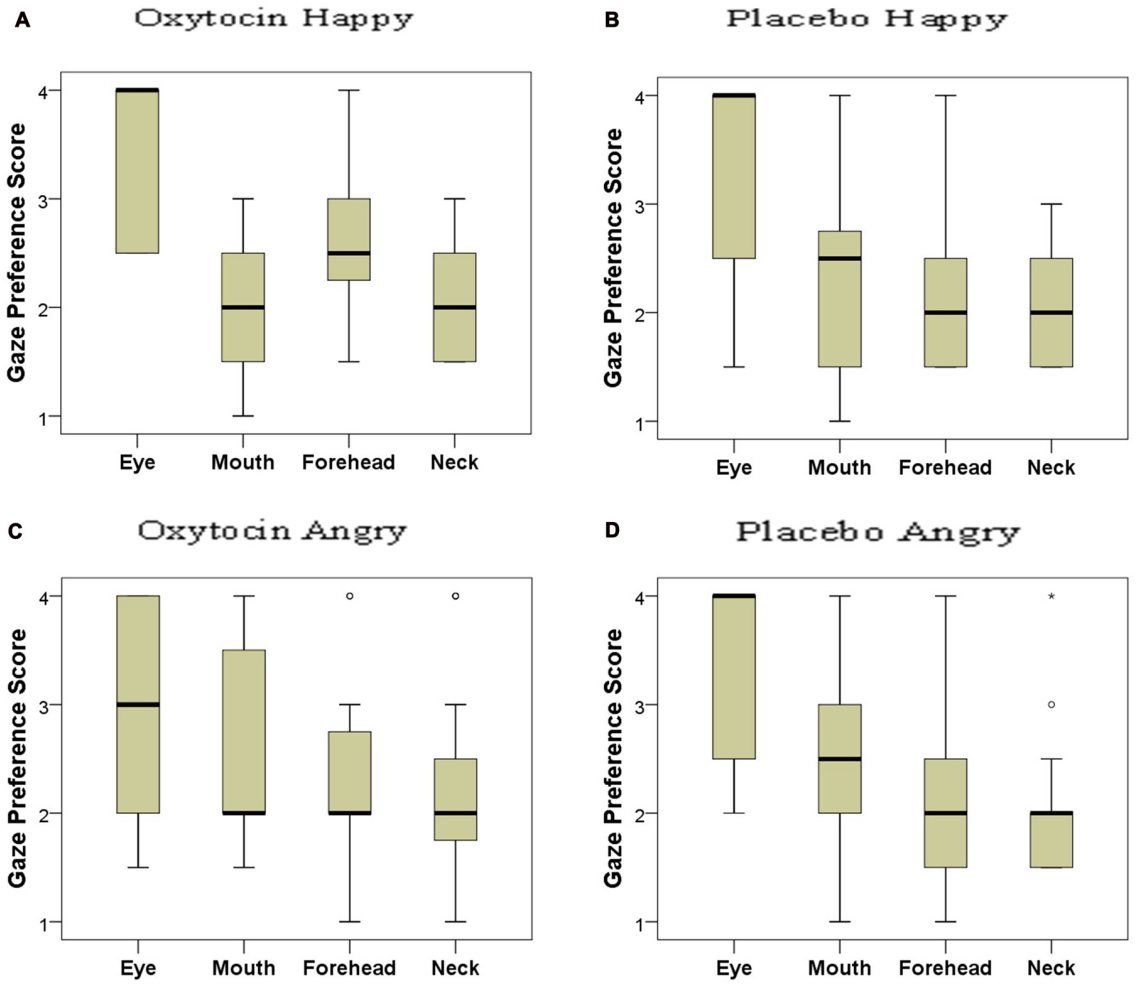

FIGURE 5 | Looking preference of subjects as reflected in their rank scores for happy (**A**: oxytocin, **B**: placebo) and angry (**C**: oxytocin, **D**: placebo) faces. A higher score indicates a higher preference. Median, quartiles, whiskers, outliers.

emotions by presenting only those parts (e.g., the eyes) of faces with different emotions. A recent touch-screen study (Müller et al., 2015) showed that dogs can generalize from upper half to lower half of the face, but more fine-scaled analysis with eye-tracking technology will add further information. A further interesting question is whether looking patterns for positive and negative faces both differ from those for neutral faces, or if the difference between negative and positive faces can be attributed to only one of them.

Although dogs often use direct gaze for the same purpose as infants do (demanding attention or initializing communicative interaction—Miklósi et al., 2003; Passalacqua et al., 2011) while interacting with their human caregivers or familiar partners, eye contact with an unfamiliar human has the potential to evoke fear (Vas et al., 2005) and to increase symptoms of anxiety (heart rate—Gácsi et al., 2013). In line with this, we may assume that in the test trials the sudden appearance of an unfamiliar human's face and his staring eyes in a very intimate, face-to-face position was conceived as threatening by the dog, and as a consequence, they showed increased attention towards the eye region of the faces regardless of the displayed emotional expression. It is also worth mentioning that the gaze of negative emotional face is a particularly effective cue to attention also in humans (Holmes et al., 2006) and this is especially true for anxious people who seem to show an attentional bias to threatening faces in an eye tracking experiment (Armstrong et al., 2010).

In the case of dogs treated with oxytocin, however, the analysis of eye gaze patterns provided a somewhat different picture: (1) subjects in OT group generally showed a weaker tendency to look at negative facial images compared to PL group, and, at the same time; (2) the preferential looking to the eye region of happy human faces disappeared. In contrast to this in a study conducted on trained dogs analyzing the number of fixations (Somppi et al., 2017) it was found that dogs after oxytocin treatment fixated less often at the eye region of angry faces and revisited more often the eye region of happy faces. These differences

might probably be attributed to the subjects in the two studies being naïve vs. trained for the eye-tracking task. Both findings support the notion that dogs' gaze bias towards the eye region of faces can be regarded as an indication of social fear, although gaze duration and fixation count showed an opposite response to oxytocin treatment in the two studies. Oxytocin is known to attenuate fear responses in many species including humans (Domes et al., 2007) thus the elimination of gaze bias toward the eye region of happy (i.e., less threatening) faces may be based on the anxiety-relieving effects of this neuropeptide. At the same time oxytocin was insufficient to eliminate the attention-getting effects of eye-region of angry faces which still kept some of its fear-evoking potential.

Previous studies have shown that male and female dogs might react differentially (or to a different magnitude) to intranasal oxytocin treatment (Oliva, Kovács). Furthermore the effect of intranasal oxytocin is also modulated by dogs' breed (and within breeds individuals with different OXTR genotype also react differently; Kovács). The present study did not address such individual variability, but further studies might investigate these together with differences in e.g., subjects' personality.

In sum our results revealed that compared to humans there are both similarities and differences in how oxytocin influences the way dogs visually explore human emotional faces. The present study also points to limitations of the sequential picture viewing paradigm for assessing cognitive- and attentional processes in dogs and highlights methodological challenges related to eye-tracking data collection.

ETHICS STATEMENT

Ethical approval was obtained from the National Animal Experimentation Ethics Committee (Ref. No. XIV-I-001/531-4-2012). Research was done in accordance with the Hungarian regulations on animal experimentation and the Guidelines for the use of animals in research described by the Association for the Study Animal Behavior (ASAB).

AUTHOR CONTRIBUTIONS

AH and JT conceived the experiment. AK, AH, OK and BM performed the experiments. AK and AH analyzed the data and wrote the manuscript. AK and JT secured funding. JT supervised the project.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnbeh.2017.00210/full#supplementary-material

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Intranasal Oxytocin Treatment Increases Eye-Gaze Behavior toward the Owner in Ancient Japanese Dog Breeds

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Dogs acquired unique cognitive abilities during domestication, which is thought to have contributed to the formation of the human-dog bond. In European breeds, but not in wolves, a dog's gazing behavior plays an important role in affiliative interactions with humans and stimulates oxytocin secretion in both humans and dogs, which suggests that this interspecies oxytocin and gaze-mediated bonding was also acquired during domestication. In this study, we investigated whether Japanese breeds, which are classified as ancient breeds and are relatively close to wolves genetically, establish a bond with their owners through gazing behavior. The subject dogs were treated with either oxytocin or saline before the starting of the behavioral testing. We also evaluated physiological changes in the owners during mutual gazing by analyzing their heart rate variability (HRV) and subsequent urinary oxytocin levels in both dogs and their owners. We found that oxytocin treatment enhanced the gazing behavior of Japanese dogs and increased their owners' urinary oxytocin levels, as was seen with European breeds; however, the measured durations of skin contact and proximity to their owners were relatively low. In the owners' HRV readings, inter-beat (R-R) intervals (RRI), the standard deviation of normal to normal inter-beat (R-R) intervals (SDNN), and the root mean square of successive heartbeat interval differences (RMSSD) were lower when the dogs were treated with oxytocin compared with saline. Furthermore, the owners of female dogs showed lower SDNN than the owners of male dogs. These results suggest that the owners of female Japanese dogs exhibit more tension during interactions, and apart from gazing behavior, the dogs may show sex differences in their interactions with humans as well. They also suggest that Japanese dogs use eye-gazing as an attachment behavior toward humans similar to European breeds; however, there is a disparity between the dog sexes when it comes to the owners' oxytocin secretion. Japanese dogs also showed different attachment behaviors from both European breeds and wolves, and they likely use additional strategies to substitute gaze when forming the human–dog bond.

Keywords: human-dog bond, oxytocin, gaze, positive loop, Japanese breeds, heart rate variability

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INTRODUCTION

The dog (*Canis familiaris*) was the first animal to be domesticated (Serpell, 2016), with hundreds of different dog breeds recognized today. During the domestication process, dogs were subjected to a strong selection according to their temperament, behavior, and cognitive abilities (Hare and Tomasello, 2005). Dogs are skilled at understanding human communicative gestures compared with wolves and chimpanzees (Hare et al., 2002). They even look back at humans when encountering unsolvable tasks, while wolves do not (Miklósi et al., 2003). These findings suggest that dogs acquired their unique cognitive abilities during domestication.

The shared communicative signals with humans, such as eye-gaze, might also be related to interspecies' bonding, namely human–dog bonding. Dogs can distinguish between individual humans (Nagasawa et al., 2009) and show distinctly different behaviors to caregivers compared with hand-raised wolves (Topál et al., 2005). Furthermore, interaction with dogs confers a social buffering effect to humans (Polheber and Matchock, 2014). Likewise, dogs also receive more social buffering effects from interacting with humans than from conspecifics (Tuber et al., 1996). Nagasawa et al. (2009, 2015) hypothesized that an oxytocin-mediated positive loop exists between humans and dogs, which is mediated by gazing, an attachment behavior observed in human mothers and infants. Indeed, they reported that dog gazing behavior to the owner, which was not observed in wolves, increased urinary oxytocin concentrations in owners, which consequently facilitated owners' affiliation and increased urinary oxytocin concentrations in dogs. Furthermore, nasally administered oxytocin increased the gazing behavior in dogs, which in turn increased urinary oxytocin concentrations in owners. These results show the existence of an oxytocin-mediated positive loop in human-dog relationships similar to that of human mother–infant relations (Feldman, 2012), and support that dogs might have acquired this interspecies bonding feature by the domestication process. The relationship between oxytocin and human-directed social behavior in dogs has recently become clearer. Kis et al. (2014) showed that the polymorphisms of the oxytocin receptor gene are related to social behavior such as proximity seeking and friendliness toward humans. Kovács et al. (2016) administrated oxytocin to different types of working dog breeds (Border Collie and Siberian Husky, the former is the cooperative-working type and the latter is the independent-working type) and compared their social behavior toward humans. They found that Border Collies looked more at their owners and the experimenter than Siberian Huskies after oxytocin administration. These studies indicated that the oxytocinergic system modulates dog social behavior as well as that of humans.

Recently, genetic characteristics among dog breeds have been studied and their genetic classifications constructed. A cladogram analysis of dog genes revealed a unique clade between European-originating breeds and wolves which was categorized as 'ancient breeds.' This category includes popular Japanese breeds such as Shiba and Akita, and a large group of breeds with presumed modern European origins (Parker et al., 2004; vonholdt et al., 2010). Since these ancient breeds are most closely related to wolves genetically, sharing the most recent common ancestor, they may show different behavioral characteristics when compared with other breed groups. Ito et al. (2004) identified a polymorphism region in the dopamine receptor D4 gene in canine breeds which was associated with human-directed aggressive traits. Moreover, through a neighbor-joining tree based on allele frequencies, breeds were divided into two main groups, whereby the group including Japanese breeds showed the highest scores in human-directed aggressive traits, assessed by a questionnaire administered to dog specialists, when compared with breeds of Occidental origin. The Japanese breed, Shiba Inu, showed the most pronounced human-directed aggression (Arata et al., 2014). Moreover, Tonoike et al. (2015) investigated whether dogs' behavioral characteristics were different among genetically clustered breed groups and found that the dogs in the ancient and spitz breed groups showed low attachment and attention-seeking behaviors to their owners. It was also found that it took longer for ancient breeds to make eye contact with humans, and they gazed at humans for shorter periods compared with other breed groups during an unsolvable situation (Konno et al., 2016). These characteristics in Japanese breeds may stem from the fact that Japanese breeds were not selected for a particular cooperative function, so they are not highly domesticated and are genetically close to wolves.

Based on these previous studies, the ancient breeds were expected to display less attachment behavior more similar to wolves when compared with European breeds. Only limited studies have examined the effects of exogenous oxytocin in ancient breeds (Kovács et al., 2016), and revealed that the responses to oxytocin were different from a European breed; Border Collies gazed more at humans than Siberian Huskies after oxytocin administration. While some reports demonstrated that human-directed behavior is modulated by experience and learning to some degree (Barber et al., 2016; D'Aniello and Scandurra, 2016), there should be genetic modulation in these behaviors as well (Nagasawa et al., 2015). Therefore, we hypothesized that Japanese dogs show intermediate bonding traits between those of wolves and European dog breeds; therefore we tested the existence of an oxytocin-mediated positive loop in ancient breeds, Japanese dogs. We investigated whether Japanese breeds showed the oxytocin-gaze bonding system with their owner using the same procedure as Nagasawa et al. (2015). We predicted that oxytocin would increase a dog's gazing behavior while simultaneously increasing the owner's urinary oxytocin levels, to a lower degree than those seen in European breeds. We also attached heart rate monitors to both dogs and owners in order to monitor physiological changes during the interaction. Interaction with dogs has been shown to be effective in reducing human stress (Odendaal and Meintjes, 2003; Nagasawa et al., 2015). Emotional status, including stress and tension, can be assessed by the balance between the sympathetic and parasympathetic nervous systems, and heart rate variability (HRV) is one of the most widely used metrics of this balance. There are many methods to evaluate HRV, and the time domain methods are the simplest ones (Task Force of The European Society of Cardiology and The North American Society of

Pacing and Electrophysiology, 1996). Although there's room for consideration, some studies showed the combination of parameters analyzed in the time domain methods could indicate the emotional states (human: Kreibig, 2010, dog: Gácsi et al., 2013; Katayama et al., 2016). It was also demonstrated that oxytocin can increase HRV (Norman et al., 2011; Kemp et al., 2012). Increase of oxytocin in humans also facilitated females' reactivity to infant crying (Riem et al., 2011). Infant crying is one of the attachment/alarming signals from infants to mothers, suggesting that attachment signals can increase tension/arousal in mothers. Therefore, if oxytocin administration increases dogs' gazing behavior toward their owners and stimulates oxytocin secretion in the human, we have the following two hypotheses; one is that mutual gazing will lead to an activation of the parasympathetic nervous system in owners, which would be reflected in a change in the HRV, and the other is that changes of the dog's behavior also lead to owners' emotional arousal or tension, thereby, activates the sympathetic nervous system.

MATERIALS AND METHODS

Participants

This experiment involved 21 volunteers (male: $n = 11$; female: $n = 10$) and their 22 dogs (5 gonadally intact male, 5 castrated, 4 gonadally intact female, 8 spayed; age: male 5.2 ± 1.0 years, female 4.3 ± 1.0 years; 18 Shiba, 2 Kai, and 2 Shikoku). Participants were recruited in dog training classes, veterinary clinics, dog runs, and through the internet. Written informed consent was obtained from the owners. We also recruited students and staff from the university who matched the owners in sex and appearance, but were unfamiliar to the dogs, to serve as unfamiliar people during the 30-min interaction. Owners and the unfamiliar people were aware of the procedure of the sessions, but blinded to its purpose and treatment. We conducted two experimental sessions, under conditions of oxytocin and saline treatment, per a dog on different days, at least 1 month apart. The order of treatment was counterbalanced.

Procedures

Experiments were conducted in an area (4.5 × 4.5 m) that was divided by partitions in a room at Azabu University. Three chairs were set in a circle in the experimental area, and marks were placed at a 0.7-m radius from each chair with vinyl tape. We attached heart rate monitors to the chests of both owner and dog to measure HRV. If a dog reacted adversely to wearing a heart rate monitor, it was exempted from the HRV measurement. This experiment consisted of three phases: resting before the interaction, a 30-min interaction between the participants and dog, and resting after the interaction. Two video cameras (GZ-HM670, Victor, Japan) were placed in the corners, and two others (PC-355micro, Sun-Mechatronics, Japan) were placed on the ceiling of the experimental room to record the behaviors of the dogs during the 30-min interaction. The owner and his/her dog avoided eating and drinking other than water from 2 h before the experiment as well as during the experiment. The dog urinated on the way to the experimental room after arrival at the university by a car. The owner also urinated 1 h before the 30-min interaction to empty the bladder, then rested on one of the chairs with the dog, but without interacting. Immediately prior to the 30-min interaction phase, the experimenter took out the dog to urinate and the dog's urine sample was collected by the experimenter using absorbent cotton. At the same time, the owner collected his/her own urine using a paper cup in the restroom and placed the sample in a cold reserving box. The experimenter removed the sample from the cold reserving box as soon as the owner left the restroom. Urine samples of both dog and owner were centrifuged at 4°C in a refrigerated centrifuge, and the supernatants were frozen at −80°C until assay. While the owner and the dog were providing urine samples, two unfamiliar people entered the experimental area and sat on the chairs. The order of seating was randomized by using the randomize function in Excel (Microsoft).

After the owner was seated, 100 μL of oxytocin (40 IU) or 100 μL of saline was intra-nasally administered to the dog by a hand-compressing air spray bottle. Following oxytocin or saline administration, the dog entered the experimental area. All participants were instructed to remain seated in their chairs, while the dog was allowed to move freely in the room. In order to prompt the dog's movement during the interaction, participants were instructed to change their seats every 10 min. They were not allowed to talk to each other or to talk to and touch the dog voluntarily; however, if the dog touched participants, the participants were allowed to pet the dog in return. After the 30-min interaction, the unfamiliar people left the experimental area. The owner and the dog remained in the room and rested. Subsequently, a second urine sample was collected from both dog and owner 30 min after the interaction. Ethical approval for this study was provided by the Ethics Committee of Azabu University (#131119-1), which follows "Guidelines for Proper Conduct of Animal Experiments" by Science Council of Japan (2006).

Outcome Measurements

Dog Behavioral Assessment

The dog's behaviors were recorded using video cameras during the 30-min interaction. We measured the total amount of time during which the dog touched the participants (*touch*), was at close proximity to participants (*proximity*; dog's head or body was within a 0.7-m radius from a participant), and has its nose oriented toward the participant's face (*gaze*). These videos were analyzed by two persons who were blinded to the details of the study. The scores obtained by both observers were highly correlated (rs = 0.92–1.00, $p < 0.01$). Additionally using stopwatches, the participants recorded the total amount of time during which they met the dog's eyes ("mutual gaze," which was defined as the orientation of the dog's nose toward the participant's face or when the dog's eyelids and eyebrows lifted to see the participant's face). These scores significantly correlated with the *gaze* duration (rs = 0.88, $p < 0.01$). The average of each behavioral variable recorded by the two unfamiliar persons was used in the analysis.

Measurement of Urinary Oxytocin in Owners and Dogs

Immediately after collection, urine samples were frozen at −80°C until the assay was performed. After thawing, urine samples were centrifuged at 4°C, and urinary oxytocin concentrations were measured by radioimmunoassay (Mitsui et al., 2011). Urinary oxytocin concentrations were corrected by using creatinine concentrations.

Heart Rate Variability

Heart rate variability was telemetrically measured using a Polar RS800CX digital system device attached to the chests of owners and dogs during the experiment. The dogs that gazed at their owner for less than 5 s or reacted adversely to wearing a heart rate monitor and the owners whose data included missing values were excluded from the analysis. We selected 15-s periods before and after the period during which the dog and its owner gazed mutually in a state of calmness using Kubios Heart Rate Variability Analysis Software 2.0 for Windows (Kubios Ltd., Finland). The heart rate during these periods was converted to inter-beat (R-R) intervals (RRI). Subsequently, we calculated the following HRV parameters: (1) the standard deviation of normal to normal R-R intervals (SDNN), which is an index of the autonomic nervous system and (2) the root mean square of successive heartbeat interval differences (RMSSD), which is an index of the parasympathetic nervous system. We compared between these parameters 15 s before (pre) and after (post) mutual-gaze under two administration conditions, oxytocin and saline. It was difficult to obtain sufficient data per dog from one period; therefore, we collected two periods for each dog and owner, and combined them together in the analysis.

Statistical Analysis

Most variables were not distributed normally; therefore, after logarithmic conversion, a linear mixed model (LMM) was conducted to compare behaviors using the factors "dog's sex," "participant" (owner and unfamiliar people), and "administration" (oxytocin and saline), and the dog's identity as a random factor for each behavioral variable. Urinary oxytocin concentrations and the parameters of HRV for both dogs and their owners were also analyzed by a LMM using "dog's sex," "administration," and "time of data collection" (pre and post) as factors, and the dog's identity as a random factor, after logarithmic conversion. If significant differences were observed, a *post hoc* analysis with a Bonferroni correction was conducted. The relationships between the variables were examined using a multiple linear regression analysis. The objective variable was the oxytocin change ratio, which was calculated by dividing the post-interaction urinary oxytocin level by that of pre-interaction, and the explanatory variables were dog's sex (male dog = 0, female dog = 1), owner's sex (male = 0, female = 1), administration (saline = 0, oxytocin = 1), duration of *touch*, duration of *proximity*, and duration of *gaze*. Results were expressed as mean ± standard error of the mean (SE). Statistical significance was considered at $p < 0.05$. All statistical analyses were performed using SPSS software v.24.0 (IBM Japan, Tokyo).

RESULTS

Differences in Behavioral Variables between the Two Treatment Groups

We observed the following significant main effects and interactions. For *gaze*, there were significant main effects of participant ($F[1,57.99] = 29.84$, $p < 0.001$) and administration ($F[1,58.92] = 4.13$, $p = 0.047$), and a significant interaction between participant and administration ($F[1,57.99] = 5.55$, $p = 0.02$). The *post hoc* tests with a Bonferroni correction showed that dogs exhibited gazing for a significantly greater amount of time toward their owners than toward unfamiliar people in both administration conditions (oxytocin administration: $p < 0.001$, saline: $p = 0.035$). Dogs also gazed at their owners significantly longer following oxytocin administration than following saline administration ($p = 0.003$, **Figure 1A**). For *touch*, there were no significant differences (dog's sex: $F[1,20.09] = 2.45$, $p = 0.13$, participant: $F[1,58.13] = 1.02$, $p = 0.32$, administration: $F[1,59.38] = 0.12$, $p = 0.73$, **Figure 1B**).

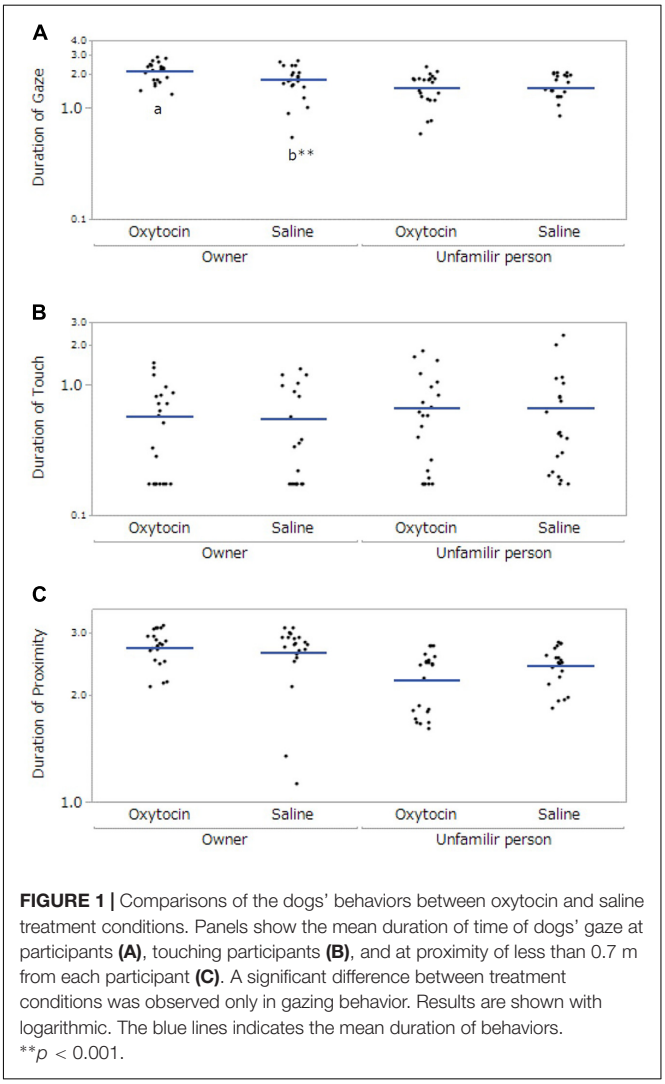

FIGURE 1 | Comparisons of the dogs' behaviors between oxytocin and saline treatment conditions. Panels show the mean duration of time of dogs' gaze at participants **(A)**, touching participants **(B)**, and at proximity of less than 0.7 m from each participant **(C)**. A significant difference between treatment conditions was observed only in gazing behavior. Results are shown with logarithmic. The blue lines indicates the mean duration of behaviors. $^{**}p < 0.001$.

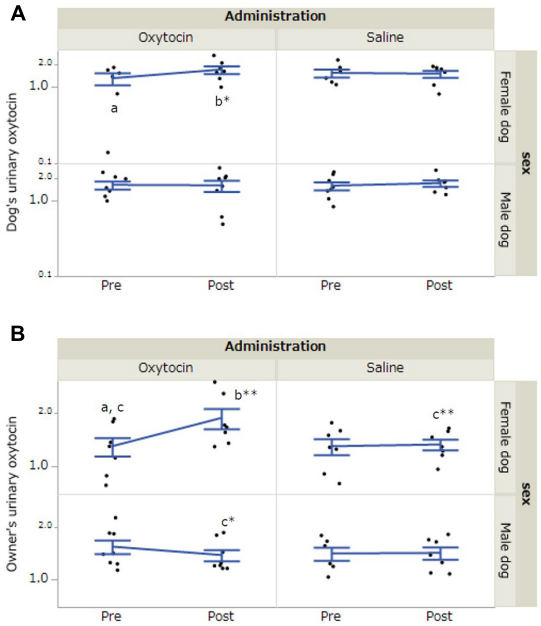

FIGURE 2 | Comparisons of urinary oxytocin concentrations between oxytocin and saline treatment conditions. **(A,B)** Significant differences depending on treatment conditions were found in both female dogs and their owners. Results are shown as mean ± SE with logarithmic. *$p < 0.05$, **$p < 0.001$.

Finally, for *proximity*, there was a significant main effect of participant ($F[1, 58.44] = 17.23$, $p < 0.001$). The *post hoc* tests with a Bonferroni correction showed that dogs kept proximity longer to their owners than unfamiliar persons ($p < 0.001$); however, we did not found the significant difference between oxytocin and saline administrations (**Figure 1C**).

Differences in Urinary Oxytocin Concentrations between the Two Treatments

In dogs, there was a significant interaction between dog's sex, administration, and time of urine collection ($F[1,36.16] = 5.80$, $p = 0.02$). A *post hoc* test with a Bonferroni correction indicated a significant increase in urinary oxytocin concentrations in female dogs after 30-min interaction in the oxytocin condition ($p = 0.014$, **Figure 2A**). In the owners, the results of LLM showed significant interactions between dog's sex and time of urine collection ($F[1,36.17] = 7.18$, $p = 0.01$) and dog's sex, administration, and time of urine collection ($F[1,36.17] = 6.03$, $p = 0.02$). A *post hoc* test with a Bonferroni correction indicated a significant increase in urinary oxytocin concentrations in the owners of female dogs after 30-min interaction in the oxytocin condition ($p < 0.001$). Post-interaction urinary oxytocin concentrations in the owners of female dogs were significantly higher in the oxytocin condition relative to the saline condition ($p = 0.001$). Furthermore, post-interaction urinary oxytocin concentrations were significantly higher in the owners of female dogs than in the owners of male dogs in the oxytocin treatment group ($p = 0.02$, **Figure 2B**). No significant differences were observed when we included the factor of "owner's sex" in LLM instead of "dog's sex." The intra-assay coefficient of variation (CV) of the oxytocin assay corresponded to 4.05%.

Multiple Regression of Urinary Oxytocin Concentrations in Owners and Dogs

To analyze the effect of dogs' behaviors on urinary oxytocin concentrations in owners and dogs, a multiple linear regression analysis was conducted to explain the oxytocin change ratio in owners using the following factors: dog's sex (male dog = 0, female dog = 1), owner's sex (male = 0, female = 1), administration (saline = 0, oxytocin = 1), duration of *touch*, duration of *proximity*, and duration of *gaze*. Results indicated that the oxytocin change ratio in owners showed a significant regression equation (Adjusted $R^2 = 0.304$, $F[6, 21] = 2.969$, $p = 0.029$). The dog's sex and the duration of *touch* were significantly related to the oxytocin change ratio in owners (dog's sex: $\beta = 0.510$, $p = 0.011$, *touch*: $\beta = -0.490$, $p = 0.019$, the oxytocin change ratio in owners $= -0.055 + 0.300$ * dog's sex $+ 0.136$ * administration $+ 0.072$ * *gaze* $+ (-0.375)$ * *touch* $+ 0.145$ * *proximity* $+ 0.159$ * owner's sex). That is, the owners of female dogs showed a higher increase in oxytocin, and the longer the duration of touch, the lower the increase ratio of oxytocin. In dogs, no significant regression equation was observed (Adjusted $R^2 = 0.133$, $F[6,19] = 0.509$, $p = 0.794$).

The Heart Rate Variability in Owners during the Interaction with Their Dogs

After excluding participants not meeting the inclusion criteria, 12 owners (five male, seven female) and 7 dogs (three male, four female) were included in the final HRV analysis. The data collected from the dogs was not sufficient for statistical analysis; therefore, only the owners' data were analyzed. A significant main effect was found in administration in RRI ($F[1,53.99] = 14.10$, $p < 0.001$), and the RRI under the oxytocin condition was lower than that under the saline condition ($p < 0.001$, **Figure 3A**). For SDNN, there were significant main effects of dog's sex ($F[1,21.68] = 11.71$, $p = 0.002$) and administration ($F[1,55.04] = 33.55$, $p < 0.001$), and a significant interaction between dog's sex and administration ($F[1,55.04] = 21.29$, $p < 0.001$). *Post hoc* tests indicated that the SDNN of the owners of male dogs was higher than that of the owners of female dogs ($p = 0.002$), and the SDNN under the oxytocin condition was lower than that of the saline condition ($p < 0.001$). Moreover, the SDNN of the owners of male dogs under the oxytocin condition was lower than that under the saline condition ($p < 0.001$), and owners of male dogs showed a higher SDNN than owners of female dogs under the saline condition ($p < 0.001$, **Figure 3B**). In RMSSD, with significant main effects found in dog's sex ($F[1,21.08] = 8.70$, $p = 0.008$) and administration ($F[1,58.35] = 18.93$, $p < 0.001$). The RMSSD of the owners of male dogs was higher than that of the owners of female dogs ($p = 0.008$), and the RMSSD under the oxytocin condition was lower than that under the saline condition ($p < 0.001$, **Figure 3C**).

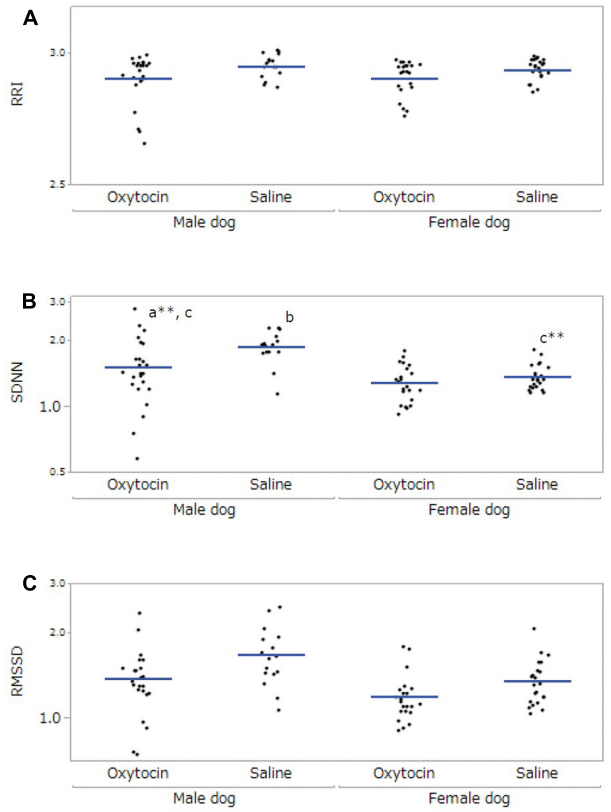

FIGURE 3 | Comparisons of heart rate variability of owners between oxytocin and saline treatment conditions. All parameters, RRI **(A)**, SDNN **(B)**, and RMSSD **(C)**, showed significant differences between oxytocin and saline treatments conditions. Significant dog-sex differences were observed in SDNN and RMSSD. A significant interaction between administration and dog's sex was found in SDNN. Results are shown with logarithmic. The blue lines indicates the mean of HRV. $^{**}p < 0.001$.

No significant main effect of "time of data collection" was found in any HRV parameter.

DISCUSSION

The aim of the present study was to investigate whether relationships based on the oxycitocinergic positive loop (discovered in European dog breeds) are also built between humans and ancient breeds, which are considered genetically closer to wolves. The results of this experiment demonstrated that, during a 30-min interaction, intranasal oxytocin did not affect the total amount of time during which dogs touched or were at close proximity to participants. However, there was an increase in the duration of the dog's gazing behavior at its owner. While Nagasawa et al. (2015) reported that intranasal oxytocin only intensified the gazing behavior of female dogs, we observed no difference between the sexes in the present study. It is also known that inter-species differences in oxytocin receptor exist (Donaldson and Young, 2008), as well as sex difference (Rilling et al., 2014). Moreover, the frequencies of the single-nucleotide polymorphisms of the oxytocin receptor gene are significantly different not only among wolves and dogs, but also among different dog breed groups (Tonoike et al., 2016). Therefore, these results suggested that sex differences may exist depending on the dog breeds, such as between European breeds and the more ancient Japanese breeds.

The urinary oxytocin in female dogs increased after 30 min interaction with owner in the current study as well as their owners, and this was not consistent with the results of Nagasawa et al. (2015) in which elevated urinary oxytocin after interaction under oxytocin treatment conditions only occurred in the owners of female dogs, but not in dogs themselves. The enhanced female dog gazing behavior due to oxytocin treatment activated the owner's oxytocin nervous system. However, since active contact between the owner and the dog was restricted during the interaction, we assume that the positive loop was interrupted and the dogs' urinary oxytocin was not elevated by 30-min experiment in the previous study (Nagasawa et al., 2015). However, the results of the present experiment suggested that the oxytocin positive loop was not inhibited in this ancient breed. The discrepancies between the current findings and those in the previous study included remarkably fewer approaches made by the dogs to their owners. The mean gaze duration of oxytocin-treated female Japanese dogs was slightly less than 80% of the previous study in European breeds only (Nagasawa et al., 2015: 244.55 ± 63.95 s, this experiment: 190.14 ± 56.60 s), and the mean skin-contact time was only 5.14 ± 2.41 s, and 1/3 of the female dogs did not touch their owners at all (Nagasawa et al., 2015: the mean skin-contact time was 78.78 ± 37.62 s, all oxytocin-treated female dogs touched their owner during 30-min interaction). While time spent in proximity to owners was significantly longer than to unfamiliar persons, it was still at only around 60% of the time recorded in the previous study (Nagasawa et al., 2015: 1011.60 ± 126.77 s, this experiment: 637.91 ± 136.05 s) (**Table 1**). This result is consistent with findings in Konno et al. (2016), which demonstrated that the gaze of the Japanese dog breed Akita was shorter than European breeds, and in Tonoike et al. (2015), which demonstrated that Japanese dog breeds followed and sought out contact with their owners much less frequently than European breeds. Moreover, the result of multiple regression analysis indicated that the elevation rate of urinary oxytocin in the owners decreased when the touch duration with owners was longer, as well as when dogs were males. Takeuchi and Mori (2006) conducted a questionnaire survey of veterinarians, and found that Japanese breeds were scored lower for playfulness and affection demand, and higher for aggression to dogs, watchdogs barking, territorial defense, and snapping at children than European breeds. Based on these characteristics, Japanese dog breeds as pet dogs would normally have less prolonged skin-contact with their owners than European breeds. Therefore, the restricted contact made by the owner during the present experiment is thought to have had no marked effect on oxytocin secretion in the Japanese dogs compared with the European breeds. However, the female dog's pre-treatment urinary oxytocin level under oxytocin treatment conditions was lower than that under saline treatment conditions, albeit not significantly different. Since the order of administration

TABLE 1 | The comparison between the previous studies and the current study.

(A) The results of oxytocin administration (inhibition of human's behavior)								
	Gaze	**Touch**	**Proximity**	**Urinary oxytocin**		**Heart rate variability**		
				Dogs	**Owners**	**RRI**	**SDNN**	**RMSSD**
European breeds [2]	F increased (OT)	N.S.	N.S.	N.S.	OW of F increased (OT)	–	–	–
Japanese breeds [3]	Both M and F increased (OT)	N.S.	N.S.	F increased (OT)	OW of F increased (OT)	OT > S	OT < S (Total and S), M > F	OT < S, M > F
(B) Dog's behavior in oxytocin administration (sec/30 min, inhibition of human's behavior)								
	Gaze		**Touch**		**Proximity**			
European breeds [2]	244.55 ± 63.95		5.14 ± 2.41		1011.60 ± 126.77			
Japanese breeds [3]	190.14 ± 56.60		78.78 ± 37.62		637.91 ± 136.05			
(C) Dog and wolf's behavior in free-interaction with owner (sec/5 min)								
	Dog's gaze to owners		**Talking to dogs**		**Touching dogs**			
European breeds [1]	57.93 ± 9.87		28.66 ± 6.34		54.51 ± 13.42			
Wolf [1]	0.21 ± 0.10		22.72 ± 5.36		33.86 ± 18.25			

OT, in oxytocin administration; S, in saline administration; M, male dogs; F, female dogs; OW, owners.
[1]Experiment 1 in Nagasawa et al., 2015; [2]Experiment 2 in Nagasawa et al., 2015; [3]The current study.

condition were counterbalanced, thus, we cannot explain this low pre-treatment urinary oxytocin level in female dogs. Therefore, it is essential to consider carefully the elevated urinary oxytocin in female dogs due to treatment with oxytocin in this experiment.

On the other hand, despite the absence of sex effects on the duration of dogs' gaze through administration of oxytocin, we observed an increase in urinary oxytocin concentration only in female dogs and their owners. Nagaswa et al. (2009) found a significant positive correlation between the number of behavioral exchanges that were initiated by dog's gazing behavior at its owner and the increase in the owner's urinary oxytocin. Therefore, although gaze plays an extremely important role as a trigger in the interaction between dogs and humans, there also needs to be other elements present, or elements that substitute gaze. In this experiment, we did not observe an effect of the sex of the owner on urinary oxytocin levels, but there may be human factors, which was subtle but different between the owners of the male and female dogs, that are involved in the elevation of urinary oxytocin levels.

This experiment was conducted based on the hypothesis that Japanese dogs are intermediates between wolves and European dog breeds concerning their bonding phenotype with humans. While wolves show almost no mutual gaze behavior with their owners, they demonstrate prolonged skin-contact. Furthermore, in previous studies (Nagasawa et al., 2015), the urinary oxytocin levels were significantly higher in wolves than dogs. This can be explained by the high degree of unity and cooperative behavior in wolf packs and their wariness of outgroups. Generally, Japanese dogs are also strongly wary and aggressive toward external parties and are not easily sociable with unfamiliar persons. Therefore, it was hypothesized that their urinary oxytocin levels would be higher than European breeds. Nevertheless, Japanese dogs did not actively touch their owners, unlike wolves, and the urinary oxytocin levels were similar to European breeds. There is a hypothesis that domestication in dogs is related to a modified stress response (Hare and Tomasello, 2005), which is regulated by glucocorticoids. Oxytocin itself antagonizes glucocorticoid secretion (Kikusui et al., 2006); therefore, these two neuroendocrine systems can be modified during the domestication process, either dependently or independently. Based on the results of the current experiment, urinary oxytocin concentrations in Japanese breeds are similar to those of European breeds and lower than that of wolves, suggesting their endocrine systems may differ substantially from that of wolves. The promoter regions of behavior-related genes were differently methylated between wolves and dogs (Banlaki et al., 2017). Investigating the differences in oxytocin, glucocorticoids, and these receptor genes, and epigenetic modifications between Japanese dog breeds and wolves is expected to provide major elucidation of the domestication process.

We also investigated the changes that occur in the autonomic nervous systems of owners during owner-dog mutual gaze. The owners' RRIs were significantly lower when the dogs were treated with oxytocin compared with saline treatment conditions, which indicated an elevation of heart rate. SDNN, which is an index of the activity of the autonomic nervous system, was also significantly lower when dogs were under oxytocin

treatment conditions compared with saline treatment, which indicates that the activity of the autonomic nervous system was decreased. RMSSD, which is an index of the activity of the parasympathetic nervous system, was also lower under oxytocin treatment conditions compared with saline treatment conditions. This indicates that the parasympathetic nervous system activity decreased. It was reported that a decrease in SDNN is related to the stress conditions when in parallel with decreased RMSSD (Task Force of The European Society of Cardiology and The North American Society of Pacing and Electrophysiology, 1996), whereas some studies showed that increase of the parameters of HRV was related to positive emotional states (Kreibig, 2010). The results of the current study correspond more to the former. In human studies, oxytocin promotes reactivity to attachment signals emitted from infants (Riem et al., 2011); thus, interaction with oxytocin-treated dogs enhanced owner's tension/arousal, probably due to the increase in eye-gaze attachment behavior. There were also sex differences in SDNN and RMSSD; the owners of female dogs displayed lower SDNN and RMSSD. Therefore, the owners of female dogs may have experienced more tension than the owners of male dogs. Further studies are needed to clarify the correlation between owners' HRV and oxytocin release. In addition, although gaze plays an extremely important role in the interaction between dogs and humans, it is possible there are elements other than gaze affecting the interaction. These yet uncharacterized signals may be modulating the owners' HRVs differentially for male and female dogs. As discussed above, the differences between the current findings and those from a previous study included remarkably fewer approaches made by the Japanese dogs toward their owners; the mean skin-contact time was only 5.14 $\pm$ 2.41 s, and 1/3 of the female dogs did not touch their owners at all (Nagasawa et al., 2015: 78.78 $\pm$ 37.62 s). Tonoike et al. (2015) also demonstrated that Japanese dog breeds followed and sought out contact with their owners much less frequently than European breeds. Moreover, the result of multiple regression analysis indicated that the elevation rate of urinary oxytocin in the owners decreased when the touch duration was longer. Based on these results, which differ from European breeds, Japanese dog breeds may use different subtle types of signals for establishing bonds with their owners. Future studies are needed to clarify this issue.

The results that Japanese dogs treated with oxytocin changed their gazing behavior toward their owners, which promoted the secretion of oxytocin in the owners, indicated that Japanese breeds, which were included in ancient breeds, also have the possibility to establish the oxytocin-gaze positive loop with humans; however, their manner of affiliative interaction with humans differed from both European breeds and wolves. Moreover, the interactions with dogs treated with oxytocin may cause the change of the owners' autonomic nervous system depending on the dog's sex although the direct effect of mutual gaze with dogs was not found. Considering the difficulty in collecting urine and monitoring their HRV with an electrocardiograph in Japanese breeds and how the mutual gaze between Japanese breeds and their owners was limited, any further discussion on this topic is difficult. In addition, the social experience and learning also modulate the human-directed behavior (Barber et al., 2016; D'Aniello and Scandurra, 2016). Therefore, further detailed analysis, including the factors such as breed, sex, social experience, and age, is needed using a larger dog sample size and direct comparisons with European breeds. These findings pave the way for future studies investigating the possibility of whether ancient breeds, including Japanese dog breeds, adopt different attachment strategies than European breeds.

AUTHOR CONTRIBUTIONS

As the first author, MN was involved in all steps of the process, and was the primary writer of the text. MO has been involved data collection and analysis. KM has been involved in the hormone assay, as well as the write up of the text. As supervisor, TK has been involved in the design and has contributed to the write-up.

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Increased Serum and Urinary Oxytocin Concentrations after Nasal Administration in Beagle Dogs

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In recent years more and more studies have revealed the effect of extraneous oxytocin on the social behavior of dogs. The distribution of administered oxytocin in different physiologically relevant compartments is important because this knowledge forms the basis for the timing of behavior tests after the administration. Most behavioral studies rely on the non-invasive intranasal application of oxytocin. The aim of this study was to determine the time course of intranasal administered oxytocin secretion into blood and urine and also establish a connection between intranasal received oxytocin and urinary cortisol in dogs. In our experiment, four dogs received three puffs, 12 IU intranasal oxytocin treatment, two dogs received three puffs intranasal placebo treatment. Blood and urine samples were collected immediately prior to the administration then regularly during 4 h. After nasal oxytocin application, the serum oxytocin concentration increased, reached a maximum 15 min after the treatment and then rapidly returned to baseline levels 45 min later. The peak urinary oxytocin concentration occurred between 45 and 60 min after administration and returned to baseline levels slowly. We found considerable differences among individuals in the secretion of oxytocin in both the serum and the urinary oxytocin concentration measurements. Our results confirm that intranasally administered oxytocin passes into the blood stream. The time course of intranasally administered oxytocin secretion is similar to the time course of intravenously administered oxytocin secretion, and the peak values are also similar in both the serum and the urinary oxytocin concentration measurements, although there are large individual differences.

Keywords: dog, intranasal, oxytocin, secretion, cortisol

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INTRODUCTION

Beside the reproductive functions, oxytocin plays an important role in the regulation of social behavior (pair bonding, sexual and maternal behavior, peer recognition, and social memory) [for reviews, see Ref. (1, 2)]. Recent studies have revealed the relevance of oxytocin in human bonding, trust and in some aspects of human social cognition including social perception, emotion recognition, sensitivity to others' experiences, and prosocial behaviors [for reviews, see Ref. (3, 4)].

The manipulation of the oxytocin system is a possible tool for changing sociocognitive performance. Human studies reported some beneficial effects of oxytocin on social attention and emotion

recognition in autistic individuals, and reduction of social anxiety in patients suffering from social phobia and borderline personality disorder (5–9).

Similar therapeutic value can be relevant for companion animals with behavioral problems. There are millions of dogs and cats relinquished to shelters after abandonment or abuse. Medical treatment as a complementary therapy to behavioral intervention may help the social integration of these companion animals to rejoin human families.

In recent years, increasing attention has been paid to the effect of extraneous oxytocin on the social behavior of dogs (10–14).

There have been some doubts whether and how intranasally administered oxytocin reaches specific brain areas. Thus, several studies attempted to measure oxytocin concentration and the time course of this effect in the brain, plasma, urine, and saliva. In male rats and mice, increased oxytocin content was measured in microdialyzates from both the left amygdala and the right dorsal hippocampus after nasal application of oxytocin (15). This study showed that nasally administered oxytocin reaches behaviorally relevant brain areas and these changes are paralleled by changes in plasma oxytocin concentrations. The pharmacokinetics of intranasally administered oxytocin was also investigated in human saliva, blood, and cerebrospinal fluid (CSF) (16–19), in dog plasma and urine (10), in rhesus macaque blood and CSF (20–22), and in pig CSF (23). Knowing the distribution of oxytocin in different physiologically relevant compartments is important because this knowledge forms the basis for the timing of behavior tests after the administration of oxytocin. So far in behavior studies intranasal administration is followed by a 40 min waiting period that is presumed to be necessary for central oxytocin levels to reach a plateau based on the vasopressin measurements in the CSF (24).

The short-term use of intranasal oxytocin administered to humans in dosage up to 40 IU results in no adverse reactions (25). Only few studies have investigated the effects of long-term administration of oxytocin in humans (26–28). Some researchers have also revealed dosage dependent effects of intranasally administered oxytocin (29, 30) that should be considered in the case of regular use. Some negative effects of oxytocin were also documented (26, 31). For example, in male prairie voles (*Microtus ochrogaster*), long-term developmental treatment with low doses of intranasal oxytocin resulted in a deficit in partner preference behavior (32).

Intracerebral oxytocin inhibits the stress-induced activity of the hypothalamic–pituitary–adrenal axis responsiveness, thus oxytocin may have an inhibitory influence on stress-responsive neurohormonal systems under physiological condition (33). Some studies found that suckling stimulation produced a significant increase in plasma oxytocin levels and a significant decrease in plasma cortisol concentrations (34–37). Human research demonstrated that oxytocin infusion decreases plasma cortisol (38, 39). In contrast, no significant alterations in cortisol were observed following intranasal oxytocin administration (16). After positive human–dog interaction both species showed significant increases in plasma oxytocin, but only human participants showed a significant decline of cortisol. No similar change occurred in dogs (40). Little increase was found in urinary cortisol levels after intravenous oxytocin injection in dogs, and exercising increased urinary oxytocin concentrations, but had no effect on urinary cortisol (41). In this study, the authors argued that increased oxytocin may have inhibited a cortisol response despite other observations that exercise increases cortisol concentration [e.g., Ref. (42, 43)]. These discrepancies suggest that the inhibitory effect of oxytocin on cortisol secretion may not always be observed.

Most behavioral studies rely, however, on the non-invasive intranasal application of oxytocin. Thus, the aim of this study is to determine the time course of oxytocin secretion into blood and urine after intranasal administration and also establish a connection between blood oxytocin and urinary cortisol in dogs. We also measured the cortisol/creatinine ratio (C/C ratio) to control the water metabolism of the dogs. Our study is a follow up experiment to the work of Mitsui et al. (41) who used i.v. administration. In general, we have expected similar pharmacokinetic effect of the drug thus we included only a restricted sample of dogs based on ethical considerations.

MATERIALS AND METHODS

Subjects

Six (three males, three females, mean age: 2.75 ± SD = 1.13; SEM = 0.46) healthy intact beagle dogs bred by the National Public Health Center, National Research Directorate for Radiobiology and Radiohygiene (OKK-OSSKI) were involved in the study. The animals were not given any medication prior to the study, and they have not previously participated in similar research. Identification of individuals has occurred on the basis of chips' last four digits. Four dogs represented randomly the experimental group (724, 233—males, 825, 827—females), two beagles were assigned to the control group (760—males, 9,695—females).

Ethical Statement

Research was done in accordance with the Hungarian regulations on animal experimentation and the Guidelines for the use of animals in research described by the Association for the Study Animal Behavior. Ethical approval was obtained from the National Animal Experimentation Ethics Committee [Ref. No.: TTK/12187/1 (2016), Cert. No.: ELTE-AWC-016/2016].

Experimental Procedure

Preparation

Hair was sheared above the *v. cephalica antebrachii*, and the skin was cleaned by alcoholic disinfectant solution. Venous catheter were placed and fixated in all dogs. Urinary bladder was emptied by urethral catheter just before initiation of testing. Urine samples of bitches were collected by Foley catheter, and samples of males were collected by Buster male catheter. Test was started within 30 min after the placement. All dogs were familiar with the experimental room.

Treatment

Four dogs (two males and two females) received three puffs, 12 IU (4 IU/puff) intranasal oxytocin (Syntocinon, Novartis) treatment. This amount is regularly applied in dogs [e.g., Ref. (11, 12)]

and it is half of the amount typically used in human studies [e.g., Ref. (44, 45)]. Two dogs (one male and one female) received three puffs intranasal placebo (0.9% NaCl solution) treatment. No force was applied during treatments. The assistant who was familiar to the dogs, held the head of the animals gently for the time of the application. We did not miss any administration. Dogs were kept in individual cages in a silent room after the administration, between the sample collections water was available *ad libitum* and animals saw each other during the examination period.

Collection of Samples

Blood and urine samples were collected immediately prior to the administration (Time 0), then every 15 min between 0 and 2 h (Time 15, 30, 45, 60, 75, 90, 105, and 120) and followed by 30 min sampling up to 4 h following the administration (Time 150, 180, 210, and 240). Sample collection did not take longer than 3–4 min per dog per occasion and was done by a trained veterinarian and assistant who were familiar to the subjects on daily basis. Blood samples (0.5–0.7 ml) were collected in tubes kept on ice without anticoagulant containing aprotinin and just after coagulation were centrifuged at 1,000 × *g* for 15 min at 4°C. Coagulation was done for about 20 min. The ice-keeping period was depending on coagulation and occupation of centrifuge but not more than 40 min. Ice was supplied as needed. Serum samples were frozen at −80°C and urine samples were kept at −20°C until the measurement.

Immunochemistry

Serum and urinary oxytocin concentrations were measured by competitive ELISA kits. Samples were extracted as it is prescribed in assay procedure (Oxytocin ELISA DE-3117, Demeditec Diagnostics GmbH, Germany; detection range: 15.6–1,000 pg/ml; reactivity: human, all animals). An equal volume of 0.1% trifluoroacetic acid in water (TFA–H_2O) was added to the sample and centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant was filtrated by Sep-Pak column (Waters, Hungary) pretreated with acetonitrile and TFA–H_2O. The sample was eluted from the column with use of acetonitrile and TFA–H_2O mixture. The sample was evaporated by centrifugal vacuum concentrator (Labconco, USA). The samples were reconstituted with assay buffer occurred just before the measurement. The extraction efficiency was determined by 200 pg/ml oxytocin spiked, extracted, paired samples. The assay procedure describes the way of sample dilution with Standard 0, and thus we accepted the linearity as it is described in the product information leaflet. Assay precision is described with 10.2% intra-assay and 11.8% inter-assay CV, sensitivity 15.0 pg/ml.

Urinary creatinine concentrations were measured by colorimetry (Creatinine, Normachem, Hungary), 2.2% intra-assay and 3.89% inter-assay CV, sensitivity 2.3 mmol/l. In the initial assays, some specimens (six) were found to contain more oxytocin and creatinine than the highest standard. Therefore, these specimens were diluted with Standard 0 in 1:4 ratio, and final concentration was calculated by four times multiplication of measured result.

Urinary cortisol concentrations were measured by Cortisol ELISA (DRG International Inc., USA), 3.2% intra-assay and 7.7% inter-assay CV, sensitivity 6.9 nmol/l.

Data Analysis

In the case of urinary oxytocin and cortisol concentration measurement, there are missing data at some time points. There was no urine in the urinary bladder in these cases. Due to missing data, the low number of subjects, and the individual differences, the possibility of statistical analysis of these data is limited. In addition to the descriptive analysis, statistical analyses were carried out using SPSS (version 22.0.0). Based on visual inspection of serum oxytocin concentration changes (see **Figure 1**), we focused on the data between 15 and 60 min and used linear mixed models including time and treatment and their two-way interaction as fixed factors, and dog ID as a random term.

RESULTS

Serum Oxytocin Concentration

The basal concentration was 58.60 ± SD = 18.27; SEM = 7.46 pg/ml ($N = 6$). After nasal oxytocin application, the serum oxytocin concentration increased, reached a maximum 15 min after the treatment, and then rapidly returned to baseline levels (Time 45) (**Figure 1**). We found treatment × time interaction to have significant effect both on absolute and relative (i.e., compared to baseline) concentrations [absolute: treatment × time $F(4,16) = 30.35$, $p < 0.001$; driven by differences between relative oxytocin levels at $T = 30$ vs $T = 60$: $b \pm$ SE = 444.60 ± 112.70 pg/ml, $t_{16} = 3.95$, $p = 0.001$ and $T = 15$ vs $T = 60$: $b \pm$ SE = 936.28 ± 112.70 pg/ml, $t_{16} = 8.31$, $p < 0.001$ in oxytocin treated as opposed to control group]. Similar results were obtained when concentrations at the start of the experiment (time = 0) were subtracted from concentrations measured at later times (15, 30, 45, and 60 min) following oxytocin or placebo treatment [relative: treatment × time $F(3,12) = 26.78$, $p < 0.001$; driven by differences between relative oxytocin levels at $T = 30$ vs $T = 60$: $b \pm$ SE = 444.60 ± 125.56 pg/ml, $t_{12} = 3.54$, $p = 0.004$ and $T = 15$ vs $T = 60$: $b \pm$ SE = 936.28 ± 125.56 pg/ml, $t_{12} = 7.46$, $p < 0.001$ in oxytocin treated as opposed to control group].

Urinary Oxytocin Concentration

Based on the existing data, the basal concentration was 429.29 ± SD = 113.76; SEM = 50.87 pg/ml ($N = 5$). The peak urinary oxytocin concentration after nasal oxytocin application in female dogs occurred between Time 45 and Time 60 and returned to baseline levels slowly. There are missing data at some data point mainly for male dogs (urinary oxytocin: 78 sample collection, 12 missing data), because the urinary bladder was empty. Large individual differences are perceivable in the decay of the peak values for the two females. The values of the placebo treated dogs' urinary oxytocin concentrations are clearly distinguishable from the values of the oxytocin treated dogs, there are not similar high peaks for the former (the concentrations remain low, under 500 pg/ml) (**Figure 2**).

Urinary Cortisol Concentration

Based on our data, the basal concentration was 189.56 ± SD = 50.90; SEM = 22.76 pg/ml ($N = 5$). Mainly in the case of

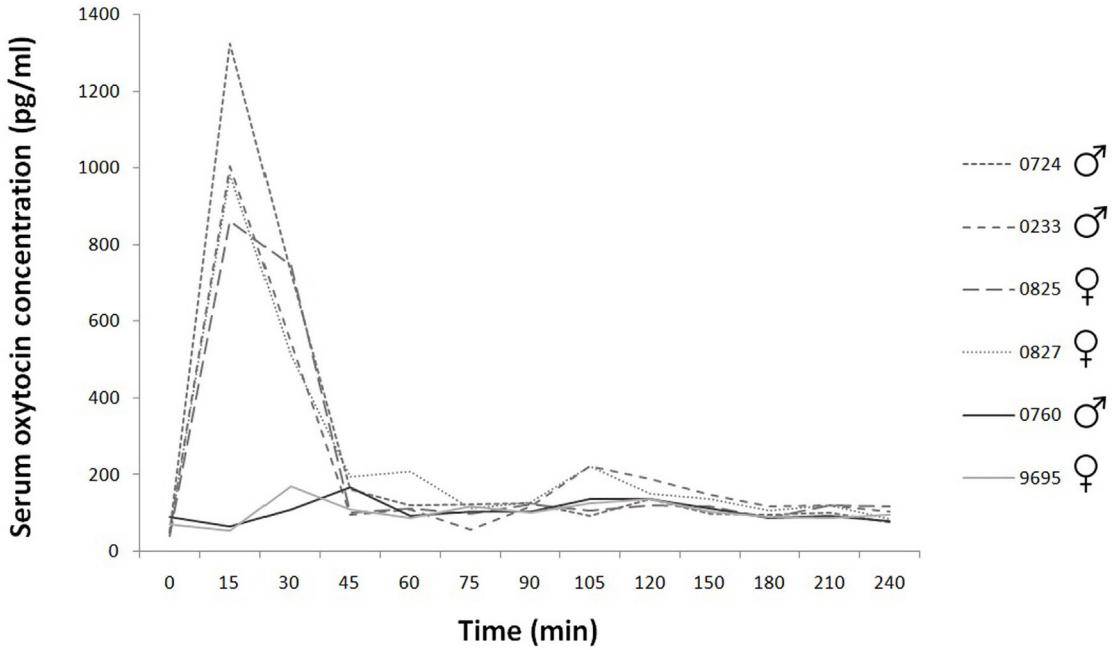

FIGURE 1 | Time course of individual serum oxytocin concentrations during the 240 min. Experimental group: dashed line; control group: continuous line.

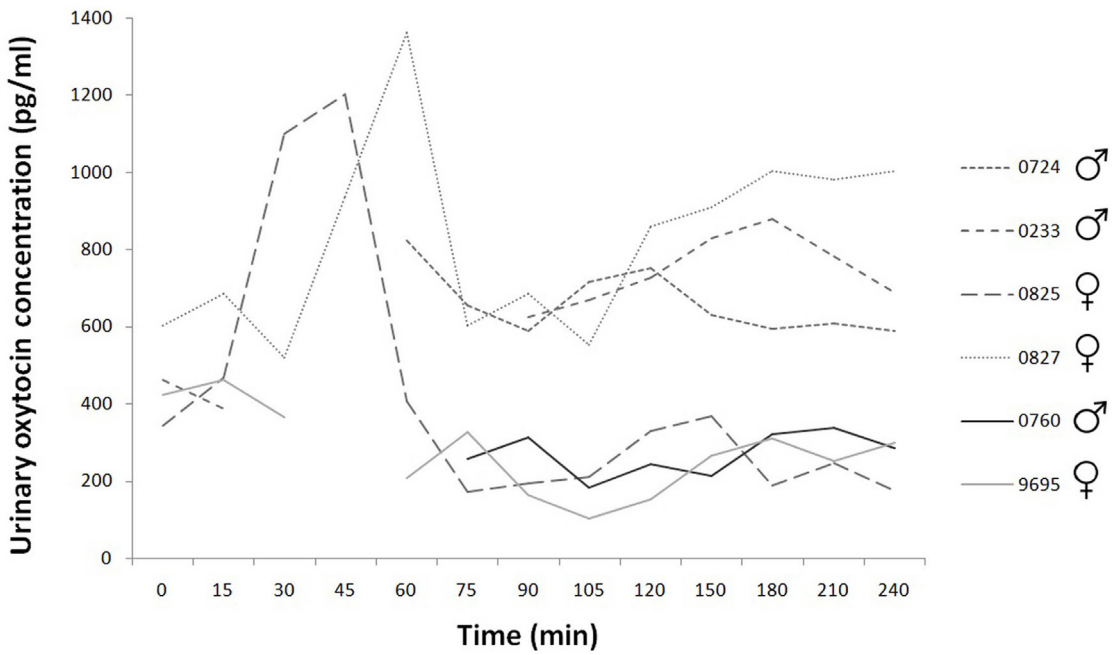

FIGURE 2 | Time course of individual urinary oxytocin concentrations during the 240 min. Experimental group: dashed line; control group: continuous line.

male dogs there are missing data points (urinary cortisol: 78 sample collection, 12 missing data), there was no urine in their bladder at these times. However, according to the available data, the concentration values are clearly higher in male dogs than female dogs (**Figure 3**).

Cortisol/Creatinine Ratio (C/C Ratio)

The C/C ratio indicated the normal water metabolism, so the measured oxytocin and cortisol concentrations were not influenced by any of the factors related to the water excretion (**Figure 4**).

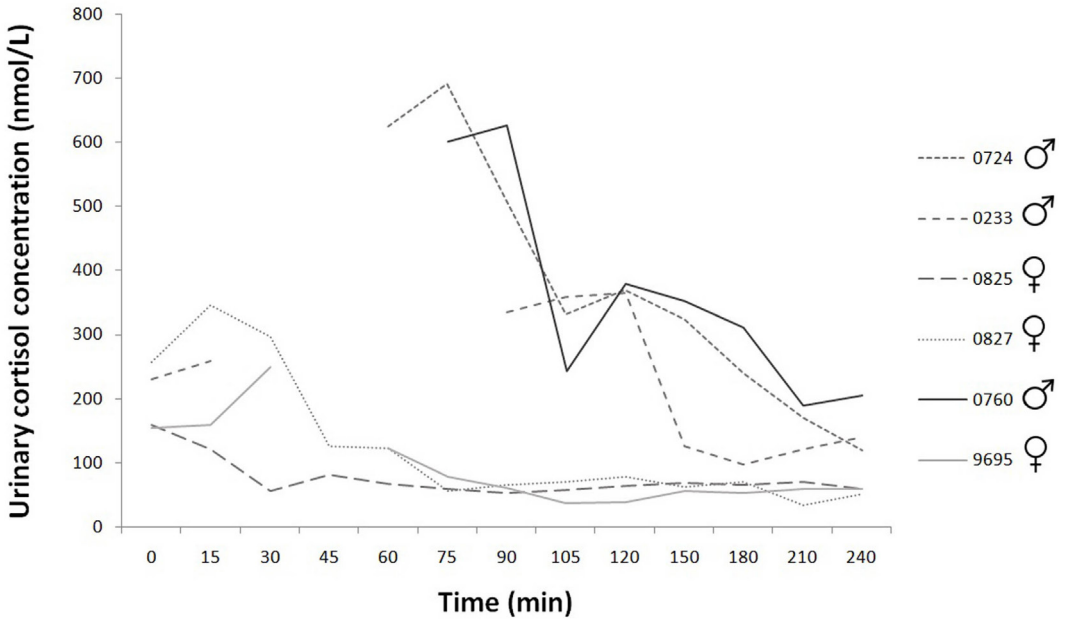

FIGURE 3 | Time course of individual urinary cortisol concentrations during the 240 min. Experimental group: dashed line; control group: continuous line.

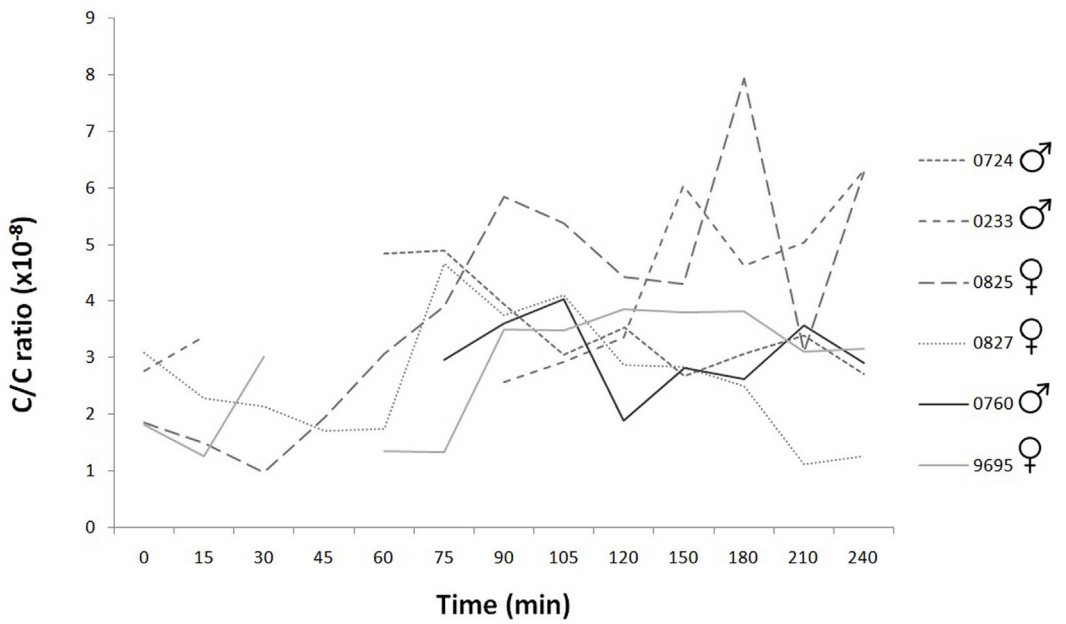

FIGURE 4 | Time course of individual cortisol/creatinine ratios during the 240 min. The C/C ratio of healthy dog is under 10×10^{-6}. The values did not exceed this limit during the examination period in any subjects.

DISCUSSION

The aim of this study was to measure the time course of intranasal administered oxytocin secretion into blood and urine and investigate the connection between intranasal received oxytocin and urinary cortisol in dogs.

After three puffs, 12 IU intranasal oxytocin, the serum oxytocin concentration increased, reached a maximum 15 min following the treatment then rapidly returned to baseline levels (Time 45). Similar findings were reported in dogs; however, in that study a higher dose of oxytocin was used (10). This explains that the oxytocin concentration was significantly higher in treated

animals than in controls even after 90 min. Our results are more likely in line with the time lapse of plasma oxytocin concentrations were measured in dogs after the animals were injected with exogenous oxytocin intravenously (41). Human studies also found sharp increase in plasma oxytocin concentrations peaking at 10–40 min after treatment, and the levels returned to baseline only at 75–150 min after administration (16, 17, 19, 46). Our results confirm that intranasally administered oxytocin passes into the blood stream. The concentration increases rapidly for a limited time but the time window differs among studies and possibly also among individuals (17).

Recent studies compared plasma oxytocin concentrations after using either intranasal spray or a nebulizer (20, 21). Only the nasal spray oxytocin administration resulted in significant increases in peripheral oxytocin. Further, the concentration returned to the baseline level sooner after nebulizer, this time course is similar to our results after treatment with nasal spray.

In the case of urinary oxytocin and cortisol concentration measurement, we could not obtain data at some time points because there the urinary bladder was empty. Each handling induces some excitation in dogs; water consumption is influenced by excitation also. Animals had free access to water but they did not drink enough in an "interesting" situation. Total emptying of urinary bladder was needed for correct measurement of excretion, but the decreased interest for drinking limited the urination. Due to these missing data, the low number of subjects, and the individual differences, the possibility of statistical analysis was limited. Nevertheless, the peak urinary oxytocin concentration occurred between Time 45 and Time 60 and returned to baseline levels slowly. Similar findings were presented after intravenous oxytocin injection (41). According to the descriptive analysis, we found considerable differences between individuals in the secretion of oxytocin in both the serum and the urinary oxytocin concentration measurements.

Many studies reported elevated oxytocin levels in the CSF after intranasal oxytocin treatment (19, 20, 23). This effect has been explained by three non-exclusive mechanisms (47–49): (1) direct passage of exogenous oxytocin through the BBB; (2) indirect feedback signals from the periphery could stimulate endogenous oxytocin secretion; and (3) oxytocin utilizes specific connections between the nasal cavity and the brain provided by the olfactory and trigeminal nerves. The first possibility was questioned by several early studies (50, 51). The contribution of the second mechanism was made less likely by showing that intranasally administered exogenous (D_5-deuterated) oxytocin increased labeled oxytocin in the CSF but did not change plasma and CSF endogenous oxytocin concentrations (22). Thus, it is most likely that intranasal oxytocin reaches the brain directly by various extracellular mechanism involving perineuronal channels, perivascular spaces, or lymphatic channels (52, 53).

Although we know that intranasally administered oxytocin passes into the CSF, further research is needed to reveal whether the central access is responsible for the neurobehavioral effects demonstrated by previous studies or peripheral pathways also contribute to the observed effects. At the moment, the distribution of administered oxytocin in the brain is also unknown (22).

Treatment did not change urinary cortisol concentration; however, according to the available limited data, the concentration values are higher in male dogs than female dogs. The venous cannula and the urethral catheter placement, fixation and saliva sampling did not cause any significant, increased pain, and stress to the normal veterinary intervention. Nevertheless, one may assume that the sexes reacted differently to the handling procedure. Similar sex difference in cortisol responses to psychological stress was also found in humans (54, 55).

The serum estrogen and progesterone concentrations of bitches vary depending on estrous stage, in contrast to testosterone in males, which is roughly constant (56). In addition, the pathway of steroid hormone metabolism in mammals is influenced and limited by different enzymatic effects. The higher cortisol levels in males can be explained by the multistep cascade mechanism of steroids, cortisol is also newly formed from its breakdown products. Males generally show an increased cortisol regeneration enzyme activity (57). This may also explain the higher cortisol levels in males. However, it is possible that there is difference between the effect of endogenous and the effect of exogenous oxytocin on cortisol secretion.

Due to the invasive nature of such research (and the follow up aspect of this study), we [see also Ref. (10, 17, 23, 41)] limited sample size following animal welfare recommendations (58). However, the sampling success—especially in complex living organisms—cannot be guaranteed. The lack of data and the small number of subjects precluded partly the statistical analysis. Large individual and methodological differences among oxytocin studies warrant further independent investigations.

In addition to the possible individual differences, the pharmacokinetics of oxytocin might also differ in females and males and in different species. More investigations are needed to determine safe and effective doses for chronic intranasal oxytocin both in different sexes and in different species.

CONCLUSION

In summary, our results confirm that similarly to i.v. application, intranasally administered oxytocin passes into the blood stream. The time course of intranasally administered oxytocin secretion is similar to the time course of intravenously administered oxytocin secretion, and the peak values are also similar in both the serum and the urinary oxytocin concentration measurements, although there are large individual differences.

ETHICS STATEMENT

Research was done in accordance with the Hungarian regulations on animal experimentation and the guidelines for the use of animals in research described by the Association for the Study Animal Behavior (ASAB). Ethical approval was obtained from the National Animal Experimentation Ethics Committee [Ref. No.: TTK/12187/1 (2016), Cert. No.: ELTE-AWC-016/2016].

AUTHOR CONTRIBUTIONS

AT: substantial contributions to the conception and design of the work; the acquisition, analysis, and interpretation of data for the

work; drafting the work and revising it critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. JT and LB: substantial contributions to the design of the work; the acquisition and analysis of data for the work; revising the work critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. ÁM: substantial contributions to the conception of the work; the interpretation of data for the work; revising the work critically for important intellectual content; final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Effects of Affiliative Human–Animal Interaction on Dog Salivary and Plasma Oxytocin and Vasopressin

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Oxytocin (OT) and vasopressin (AVP) are neuropeptides with diverse effects on social behavior, cognition and stress responses. Recent studies suggest that OT facilitates and responds to affiliative forms of human–animal interaction (HAI). However, previous studies measuring OT and AVP in dogs have been limited to measures from blood or urine, which present concerns related to the invasiveness of sample collection, the potential for matrix interference in immunoassays, and whether samples can be collected at precise time points to assess event-linked endocrine responses. Previous studies from our laboratory validated salivary measures of OT and AVP in dogs, however, it is currently unknown whether these measures respond dynamically to aspects of HAI. Here, we investigated the effects of affiliative forms of HAI on both plasma and salivary OT and AVP in dogs. We employed a within- and between-subjects design with a group of Labrador retrievers and Labrador retriever × golden retriever crosses (23 females, 15 males). Half of the dogs engaged in 10 min of free-form friendly interaction with a human experimenter (HAI condition), and the other half rested quietly in the same environment, without human interaction (control condition). We collected blood and saliva samples before, and immediately following both experimental conditions, and all samples were analyzed using enzyme-linked immunosorbent assays (ELISAs) following previously validated protocols. Dogs participating in HAI exhibited a significant increase in both salivary OT (+39%) and plasma OT (+5.7%) whereas dogs in the control group did not. Salivary AVP showed no change in the HAI group but increased significantly (+33%) in the control group. Plasma AVP decreased significantly following HAI (−13%) but did not change across time in the control condition. Within the dogs exposed to HAI, increases in salivary OT, and decreases in plasma AVP, were predicted by the extent of affiliative behavior between the dog and human (indexed by scores from a principal components analysis of social behaviors between the dog and human). Collectively our results suggest that measures of salivary OT and AVP provide useful biomarkers in studies of HAI, and afford a flexible and non-invasive toolkit than can be employed in diverse research contexts.

Keywords: oxytocin, vasopressin, dog, human–animal interaction, ELISA, saliva, plasma

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INTRODUCTION

Studies throughout the last three decades have explored the psychological and physiological effects of human–animal interaction (HAI). Often the aim of such studies is to characterize the mechanisms through which non-human animals affect human health and wellbeing, and in turn, how interaction with humans affects animal participants. Recently such studies have begun to focus on the neuropeptide oxytocin (OT), which, together with (structurally related) arginine vasopressin, is well known for its roles in facilitating selective social bonds, and regulating various aspects of social behavior and cognition in mammals (Carter et al., 2008). For example, central OT administration can facilitate maternal behavior in sexually naïve rodents (Pedersen and Prange, 1979; Caldwell and Young, 2006), and both OT and AVP are critical to the formation of partner preference in pair-bonded species (Carter et al., 1992). With regard to the establishment of selective social attachment, the OT receptor (OXTR) is highly expressed in the nucleus accumbens of some pair-bonded species, suggesting that OT may help to encode social reward through modulation of the mesolimbic dopamine pathway (Insel and Shapiro, 1992; Lim and Young, 2006).

Research on the role of OT in HAI has been conducted by measuring OT release during interactions between humans and dogs, and evaluating the effects of exogenous OT administration on dog behavior in this context. Studies of endogenous OT have revealed increases in blood or urinary OT concentrations in both humans and dogs following affiliative interaction in dog-human dyads (Miller et al., 2009; Nagasawa et al., 2009; Handlin et al., 2011; Rehn et al., 2014; Nagasawa et al., 2015). Additionally, preliminary evidence suggests that dogs bred for friendly and non-aggressive temperaments are characterized by high levels of plasma OT, relative to pet dogs (MacLean et al., 2017b). Studies using intranasal OT administration in dogs suggest that exogenous OT can facilitate social play (Romero et al., 2015), and promote bonding with both conspecifics and humans (Romero et al., 2014). Additionally, recent studies have demonstrated that exogenous OT can improve dogs' sensitivity to human communication (Oliva et al., 2015; Macchitella et al., 2016). Thus, a rapidly growing body of research suggests that OT pathways may be centrally involved in affiliative forms of HAI (Beetz et al., 2012; MacLean and Hare, 2015; Carter and Porges, 2016).

We are not aware of any studies investigating AVP in the context of HAI, however, many of the effects of OT are also critically dependent on AVP (Carter, 1998; Landgraf and Neumann, 2004). Although AVP plays important roles in selective sociality, particularly among males (Caldwell et al., 2008), its effects are often antagonistic to those of OT. For example, many of the social effects of OT during HAI may be facilitated by OT's attenuation of sympathetic arousal (Kis et al., 2014; Buttner, 2016) through actions in the hypothalamus (Dabrowska et al., 2011), and on the vagus nerve (Porges, 2003, 2007, 2011). In contrast, AVP activates the hypothalamic-pituitary-adrenal (HPA) axis, and is more strongly linked to anxiety and aggression (Coccaro et al., 1998; Neumann and Landgraf, 2012). We are aware of two studies investigating relationships between AVP and behavior in dogs, both of which revealed positive associations with anxiety or aggression (Hydbring-Sandberg et al., 2004; MacLean et al., 2017b). Given its structural and functional relationships with OT, we expect that AVP may also regulate or respond to aspects of HAI, although these effects are likely to differ from those for OT.

To date, all studies of OT and AVP in dogs engaged in HAI have quantified peptide concentrations in blood or urine samples. Although urine sampling can be performed non-invasively, it yields poor temporal resolution and characterizes long periods of peptidergic activity. In contrast, blood sampling can capture acute changes in peptide release, but is an invasive procedure that may induce stress and acute pain, and is not well suited to many of the contexts in which HAI studies are conducted. Additionally, OT and AVP rapidly bind to other molecules in blood (and likely urine) which can lead to matrix interference in assays, or erroneously low estimates of peptide concentrations (Martin and Carter, 2013; Brandtzaeg et al., 2016). We recently validated methods for quantifying OT and AVP in dog saliva samples, which can be collected non-invasively, and at precise time points during HAI. Enzyme-linked immunosorbent assays (ELISAs) yielded good parallelism and accuracy, and did not require an extraction procedure. Lastly, we previously measured salivary OT in nursing dams and detected an acute rise in OT associated with milk letdown, providing an initial biological validation of this measure (MacLean et al., 2017a).

In the current studies, we evaluated both plasma and salivary OT and AVP concentrations in dogs, before and after affiliative interaction with a human, or in a control condition. Because the time course of salivary OT/AVP release during HAI was unknown, we first conducted a short pilot study to identify time points associated with changes in salivary OT/AVP concentrations during HAI. We then conducted an experiment with dogs assigned to an HAI or control condition, and assessed OT/AVP changes over time in both groups, and as a function of behavior during the test period. Based on the studies described above, we hypothesized that dogs in the HAI condition would exhibit increases in OT across the study period, and that any such changes would be larger in the HAI group than the control group. We also hypothesized that within the HAI group, changes in OT would be predicted by the extent of affiliative behavior between the human and the dog during the study. Because no studies have investigated effects of HAI on AVP, we had no specific hypotheses for the nature of this response. However, given that AVP also plays important roles in social emotions and behavior, we expected that dogs in the HAI group would exhibit changes in AVP that differed from those of dogs in the control group.

PILOT STUDY

Prior to the main HAI study, we conducted a short pilot study with 10 dogs to identify the optimal sampling periods for

detecting changes in salivary OT and AVP. Based on our studies with nursing dams (MacLean et al., 2017a), and recent studies measuring salivary OT in humans (de Jong et al., 2015), we expected changes in saliva to be rapid. Therefore, we assessed both plasma and salivary OT and AVP at 5 and 10 min following the start of HAI. In both the pilot study and Experiment 1, we opted to collect both blood and saliva, despite the possibility that blood draws may impose a mild stressor with potential to influence hormonal changes detected saliva. We adopted this strategy because no studies have measured salivary OT/AVP in dogs during HAI, and consequently any effects (or lack thereof) in saliva would be challenging to interpret without knowledge of how blood concentrations had changed across the same period.

Method

Subjects

We tested dog subjects from the breeding colony at Canine Companions for Independence (CCI) in Santa Rosa, CA, United States. The pilot sample included 10 dogs (eight female, two male, four Labrador retrievers, six Labrador retriever × golden retriever crosses, mean age = 1.7 years (range = 1.6–1.8 years)). All dogs were pair-housed in indoor–outdoor kennels with *ad libitum* access to water and daily access to large outdoor play yards. Subjects were tested in a quiet room inside a familiar building on CCI's campus. Client consent was obtained for participation of all dogs and all animal procedures were approved by the Duke University IACUC (protocol #A138-11-06).

Procedure

Prior to the test, each dog was allowed to rest quietly in a crate (outside the test room) for 30 min. The dog was then taken to a nearby room and we collected a baseline blood and saliva sample. Saliva samples were collected using the Salimetrics Children's swab as described in MacLean et al. (2017a). Blood samples were collected from the cephalic vein into vacutainers (3 mL) containing ethylenediaminetetraacetic acid (EDTA). Blood and saliva samples were collected concurrently to minimize the time required for these procedures. Specifically, at the start of the collection period, one experimenter placed the swabs between the dog's cheek and mandibular teeth and gently held the dog's mouth closed while the second experimenter performed the blood collection. All samples were immediately placed on ice after collection.

Following this initial sample, dogs were allowed to rest in a crate inside the test room for 5 min prior to the start of the behavioral interaction. After 5 min, dogs were released from the crate and allowed to interact freely with the experimenter. The experimenter attempted to engage the dog in friendly interaction, including gently petting the dog, and speaking to the dog in a friendly tone while making eye contact. However, the experimenter allowed the dog to lead these interactions, and dogs were always free to disengage and move away from the experimenter at any point during the interaction. If subjects attempted to engage the experimenter in play (e.g., performing a play bow, chasing, or nuzzling the experimenter), the experimenter engaged with the dog in these more active forms of interaction. After 5 min of HAI we collected a second blood and saliva sample from the dog, and immediately resumed HAI for another 5 min.

The final blood and saliva samples were collected 10 min after the start of HAI and dogs received a food reward at the conclusion of this period (following the final saliva collection). The second and third blood samples were collected from (1) the cephalic vein on the opposite forelimb from the initial draw, and (2) the jugular vein, in order to avoid repeated needle punctures in the same location. We did not use a catheter for repeated collections from the same site because pilot tests revealed that catheters did not reliably maintain access to the vein when dogs were allowed to move freely between collection periods, and the procedures required to position, wrap, and access the catheter repeatedly were deemed more likely to cause discomfort for the dog than single collections from different sites. All biological samples were immediately frozen at −20°C.

Hormone Analysis

All samples were analyzed by enzyme-linked immunoassay (ELISA) following protocols previously validated in our laboratory (MacLean et al., 2017a,b). OT samples were measured using a commercially available kit from Cayman Chemical (Item #500440) and AVP samples were measured using a commercially available kit from Enzo Life Sciences (ADI-900-017A). Saliva samples were not extracted based on the results of validation studies (MacLean et al., 2017a) but plasma samples were processed using solid phase extraction (to isolate free peptide concentrations) with the protocols described in MacLean et al. (2017b).

Statistical Analysis

All statistical analyses were performed in the R programming environment for statistical computing (R Core Team, 2017). For each matrix (saliva, plasma) and hormone (OT, AVP), we used linear mixed models to predict the log transformed hormone concentration as a function of a fixed effect of time (baseline, +5 min, +10 min) and a random effect of subject ID. We used planned Dunnett contrasts to assess mean differences between hormone concentrations at baseline and +5 min, and between baseline and +10 min.

Results and Discussion

Table 1 shows the results of linear mixed models predicting changes in hormone concentrations as a function of time. We inspected the data for time points associated with the largest deviations from baseline values.

Salivary OT increased slightly from baseline to +5 min, with a further increase at +10 min that was marginally different from baseline ($p = 0.05$; **Table 1**). Changes in plasma OT and both salivary and plasma AVP were minimal, with no clear deviations from baseline. Therefore, we identified the +10-min measure as the most likely to show an HAI related effect in salivary OT, and collected samples at this time point in Experiment 1.

TABLE 1 | Pilot study results.

	T5–T0			T10–T0		
	β	*SE*	*p*	β	*SE*	*p*
Salivary OT	0.14	0.17	0.40	0.33	0.17	0.05
Plasma OT	0.03	0.04	0.48	0.01	0.04	0.83
Salivary AVP	−0.19	0.15	0.22	−0.05	0.15	0.73
Plasma AVP	−0.05	0.12	0.67	−0.07	0.12	0.56

Baseline (T0) was used as the reference value and the T5-T0 and T10-T0 Dunnett contrasts show the estimates, and standard errors of estimates, for changes in hormone concentrations at each time point, relative to T0 (β = change in log pg/mL from T0).

TABLE 2 | Subject demographics by condition for Experiment 1.

Condition	Breed	Female	Male
HAI	LAB	5	1
	LGX	6	7
Control	LAB	4	1
	LGX	8	6

HAI, human–animal interaction; LAB, labrador retriever; LGX, labrador × golden retriever cross.

EXPERIMENT 1

Method

Subjects

We tested 38 dogs (11 Labrador retrievers, 27 Labrador retriever × golden retriever crosses, 23 female, 15 male, mean age = 1.8 years, range = 1.6 – 2.2 years). All subjects were assistance dogs in training at Canine Companions for Independence and were housed and cared for as described above. Half of the subjects were assigned to the HAI condition, and half were assigned to the control condition (see below). Groups were matched closely based on sex and breed (**Table 2**).

Procedure

The HAI procedure was identical to that in the pilot study with the exception that we collected blood and saliva samples at only one time point (+10 min) after the baseline measure, based on preliminary data suggesting the greatest changes in salivary OT at this time. Therefore, the experimenter and dog had 10 continuous minutes of interaction prior to the post-test sample collection. In the control condition, dogs were placed inside an exercise pen (∼3 m × 3 m) in the test room. The experimenter remained in the room with the dog but did not interact, speak to, or make eye contact with the dog during the 10-min test period. We opted to use an exercise pen because this structure provided dogs with opportunities to locomote similarly to dogs in the HAI condition, but prevented dogs from physically accessing the experimenter. Additionally, because all subjects were accustomed to resting quietly in novel environments during their training for assistance work, it was unlikely that containment inside the exercise pen would impose a significant stressor. As in the HAI condition, after 10 min we collected a post-test blood and saliva sample. After the study, dogs in both conditions received a food reward.

Behavioral Coding

To assess whether specific behaviors or forms of social interaction (SI) were related to individual differences in hormonal response, we coded the duration (s) of several behaviors from video. Prior to coding, we reviewed all videos to determine which behaviors could be meaningfully coded, considering both the prevalence and observability for a range of theoretically relevant measures. Locomotion and postural variables were coded for subjects in both the control and HAI conditions, and consisted of the following: (1) *locomotion*: time walking, running, or jumping, with the onset of the behavior marked by three consecutive steps (to disambiguate locomotion from minor bodily repositioning), and of the offset of the behavior marked by a lack of movement for ≥ 1 s; (2) *upright*: time standing, walking, running, or jumping; (3) *lying (prone)*: time lying with abdomen against the floor; (4) *lying (supine)*: time lying on back or side with abdomen exposed; (5) *sitting*: time with rump on ground and forelegs extended.

For subjects in the HAI condition we also coded the following behaviors relating to interaction with the experimenter: (6) *physical contact*: time during which any part of the experimenter's body was in physical contact with any part of the dog's body; (7) *licking*: time in which the dog's tongue was in contact with the experimenter's body; (8) *play*: time in which the dog engaged in play bows, chasing, or gentle mouthing with the experimenter, with the onset marked by the first occurrence of any of these behaviors, and the offset marked by a period of ≥2 s without the dog engaging in any of these behaviors. A second independent rater scored all behaviors for random sample of ∼50% of observations, and inter-rater reliability was excellent for all measures (Pearson correlation, mean: $R = 0.997$) (min = 0.993, max = 1.0).

Hormonal Analysis

Plasma and salivary OT and AVP were assayed using the same ELISA kits and protocols employed in the pilot study. Inter-assay coefficients of variation were 11.1% (salivary OT), 4.2% (salivary AVP), 11.7% (plasma OT) and 11.2% (plasma AVP).

Statistical Analysis

All statistical analyses were performed in the R programming environment for statistical computing (R Core Team, 2017). To compare changes in OT/AVP across time between conditions, we used linear mixed models with the log transformed OT/AVP concentrations predicted as a function of fixed effects for sex (male, female), time (pre, post), condition (control, HAI), the time × condition interaction, and a random effect for subject ID. This model was fitted separately with each endocrine measure as the dependent variable. Because we predicted differential effects over time between groups, we conducted planned contrasts from these models assessing the effect of time within condition, and differences between conditions at each time point. Due to the exploratory nature of the study, we used an alpha value of

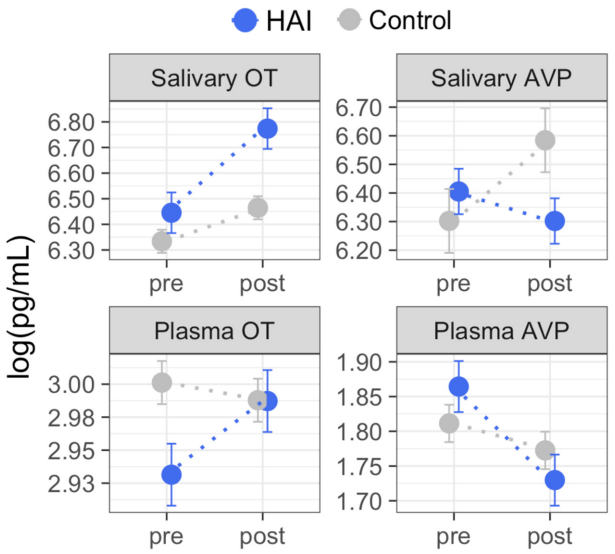

FIGURE 1 | Group means for oxytocin (OT) and arginine vasopressin (AVP) measured in plasma and saliva before and after Experiment 1. Using between-subjects design, dogs were assigned to conditions consisting of 10 min of affiliative interaction with a human (HAI, $N = 19$) or 10 min of rest without social contact with the experimenter (Control, $N = 19$). Dogs in the HAI condition exhibited significant increases in both salivary and plasma OT, and a significant decrease in plasma AVP. Dogs in the control group exhibited no significant changes in salivary or plasma OT, but showed increases in salivary AVP across time. Error bars represent the within-subjects standard error (Cousineau, 2005) and should be interpreted with regard to within-group, but not between group differences. The results of planned between-group, and within-group contrasts are reported in **Table 4**. Non-transformed concentration means: Salivary OT – HAI group: pre = 630 pg/mL, post = 874 pg/mL; Control group: pre = 563 pg/mL, post = 642 pg/mL; Plasma OT – HAI group: pre = 18.75 pg/mL, post = 19.83 pg/mL; Control group: pre = 20.11 pg/mL, post = 19.84 pg/mL; Salivary AVP – HAI group: pre = 605 pg/mL, post = 546 pg/mL; Control group: pre = 546 pg/mL, post = 724 pg/mL; Plasma AVP – HAI group: pre = 6.45 pg/mL, post = 5.64 pg/mL; Control group: pre = 6.12 pg/mL, post = 5.89 pg/mL.

$p \leq 0.05$ for significance testing, without correction for multiple comparisons.

Due to the number of behavioral variables, and substantial correlations between them, we conducted principal components analyses (PCA) to derive a smaller set of behavioral measures for statistical modeling. Because the postural and locomotion variables were coded for subjects in both the control and HAI conditions, we performed a PCA with these measures including data from all subjects. For subjects in the HAI condition, we conducted a second PCA including measures related to SI. The postural variable "lying (supine)" was observed predominantly in conjunction with abdomen petting in the HAI condition and was rarely observed in the control group (median duration in control group = 0). Thus, we omitted this measure from the PCA with variables related to locomotion, and included it in the PCA with variables related to SI. To limit the impact of outliers and skew, all behavioral variables were Yeo-Johnson transformed (Yeo and Johnson, 2000), centered, and scaled prior to fitting the PCA. To determine the number of components to retain, we conducted parallel analysis (Horn, 1965) comparing eigenvalues from the actual data to randomly resampled (with replacement, and dimensions equal to those of the original data) and simulated data (random data from a normal distribution). These analyses suggested retention of two components for both the locomotion and SI PCAs. To assess relationships between these behavioral variables and changes in OT/AVP across the experiment, we fitted linear models predicting the log transformed percent change in OT/AVP concentration as a function of fixed effects for sex (male, female) and scores from the first two principal components. To accommodate negative values in the dependent measure, we first added a constant to all values (the absolute value of the smallest observation +1) prior to log transformation. Because the control group did not have component scores for the SI variables, models for this group included only sex and principal component scores for the PCA on locomotion/postural variables. Coefficients for fixed effects were tested with likelihood ratio tests comparing the full model, to nested models with the removal of individual terms.

Results

Between Group OT/AVP Effects

Figure 1 shows the mean OT/AVP concentrations across time in the HAI and control groups. Results from the full mixed model for each matrix are shown in **Table 3**, and the results of planned contrasts comparing OT/AVP concentrations between groups, and assessing within group changes over time, are shown in **Table 4**.

For salivary OT, planned comparisons revealed that whereas the control and experimental groups had comparable mean salivary OT levels at baseline, dogs in the experimental group had significantly higher post-test salivary OT than controls. Similarly, salivary OT exhibited a significant increase from baseline to post-test in the HAI, but not the control group (**Figure 1** and **Table 4**). For salivary AVP, there were no between group differences at either time point, however, the control group exhibited a significant increase in salivary AVP whereas the HAI group did not (**Figure 1** and **Table 4**).

Plasma OT did not differ between groups at either time point, however, only the HAI group exhibited a modest, but significant, increase in plasma OT over the course of the study (**Figure 1** and **Table 4**). Lastly, there were no group differences in plasma AVP at either time point, however, the HAI group exhibited a significant decrease in plasma AVP across time, whereas the control group did not (**Figure 1** and **Table 4**).

Behavioral Predictors of Changes in OT and AVP

We retained two components from the PCA including variables related to locomotion and posture (LP), which collectively explained 80% of variance in these behaviors. Variable loadings from this model are shown in **Table 5**. The first component (LP-PC1, 50% variance explained) had strong positive loadings for locomotion and upright posture and moderate negative loadings for lying in in a prone position. The second component (LP-PC2, 30% variance explained) had a strong positive loading for sitting and moderate negative loadings for locomotion, upright posture, and lying (prone).

TABLE 3 | Results of linear mixed models predicting hormone concentrations as a function of time, condition, sex, and the time by condition interaction.

	Oxytocin						Vasopressin					
	Saliva			Plasma			Saliva			Plasma		
	β	X^2	*p*	β	X^2	*P*	β	X^2	*p*	β	X^2	*p*
Time	0.13	1.95	0.16	−0.01	0.22	0.64	0.28	4.19	0.04	−0.01	0.22	0.64
Condition	0.11	0.73	0.39	−0.07	0.88	0.35	0.12	0.31	0.58	−0.07	0.88	0.35
Sex	0.01	0.00	0.95	0.06	0.70	0.40	0.19	0.95	0.33	0.06	0.70	0.40
Time × Condition	0.20	2.28	0.13	0.07	2.93	0.09	−0.39	4.01	0.05	0.07	2.93	0.09

Planned contrasts from these models are shown in ***Table 4****.*

TABLE 4 | Results of planned contrasts between the HAI and control groups at each time point, and across time within groups.

	Group comparison (HAI vs. Control)						Within group change (Pre vs. Post)					
	Pre-test			Post-test			HAI group			Control group		
	β	*SE*	*p*	β	*SE*	*p*	β	*SE*	*p*	β	*SE*	*p*
Salivary OT	0.11	0.13	0.39	0.31	0.13	0.02	0.33	0.09	<0.01	0.13	0.09	0.16
Salivary AVP	0.12	0.21	0.58	−0.27	0.21	0.20	−0.10	0.13	0.44	0.28	0.13	0.04
Plasma OT	−0.07	0.07	0.35	0.00	0.07	0.97	0.06	0.03	0.05	−0.01	0.03	0.64
Plasma AVP	0.05	0.07	0.49	−0.05	0.07	0.47	−0.13	0.05	<0.01	−0.04	0.05	0.39

All tests had 1 degree of freedom. For group comparisons, β indicates the mean difference (HAI – Control). For change over time, β reflects the mean change from pre to post.

TABLE 5 | Variable loadings from principal component analyses including variables related to locomotion and posture (all subjects) and social interaction (HAI condition only).

		Loadings	
	Variable	PC1	PC2
Locomotion/PosturePCA (all subjects)	locomotion	0.62	−0.17
	upright	0.64	−0.16
	sitting	−0.02	0.83
	lying (prone)	−0.44	−0.50
Social interaction PCA(HAI subjects)	physical contact	−0.62	0.23
	play	0.55	0.32
	licking	0.32	0.71
	lying (supine)	−0.46	0.58

For dogs in the HAI condition, we retained two components from the PCA with variables relating to SI, which collectively explained 81% of variance in these behaviors. Variable loadings from this model are shown in **Table 5**. The first component (SI-PC1, 53% variance explained) was loaded positively by play and licking, and negatively by physical contact and lying (supine). The second component (SI-PC2, 28% variance explained) was loaded positively by all four SI variables (physical contact, play, licking, and lying supine).

Associations between changes in OT/AVP concentrations and behavior during the test are shown in **Table 6**. In the HAI condition, the percent increase in salivary OT was positively associated with SI-PC2 scores, which reflect longer durations of physical contact, play, licking, and lying (supine) with the abdomen exposed to the experimenter. Despite this positive association with salivary OT, there were no associations between changes in plasma OT concentrations and any of the behavioral variables (**Table 6**). There were also no associations between changes in salivary AVP and any of the behavioral variables, however, SI-PC2 scores were negatively related to the percent change in plasma AVP. On average, subjects in the HAI group exhibited a 10% decrease in plasma AVP across the study. However, subjects with SI-PC2 scores in the upper 50th percentile (high levels of affiliative behavior with experimenter) exhibited a larger decrease in plasma AVP (mean change = −18%, SEM = 6.89%) than subjects with SI-PC2 scores in the lower 50^{th} percentile (mean change = −4%, SEM = 4.87). Within the HAI group, LP-PC2 scores were also negatively associated with the change in plasma AVP. Thus, subjects who engaged in more sitting, and less standing, active locomotion and lying (prone) exhibited larger decreases in plasma AVP across time. Within the control group, there were no significant associations between any of the behavioral measures and changes in peptide concentrations, in saliva or plasma (**Table 6**).

Discussion

We assessed changes in dog salivary and plasma OT and AVP in response to HAI, or a control condition. Dogs in the HAI group exhibited increases in both salivary and plasma OT, whereas neither of these effects were observed in the control group. Dogs in the HAI group exhibited a decrease in plasma (but not salivary) AVP across time, whereas dogs in the control group exhibited an increase in salivary AVP, with no significant changes in plasma AVP. Within the HAI group, the extent of the increase in salivary OT, and the decrease in plasma AVP were predicted by the

TABLE 6 | Associations between changes in hormone concentrations in plasma and saliva and behavior during the test.

		Social interaction -PCI			Social interaction -PC2			Locomotion and Posture -PCI			Locomotion and Posture -PC2		
	Matrix / hormone	β	x^2	*p*	β	x^2	*p*	β	x^2	*p*	β	x^2	*p*
HAI group	Salivary OT	−0.60	1.55	0.21	0.61	4.37	0.04	0.72	2.70	0.10	0.22	0.60	0.44
	Salivary AVP	−0.02	0.00	0.97	0.16	0.22	0.64	0.10	0.04	0.84	0.18	0.26	0.61
	Plasma OT	−0.26	0.44	0.51	0.04	0.04	0.84	0.25	0.51	0.48	0.18	0.60	0.44
	Plasma AVP	−0.15	0.22	0.64	−0.49	6.15	0.01	0.21	0.52	0.47	−0.40	3.96	0.05
Control group	Salivary OT	–	–	–	–	–	–	−0.06	0.07	0.80	−0.05	0.03	0.85
	Salivary AVP	–	–	–	–	–	–	−0.36	1.80	0.18	0.20	0.35	0.56
	Plasma OT	–	–	–	–	–	–	0.12	0.89	0.35	0.13	0.58	0.45
	Plasma AVP	–	–	–	–	–	–	0.13	2.49	0.12	−0.04	0.15	0.70

PC, principal component. Refer to ***Table 4*** *for behavioral variables loading each component. β represents the association between a one unit increase in PC scores, and the log transformed percent change in hormone concentrations across time.*

degree of affiliative contact between the dog and experimenter. Therefore, both the between and within group differences are most likely attributable to the SIs between the experimenter and dog, and are unlikely to be accounted for by alternative explanations such as a stress response following the initial sample collection.

Although we observed a significant increase in both plasma and salivary OT, the effect was much more pronounced in saliva, echoing the findings of our pilot study. There are at least three reasonable explanations for this finding. First, plasma OT responses can occur extremely rapidly, and may be most evident within 90 s of a triggering event (Jonas et al., 2009). In contrast, time series analyses suggest a delay in the transfer of hormones from plasma to saliva, and hormonal peaks in saliva have been documented to occur ∼10 min after those in blood (Hernandez et al., 2014). Although the timing, and specific mechanisms through which OT and AVP reach saliva in dogs are unknown, it is probable that changes in salivary concentrations lag behind those in plasma, despite the fact that changes in salivary OT occur quickly relative to other salivary hormones (de Jong et al., 2015). Because this study was designed predominantly for salivary measures, we collected samples at the time point characterized by the highest salivary OT levels in our pilot study. Therefore, is it possible that the largest changes in plasma OT occurred quickly, as has been documented in previous studies (Handlin et al., 2011). Nonetheless, we detected a small but significant increase in plasma OT within the HAI group, suggesting similar effects in both blood and saliva.

Second, whereas OT can be measured without interference in non-extracted dog saliva, other components of dog plasma interfere with OT ELISAs, necessitating additional preparatory procedures such as solid phase extraction for the measurement of free OT (MacLean et al., 2017b). Although extraction procedures can eliminate interfering substances (e.g., albumin proteins), they are often characterized by poor recovery of the target analyte, and thus may eliminate 'signal' as well 'noise.' Unpublished data from our laboratory suggest that common extraction procedures fail to recover substantial amounts of free OT in plasma, despite performing well with kit standards. Therefore, in addition to benefits related to welfare, by virtue of not requiring extraction procedures, salivary measures of OT also have methodological advantages relative to blood sampling.

Third, it has recently been demonstrated that OT rapidly binds to other molecules in plasma, and bound OT is likely to evade detection (Brandtzaeg et al., 2016). Although the binding properties of OT in saliva are unknown, it is possible that OT remains free – and consequently detectable – in saliva, more so than in plasma. This possibility is supported by the high concentrations of OT that we observed in saliva relative to plasma, an effect that persists even with solid phase extraction of dog saliva samples (MacLean et al., 2017a).

In addition to these effects on OT, dogs in the HAI condition also exhibited a decrease in plasma AVP, whereas dogs in the control group exhibited an increase in salivary AVP. Because AVP can activate the HPA axis (Scott and Dinan, 1998; Aguilera and Rabadan-Diehl, 2000), one plausible explanation is that these differences reflect a short-term reduction in stress reactivity in the HAI group, and increased stress reactivity in the control group. This possibility is consistent with many other studies documenting that positive SIs can buffer stress responses (DeVries et al., 2003) – including interspecies interactions (Schöberl et al., 2016) – whereas social isolation can have the opposite effect. Although dogs in the control group remained in the same room with the experimenter, they were physically separated from him, and this separation may have imposed a mild psychological stressor. Despite this possibility, we observed minimal behavioral indications of stress or anxiety in this population of dogs, who are accustomed to resting in novel environments without physical contact from humans as a part of their training for assistance work. A second possibility is that the initial blood and saliva collection imposed an acute stressor in both groups, but that this event was socially buffered by OT in the HAI condition. The latter possibility is consistent with many studies documenting OT's ability to supress HPA activity in response to a stressor (Carter and Altemus, 1997). Both possibilities invoke differential stress responses between the two groups, but differ regarding the hypothesized cause of this stress. In the former scenario,

physical separation from the experimenter acts as a mild stressor for subjects in the control, but not the HAI condition. In the latter scenario, both groups are exposed to the same mild stressor (i.e., initial blood draw), but stress is buffered more effectively in the HAI group. Although we cannot distinguish between these, and other explanations at present, future studies will benefit by exploring the dynamics between OT, AVP and HPA activity in the context of HAI. Additionally, the differential changes in AVP between groups were observed in different matrices (increased salivary AVP in the control group, decreased plasma AVP in the HAI group), and the explanation for this phenomenon remains unknown. Given that AVP is released in response to both acute and chronic stress, it will be important to investigate the significance of both plasma and salivary vasopressin in future studies explicitly focusing on dogs' biological responses to potentially stressful events.

Lastly, in addition to the between group differences that we observed, within the HAI group the extent of the increase in salivary OT, and decrease in plasma AVP, were predicted the nature of interactions between the dog and experimenter. Specifically, dogs who engaged in higher levels of physical contact, play, licking and lying in a supine position with the experimenter exhibited the largest increases in OT, and decreases in AVP. Thus, as in previous studies, changes in OT and AVP concentrations depended not only on human contact, but also on the nature of these interactions (Rehn et al., 2014; Nagasawa et al., 2015).

Collectively, our results corroborate previous findings suggesting that OT responds dynamically to HAI, and provide the first data on HAI's effects on AVP in dogs. Notably, although HAI-related effects on OT have also been observed in humans, we are not aware of any studies examining AVP in humans in the context of interaction with animals. Given that we observed significant decreases in dogs' plasma AVP concentrations following HAI, we expect that future studies will benefit by incorporating this measure in humans as well. Collectively, these studies suggest that salivary measures of OT and AVP provide non-invasive biomarkers which respond to aspects of affiliative social behavior, and provide researchers with a new set of tools for exploring the roles of OT and AVP in the biology of HAI.

AUTHOR CONTRIBUTIONS

EM, NG, KL, and CSC designed the experiments. EM and KL collected the data. EM performed immunoassays and analyzed the data. EM, LG, NG, KL, WLM, and CSC wrote the paper.

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Oxytocin and Cortisol Levels in Dog Owners and Their Dogs Are Associated with Behavioral Patterns: An Exploratory Study

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We have previously shown that dog–owner interaction results in increasing oxytocin levels in owners and dogs, decreasing cortisol levels in owners but increasing cortisol levels in dogs. The present study aimed to further investigate whether oxytocin and cortisol levels in the previously tested owners and dogs were associated with their behaviors during the interaction experiment. Ten female volunteer dog–owners and their male Labrador dogs participated in a 60 min interaction experiment with interaction taking place during 0–3 min and blood samples for analysis of oxytocin and cortisol were collected at 0, 1, 3, 5, 15, 30, and 60 min. The entire experiment was videotaped and the following variables were noted; the different types (stroking, scratching, patting and activating touch, i.e., scratching and patting combined) as well as the frequency of touch applied by the owner, the number of times the owner touched her dog, the dog's positions and time spent in each position. Correlations were analyzed between the behavioral variables and basal oxytocin levels, maximum oxytocin levels, delta oxytocin levels, basal cortisol levels and cortisol levels at 15 min. Owners with low oxytocin levels before and during the interaction touched their dogs more frequently (0 min: $R_S = -0.683$, $p = 0.042$; oxytocin maximum: $R_S = -0.783$, $p = 0.013$). The lower the dogs' oxytocin levels during the interaction, the more stroking they received ($R_S = -0.717$, $p = 0.041$). The more frequently activating touch was applied by the owner, the higher the dogs' cortisol levels became (15 min: $R_S = 0.661$, $p = 0.038$). The higher the owners' maximum oxytocin level the fewer position changes the dogs made ($R_S = -0.817$, $p = 0.007$) and the shorter time they spent sitting ($R_S = -0.786$, $p = 0.036$), whereas the higher the owners' basal cortisol levels, the longer time the dogs spent standing (0 min: $R_S = 0.683$, $p = 0.041$). In conclusion, oxytocin and cortisol levels, both in dogs and in their owners, are associated with the way the owners interact with their dogs and also with behaviors caused by the interaction.

Keywords: oxytocin, cortisol, dog–human interaction, behavior

INTRODUCTION

In some human societies dogs have become a central part to family life and can even be considered as family members (Walsh, 2009a,b). The attachment relationship between a dog owner and its dog can be regarded as functionally similar to that seen between a parent and child (Topál et al., 1998; Palmer and Custance, 2008). Several studies have demonstrated that this type of relationship shows behavioral and neuroendocrine similarities to that described for mothers and infants (Serpell, 2004; Stoeckel et al., 2014; Nagasawa et al., 2015).

The neuropeptide oxytocin is produced in the paraventricular and supraoptic nuclei in the hypothalamus and is known to stimulate milk ejection during breastfeeding and uterine contractions during labor (Burbach et al., 2006). However, oxytocin is not only released during labor and breastfeeding, but may also be released by non-noxious sensory stimulation such as gentle touch. Both animals and humans respond to this type of stimulation, which induces, for example, anti-stress effects (e.g., decreased cortisol levels and blood pressure) (Uvnäs-Moberg, 1998), increased function of the gastrointestinal tract (Petersson et al., 1999), as well as increased pain threshold (Petersson et al., 1996).

In addition, non-noxious sensory stimulation in the context of intraspecies friendly social interaction, as for example in pair bonding, maternal behavior and attachment, is associated with activation of the oxytocinergic system (e.g., Carter, 1998; Uvnäs-Moberg et al., 2005). Oxytocin also facilitates bonding between mothers and young (e.g., the prairie vole: Carter, 1998; Insel et al., 1998; Sheep: Keverne and Kendrick, 1994; Humans: Uvnäs-Moberg, 1996; Feldman et al., 2007).

Studies in humans have shown that oxytocin, when applied through nasal spray, can stimulate certain aspects of social interactions, such as increase the ability to interpret tone of voice (Hollander et al., 2007) and facial expression (Domes et al., 2007a) and facilitate friendly social interactions (Domes et al., 2007b). It also increases trust (Kosfeld et al., 2005) and causes anti-stress and anxiolytic effects (Heinrichs et al., 2003). In similar ways, it has been shown that high endogenous oxytocin levels in mothers is related to the mothers being more interactive with their children, less anxious and more sensitive to their children's cues (Uvnäs-Moberg et al., 1990; Feldman et al., 2007).

Also interaction between humans and dogs, which include pleasant non-noxious sensory stimulation, can induce oxytocin release in both humans and dogs and generate effects such as decreased cortisol levels and blood pressure (Odendaal and Meintjes, 2003; Miller et al., 2009; Handlin et al., 2011). In addition, dogs have been shown to be able to interpret their owner's cues in different situations (Miklósi, 2009). Recent research has indicated that oxytocin can influence the social behavior of dogs toward humans, for example polymorphisms in the oxytocin receptor gene in dogs have been associated with differences in human directed behavior (Kis et al., 2014), nasally administered oxytocin increased gazing behaviors in dogs (Nagasawa et al., 2015) and oxytocin enhanced performance using momentary distal pointing cues (Oliva et al., 2015).

We have previously shown that interaction between dog owners and their dogs results in increasing levels of oxytocin in both owners and dogs, whereas cortisol levels decrease in the owners but increase in the dogs (Handlin et al., 2011). In addition, higher oxytocin levels in both owners and dogs, and lower levels of cortisol in the owners, are related to the owner's description of the owner-dog relationship as being pleasant and interactive and associated with fewer problems. We could also show that the owners' and the dogs' oxytocin levels are closely related (Handlin et al., 2012). Based on our previous results we expected that touch and behaviors related to calm and anti-stress would be positively related to oxytocin levels in both dogs and owners, whereas behaviors related to activation or stress would be associated with cortisol levels. The overall aim of the study was therefore to investigate whether oxytocin and cortisol levels in the previously tested owners and dogs were associated with their behaviors and more specifically we wanted to address the following questions: (1) Is the frequency and type of touch initiated by the owner associated with oxytocin levels in owners and dogs? (2) Is the frequency and type of touch initiated by the owner associated with cortisol levels in owners and dogs? (3) Are owners' oxytocin and cortisol levels associated with the dogs' behavior?

MATERIALS AND METHODS

The results of the present paper is based on data described in detail in previous manuscripts (see Handlin et al., 2011, 2012). Those parts of materials and methods, which are of relevance for the present paper will be summarized below.

Setting and Participants

Ten privately owned male Labrador retrievers, older than 1 year (mean age = 4.7 years; *SD* = 3 years) and their female middle-aged owners (mean age = 53 years; *SD* = 10 years), with whom they had been living together with during their entire lives, were recruited to the study by information given at veterinarian clinics and local workplaces. The owners were informed that the overall aim of the study was to investigate positive consequences of the human-dog relationship. The owners were to take part in an interaction experiment during which both owners and dogs would be exposed to blood sampling for analysis of hormones, such as oxytocin and cortisol. In addition the experiment would be videotaped for behavioral analysis. The owners were also informed about some additional measurements (such as heart rate etc.) that are not described in the present paper.

The study was conducted in an ordinary room (∼4 by 5 m) with four chairs, a desk and a bookcase. Dogs had *ad libitum* access to water during the testing. The experiments were performed in either the mornings or the evenings, depending on the participants' work schedules. The study was conducted at the Swedish University of Agricultural Sciences in Skara, Sweden.

Interaction Experiment

The owner was sitting in a chair with her dog lying or sitting beside her before the start of the experiment. At time-point zero the owner approached her dog and started to interact with him by talking to the dog and by touching different parts of the dog's body for 3 min. The owner was instructed to interact with her dog in the same way as they usually do at home. After the 3 min of interaction the owner was instructed not to touch or talk to the dog for the rest of the experiment and to remain seated in her chair. The whole experiment lasted for 60 min. However, in most cases, the owner occasionally touched and talked to her dog in order to correct his behavior during the remaining part of the experiment.

Blood Sampling and Hormone Analysis

As previously described in Handlin et al. (2011), an indwelling catheter was inserted into the cubital vein of the dog owners and an intravenous catheter was inserted into the cephalic vein of the dogs immediately upon arrival at the testing facility. An experienced nurse inserted the catheters in the owners and an animal caretaker inserted the catheters in the dogs.

Thirty minutes after insertion of the catheters and immediately before the owner started to interact with her dog the first blood sample (representing basal levels) was collected. The following samples were collected at 1, 3, 5, 15, 30, and 60 min after start of interaction. All blood samples were collected by an experienced nurse and an animal caretaker, respectively. They were both present in the room during the entire experiment but were instructed to ignore the participants except during the blood sampling. When not performing blood sampling they were sitting in chairs placed in one of the corners of the room.

All blood samples were collected into EDTA tubes (4 mL) containing Trasylol® (aprotinin) (Bayer AB) and they were taken simultaneously from dog and owner. The samples were immediately put on ice, centrifuged, and the plasma was collected and stored at −20°C until analysis.

Plasma levels of oxytocin in both the dogs and the owners were determined using Correlate-EIA TM Oxytocin Enzyme Immunoassay kit (sensitivity 11.7 pg/mL, Intra Assay precision 9.1% and Inter Assay precision 14.5%) (Assay designs, Inc. Ann Arbor, MI, United States). Cortisol levels were determined using DSL-10-2000 ACTIVE Cortisol Enzyme Immunoassay kit (sensitivity 2.76 nmol/L, Intra Assay precision 10.3% and Inter Assay precision 8.0%) (Diagnostic Systems Laboratories, Inc., Webster, TX, United States). The procedures were performed according to the manufacturers' instructions and the recommended standards and controls were always included. Extraction of blood samples prior to oxytocin analysis was not performed instead the samples were diluted five times in the assay buffer before analysis. The samples from the dogs were diluted two times in the zero standard buffer before analysis of cortisol levels.

An Anthos Fluido microplate washer (Anthos Labtec Instruments GmbH) was used for all washing procedures, a Multiskan Ex microplate photometer (Thermo Electron Corporation) was used for reading the absorbance. The color development was read at 405 nm with background correction at 580 nm for oxytocin, and at 450 nm with background correction at 620 nm for cortisol. Creation of standard curves, curve fitting, and calculation of concentrations was done by using Ascent software (Ascent software ver. 2.6 for iEMS Reader MF and multiskan).

One dog was excluded from the analysis of oxytocin levels, since oxtocin levels in all samples were below the range of detection for this dog.

Video Recordings and Analysis

The entire experiment (60 min) was videotaped and the dogs' behaviors were analyzed from the tapes. The ethogram is presented in **Table 1**. The different ways in which the owner touched her dog (stroking, scratching, or patting), and the frequency of the different types of touch as well as the total number of touching were noted. In addition the quality of the owner's verbal interaction with her dog (rewarding or reprimanding) and the frequency of these interactions were

TABLE 1 | Ethogram describing the behaviors observed in the present study.

Behavior	Definition	Sampling method
Owner stroking	Owner strokes the dog using her palm	Continuous[a]
Owner scratching	Owner scratches the dog	Continuous[a]
Owner patting	Owner pats the dog	Continuous[a]
Owner touching	The owners' total amount of touch (Stroking + scratching + patting)	Continuous[a]
Activating touch	The total amount of scratching and patting	Continuous[a]
Verbal rewarding	Owner reward the dog verbally (e.g., "good dog")	Continuous[a]
Verbal reprimanding	Owner reprimands the dog verbally (e.g., "here")	Continuous[a]
Verbal instructions	Owner give the dog verbal instructions (e.g., "sit")	Continuous[a]
Dog sitting	Dog is sitting with front legs extended and hind legs curved	Continuous[b]
Dog standing	Dog is standing up on all four paws	Continuous[b]
Dog lying down	Dog is lying down	Continuous[b]
Dog changing position	Dog changing position (changing from sitting, lying down, and standing)	Continuous[a]

[a]*Continuous sampling by registering frequency.* [b]*Continuous sampling by registering duration.*

noted, as well as the total number of verbal instructions given. The dog's positions (sitting, standing, or lying down), and how long time they spent in each position were also noted. The frequency of the dogs' position changes was also measured and used as an index for stress. The behavioral analysis was divided into two parts, the interaction part, i.e., 0–3 min and remaining part, i.e., 4–60 min. The frequencies of the interactive behaviors studied are summarized in **Table 2**.

Ethics Statement

Before start of the experiment, the owners were once more informed about the study. They were then given the opportunity to ask questions and were informed that they could end their participation in the study at any time. Written consent was obtained from all subjects in accordance with the Declaration of Helsinki.

The protocol was approved by the Local Ethics Board in Uppsala (ref. number. 2005/377).

This study was carried out in accordance with the recommendations of the Swedish Board of Agriculture. The protocol was approved by the Animal Ethics Committee in Uppsala (ref. number. 296-2005). The National Board of Agriculture approved the use of privately owned dogs.

Statistical Analysis

The Statistical Package for the Social Sciences (SPSS, version 22.0, IBM software) was used for performing statistical calculations.

The data was not normally distributed and hence the Spearman rank coefficient was used for calculating correlations between hormone levels and behavioral data for both dogs and owners. p-values < 0.05 were considered significant.

The included oxytocin variables for both dogs and owners were: basal oxytocin levels, maximum oxytocin levels recorded at 1, 3, or 5 min and the delta value between basal and maximum oxytocin levels, as a measure of the increase in oxytocin levels. Cortisol variables included for both dogs and owners were: basal cortisol levels and cortisol levels at 15 min.

The following behavioral variables were included in the statistical analysis: the frequency of the owners' stroking, scratching, patting, the total number of times the owner touched her dog, the frequency of rewarding or reprimanding verbal interaction, the total number of verbal instructions given by the owner, the time the dog spent sitting, standing, or lying down and the frequency of the dogs' position changes. In addition, the frequency of scratching and patting were combined into one new variable, "activating touch," which was also included in the statistical analysis (**Table 1**).

RESULTS

The dogs' and the owners' oxytocin and cortisol levels which were used in the present study have been published previously (Handlin et al., 2011) but are summarized in **Table 3**.

Is the Frequency and Type of Touch Initiated by the Owner Associated with Oxytocin Levels in Owners and Dogs?

The Owners

None of the different types of touch affected the owners' oxytocin levels in specific ways but there were significant negative correlations between the owners' basal and maximum oxytocin levels and the total number of times they touched their dogs during the 3 min of interaction; that is, owners with low oxytocin levels before and during the interaction, touched their dogs more frequently (0 min: $R_s = -0.683$, $p = 0.042$; oxytocin maximum value 1–5 min: $R_s = -0.783$, $p = 0.013$) (**Table 4**). However, the higher their oxytocin levels became during the interaction, i.e., the more the oxytocin levels increased, the less time they touched their dog during the 4th and 60th minutes (following the interaction) of the experiment ($R_s = -0.820$, $p = 0.046$) (**Table 4**).

TABLE 2 | The frequency of the interaction behaviors studied.

	Total touch (number of times/time period)	Stroking (number of times/time period)	Petting (number of times/time period)	Scratching (number of times/time period)	Activating touch* (number of times/time period)
0–3 min	170 (146–206)	84 (26–111)	22 (5–55)	88 (47–141)	94 (34-150)
4–60 min	6 (1–11.5)	–	–	–	–

*Data is presented as median and quartiles (Q25-Q75). *Activating touch, the total amount of scratching and patting (**Table 1**). –, the behavior was not displayed.*

TABLE 3 | The owners' and the dogs' oxytocin and cortisol levels during the experiment (data from the 10 female owners for both oxytocin and cortisol, and from nine dogs for oxytocin and ten dogs for cortsiol).

		0 min	1 min	3 min	5 min	15 min	30 min	60 min	OT max
Oxytocin levels (pmol/l)	Dogs	155.8 (26.9)	211.2 (30.7)	236.9 (38.7)	178.6 (29.6)	163.5 (34.5)	157.5 (36.0)	157.5 (41.1)	251.8 (34.5)
	Owners	168.5 (34.6)	169.8 (34.1)	180.6 (34.4)	170.2 (27.8)	146.4 (34.7)	171.3 (34.2)	165.1 (26.3)	187.0 (33.6)
Cortisol levels (nmol/l)	Dogs	168.4 (14.8)	169.4 (16.1)	168.1 (15.3)	180.1 (17.8)	224.1 (32.5)	202.8 (18.3)	190.2 (18.8)	
	Owners	389.8 (119.7)	382.7 (107.4)	382.7 (109.9)	387.6 (119.6)	362.1 (107.9)	331.6 (80.1)	305.2 (62.6)	

Means and SE values (in brackets) are shown. These data have been published previously in Handlin et al. (2011).

TABLE 4 | Correlation table of hormone levels and behaviors.

Hormone	Behavior	R_s	*p*-value
Dog oxytocin max	Frequency of stroking 0–3 min	$R_s = -0.775$	$p = 0.041$*
Dog cortisol 0 min	Frequency of activating touch 0–3 min	$R_s = 0.648$	$p = 0.043$*
Dog cortisol 15 min	Frequency of activating touch 0–3 min	$R_s = 0.661$	$p = 0.038$*
Owner oxytocin 0 min	Frequency of total touch 0–3 min	$R_s = -0.683$	$p = 0.042$*
Owner oxytocin max	Frequency of total touch 0–3 min	$R_s = -0.783$	$p = 0.013$*
Owner oxytocin increase	Frequency of total touch 4–60 min	$R_s = -0.820$	$p = 0.046$*
Owner cortisol 0 min	Time dog standing up 4–60 min	$R_s = 0.683$	$p = 0.041$*
Owner oxytocin max	Time dog sitting down 4–60 min	$R_s = -0.786$	$p = 0.036$*
Owner oxytocin max	Frequency of the dogs' position changes	$R_s = -0.817$	$p = 0.007$**
Owner oxytocin increase	Frequency of verbal reprimands	$R_s = -0.851$	$p = 0.004$**

*$p < 0.05$, **$p < 0.01$.

The Dogs

There was a significant negative correlation between the dogs' maximum oxytocin levels and how many times the owners stroked their dogs; that is, the lower the dogs' oxytocin levels during the interaction, the more stroking they received ($R_s = -0.775$, $p = 0.041$) (**Table 4**). Besides stroking there were no significant relationships between the other forms of touch studied and the dogs' oxytocin levels.

Is the Frequency and Type of Touch Initiated by the Owner Associated with Cortisol Levels in Owners and Dogs?

The Owners

There were no significant relationships between the frequency and type of touch initiated by the owners and their cortisol levels.

The Dogs

There were several significant positive correlations between the frequency of activating touch (scratching and patting) during the first 3 min of the experiment and the dogs' cortisol levels at start of the experiment but also during the remaining part of the experiment; that is, the higher the dogs' cortisol levels were at start of interaction the more activating touch they received and the higher their cortisol levels became (0 min: $R_s = 0.648$, $p = 0.043$; 15 min: $R_s = 0.661$, $p = 0.038$) (**Table 4**). Besides for activating touch there were no significant relationships between the other forms of touch studied and the dogs' cortisol levels.

Are the Owners' Oxytocin and Cortisol Levels Associated with the Dogs' Behavior?

The owners' maximum oxytocin levels correlated negatively to the number of position changes the dog performed during the entire experiment ($R_s = -0.817$, $p = 0.007$) and with the time the dogs were sitting between the 4th and 60th minutes (following the interaction) of the experiment ($R_s = -0.786$, $p = 0.036$) (**Table 4**); that is, the higher the owners' maximum oxytocin level, the fewer position changes the dogs made during the experiment and the shorter time they spent in a sitting position.

In addition, the higher the owners' increase in oxytocin was during the interaction, the less verbal reprimands they gave the dogs during the experiment ($R_s = -0.851$, $p = 0.004$) (**Table 4**).

There was a positive correlation between the owners' basal cortisol levels and the time the dogs spent standing up during the 4th and 60th minutes (following the interaction) of the experiment; that is, the higher the owners' cortisol in the beginning of the experiment, the longer time the dogs were in a standing position (0 min: $R_s = 0.683$, $p = 0.041$) (**Table 4**).

DISCUSSION

Based on previous data from the experiment presented in this manuscript, we have shown that interaction between owners and their dogs results in increasing levels of oxytocin in both owners and dogs, whereas cortisol levels decrease in the owners, but increase in the dogs (Handlin et al., 2011). In addition, the owners' and the dogs' oxytocin levels are closely related (Handlin et al., 2012). We have also shown that high oxytocin levels in both owners and dogs, and low levels of cortisol in the owners, are associated with the owner's description of the owner-dog relationship as being pleasant, interactive and associated with fewer problems (Handlin et al., 2012).

Based on these previous results, findings from other studies, and the fact that behavioral data was also recorded in our study, we were interested in investigating whether the previously obtained oxytocin and cortisol levels in the owners and the dogs were associated with their behaviors during the interaction.

The first question addressed was whether the frequency and type of touch initiated by the owner were associated with oxytocin levels in owners and dogs. The results indicate that this is true for the owners and probably also for the dogs. Owners with lower oxytocin levels touched their dogs more frequently and dogs with lower oxytocin levels received more stroking. Since we know from previous results that the oxytocin levels in the dogs and their owners are closely related (Handlin et al., 2012) it is very likely that it was the dog–owner dyads with the lowest

oxytocin levels who engaged in the most frequent interactions. It can be speculated that owners with lower oxytocin levels have a stronger need of interaction to increase their oxytocin levels and generate oxytocin mediated effects, whereas owners with higher oxytocin levels already experience oxytocin mediated effects and do not have the same need of physical interaction and hence do not interact as frequently. This interpretation is supported by the fact that the higher their oxytocin levels became during the interaction, i.e., the more the oxytocin levels increased, the less time the owners touched their dog during the 4th and 60th minutes (following the interaction) of the experiment.

The second question addressed was whether the frequency and types of touch initiated by the owner were associated with cortisol levels in owners and dogs. As presented previously in Handlin et al. (2011) the interaction decreased the owners' cortisol levels. Since oxytocin is known to inhibit cortisol release (Neumann et al., 2000), this decrease in cortisol levels is probably a result of the increased oxytocin levels as a consequence of the tactile interaction. The results from the present analysis did, however, not show any significant relationships between the frequency and types of touch and cortisol levels in the owner.

In contrast, the dogs' cortisol levels correlated positively and significantly with the amount of activating touch they received from their owners. In everyday-life interactions between dogs and owners, the activating type of touch is probably used more frequently during play. On the other hand, the stroking type of touch is probably used more frequently during calm interaction between owners and their dogs and hence it might have a more calming effect on the dogs. The activating touch applied to the dogs in this study might therefore have triggered an expectation of play in the dogs.

It is important to point out that the observed increase in the dogs' cortisol levels does not appear to have anything to do with stress, since they did not display behaviors related to stress (looking at frequency of position changes). The increasing cortisol levels are therefore probably a reflection of positive arousal (Lewandowski et al., 2014) and preparation for and expectation of activity in the dogs (Horváth et al., 2008).

The third question addressed was whether the owners' oxytocin and cortisol levels were associated with the dogs' behavior. According to the results from the present analysis it appears as if there are associations between the owners' oxytocin levels and the dogs displaying calm behaviors. This was demonstrated by the findings showing that the higher the owners' maximum oxytocin level, the fewer position changes the dogs made during the experiment and the shorter time they spent in a sitting position. Together these data suggest that high oxytocin levels in the owners are associated with a friendly and calm behavior toward the dog and hence with calming effects in the dogs.

It is also possible that the owners with high cortisol displayed a more active, or even stressed, behavior which influenced the dogs, as demonstrated by the finding showing that the higher the owners' cortisol were in the beginning of the experiment, the longer time the dogs spent in a standing position.

It appeared as if the dogs and their owners responded to the interaction in similar ways with regard to oxytocin. The interaction induced oxytocin release in the owners who displayed behaviors that are associated with anti-stress effects. The dogs seemed to sense this and responded in a similar way. The calmer behaviors displayed by the dog then enhanced the calming effect in the owners. It appears as if the owners and the dogs could mutually sense the other's emotional state based on an increased ability to read the other's behavioral cues. As previously described oxytocin can facilitate and stimulate friendly social interactions, induce anti-stress and anxiolytic effects and increase trust. It has been shown that oxytocin relates to the level of maternal interaction and sensitivity to the infant cues (Feldman et al., 2007), but also to more frequent interaction between dog owners and dogs (Handlin et al., 2011, 2012).

The results from the present and previous studies suggests that the activity and effect of the oxytocinergic system are probably part of a "mammalian heritage" that can be activated by individuals from different species, as in the interspecies relationship seen between dogs and their owners, and not only by individuals from the same species. Due to their evolutionary history (Miklósi, 2009), dogs and humans have been suggested to be especially good at activating each other's oxytocinergic systems and generating oxytocin-linked effects (Beetz et al., 2012).

Even though both dogs and owners responded in similar ways with regard to oxytocin some differences were noticed in the responses related to cortisol. An explanation to the mismatch in cortisol responses between the owners and the dogs might be that the interaction was driven by the owners, rather than a reciprocal, two-way decision process. The owners knew that it was going to be a calm interaction and were prepared for this and initiated the contact. The dogs on the other hand were unaware of what type of interaction that was going to take place and since the interaction was driven by the owners it might have caused some confusion for the dogs. This could explain the difference in responses between dogs and their owners.

To keep variations due to breed and gender to a minimum we chose to study male Labrador dogs and their middle-aged female owners. Labradors are friendly and easy to work with and is one of the most common types of companion dogs. However, in future studies it would be interesting to study both female and male dogs and dog owners but also other breeds.

We are aware that the number of participants in this study is low (10 dog–owner pairs) and that the results need to be interpreted with caution but still the results may serve as proof of a concept. One of the strengths of the study is the study design which included repeated sampling. This made it possible to detect the interplay between oxytocin concentrations and physical contacts. Studies with a larger number of participants and performed under even more standardized conditions need to be done to help gaining a better understanding of the responses in dogs and their owners as a consequence of interaction.

CONCLUSION

The present study showed that oxytocin and cortisol levels, in both dogs and their owners, are associated with the way the owners interact with their dogs and also with behaviors caused by the interaction.

AUTHOR CONTRIBUTIONS

MP: involved in study design and writing manuscript. KU-M: main applicant for funding, responsible for study design, involved in writing manuscript. AN: involved in data collection and writing manuscript. L-LG: involved in data analysis and writing manuscript. EH-S: involved in funding, study design and writing manuscript. LH: involved in study design, main responsible for data collection, statistical analysis and writing manuscript.

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Hormonal Correlates of Exploratory and Play-Soliciting Behavior in Domestic Dogs

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Exploration and play are considered to be crucial behaviors during mammalian development. Even though the relationship between glucocorticoids and exploratory behavior, stress, and anxiety is well described in the literature, very little is known about their role in play behavior in non-rodents. Likewise, the functional role of the "social hormone" oxytocin in exploration, play, stress, and anxiety is still unknown. The present work addresses this literature gap by studying plasma hormone profiles for cortisol (CORT) and oxytocin (OT) of domestic dogs exposed to a novel arena containing two unfamiliar trainers who did not interact with the dogs. We provide evidence suggesting a functional relationship between hormonal measures of cortisol and oxytocin and adaptive behavior (play-soliciting and exploration) in freely behaving domestic dogs. We have taken into account several possible factors in our analyses and interpretations, from the nature and quality of the measurements to demographic factors to statistical robustness. Our results indicate that reduced CORT levels are associated with increments of both play-soliciting behavior frequency and exploratory behavior duration. Furthermore, taken together, our data and our simulations suggest a relationship between OT and the enactment of play-soliciting behaviors by freely behaving domestic dogs that must be further investigated. Future studies should consider naturalistic structured and semi-structured experimental approaches linking behavior with (neuro) physiological measures, taking into account demographic factors such as age and relevant interphase factors such as the sex of the dog; and socio-historic factors such as the playfulness of the dog, history of interaction with young humans, among others, to take full account of interaction between humans and animals in comparative studies (Parada and Rossi, 2018).

Keywords: exploration, play, oxytocin, cortisol, domestic dog

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INTRODUCTION

Exploration and play are considered linked behavioral strategies for dealing with novelty, particularly during early mammalian development (Fiske and Maddi, 1961; Power, 2000; Burghardt, 2005). Their relevance to learning, predation strategies, and even tool use in species that use tools are long lasting (Rumbaugh et al., 1972; Hall and Bradshaw, 1998; Kramer and Burghardt, 1998). They have been conceptualized as an intertwined construct that shapes the way individuals

face novelty during their life, and tending to occur when no other behavioral tendencies are active (Immelmann and Beer, 1989). Furthermore, they might be functionally similar, since they provide opportunities for adaptively shaping individuals' knowledge and behaviors to the world.

In domestic dogs, both exploratory and play behavior tendencies may have facilitated the speciation process, since wild canids displaying more exploratory and playful behaviors (i.e., decreased flight and increased sociality) might have been taken as pets and socialized into human groups (Belyaev et al., 1985; Clutton-Brock, 1995; Driscoll et al., 2009; Driscoll and Macdonald, 2010; Miklósi and Topál, 2013; Range and Virányi, 2014). The fact that social play in dogs persists into adulthood contributes to their appeal as human companions (Bekoff, 1995). A developmental perspective on young animals' exploratory responses to novelty suggests that not only do such responses determine survival but, over time, they lay the foundations for individual differentiation in how animals adapt to their environments (Parada and Rossi, 2018). Exploration, which involves the active investigation of the environment by an individual in the absence of pressing physiological needs (Immelmann and Beer, 1989), entails a particular interaction between the organism and its surrounding area, potentially shaping both of them. This probably makes exploration one of the driving forces in evolution (Greenberg and Mettke-Hofmann, 2001; Lefebvre et al., 2004). Exploration can be viewed as a type of information seeking about sources of food, mates or any unknown resource that might bridge the gap between an organism's current state and states that are better adapted to current conditions. However, exploring novel environments can increase environmental risk factors, such as predation and aggression (Brown and Nemes, 2008). Exploration thus involves tradeoffs between benefits and risks in the course of encountering new situations and new potential play partners.

Play can be classified into either locomotor play, social play, individual play, or object play, although these are not necessarily mutually exclusive categories (Mehrkam et al., 2017). Social play, an apparently purposeless motor activity directed toward another agent and varied in both form and temporal sequencing (Bekoff and Byers, 1981, 1998), is built upon cooperation with the play partner, thus it is to be expected that pro-social mechanisms are important to sustaining it (Spinka et al., 2001; Bekoff, 2018). Social play is performed more frequently and for longer periods than either object or solitary play in many species (Burghardt, 2005). In common with other mammals, canids have a number of play-soliciting behaviors, such as approaching with an exaggerated, high-amplitude gait that is sometimes referred to as "loose" or "bouncy." In addition, canids have evolved their own easily recognized social play-soliciting signals, such as play bows in which the shoulders are lowered below the level of the hips, and face pawing in which a dog lifts one of its front paws from the ground and directs it at the face of another dog, sometimes making contact (Bekoff, 1972, 1974).

In the domestic dog (*Canis lupus familiaris*), social play is very common, although unlike other canids the repertoire has expanded from conspecific play to dog-owner play, which is the more commonly seen form of social play. Dog–dog play and dog-owner play are possibly not homologous because they appear to be motivationally distinct (Rooney et al., 2000). Nevertheless, dogs direct many of the play-soliciting behaviors to humans just as easily as to other dogs. Practically all the studies on dog social play show similar results, generally indicating that social play in dogs is a marker of healthy development and positive affect, with long lasting effects on human-dog social cohesion (Horowitz and Hecht, 2016; Sommerville et al., 2017). Social play behavior has been described as an essential component of social development of animals, seemingly equipping animals with skills and strategies to deal with a variety of behaviors expressed in adulthood (Wang et al., 2012). Therefore, social play might be understood as a part of a "prosocial toolkit" that needs to be rehearsed and developed in order to facilitate the establishment of longer-term social ties (Vanderschuren et al., 1997; Vanderschuren, 2011).

Investigation of physiological mechanisms is an important element of integrated explanations in ethology (Tinbergen, 1963). Physiological measures associated with exploratory behavior, frequently related to stress and anxiety responses, have been thoroughly studied in many species (Greenberg, 1985; Koolhaas et al., 1997, 1999; Dingemanse and de Goede, 2004; Becker et al., 2007). Glucocorticoid response during exploration has been particularly well characterized in mammals, showing in general, that steroid hormone levels are negatively associated with exploratory behavior (e.g., Pellow et al., 1985; Carlstead et al., 1993; Conrad et al., 1997; Kunzl et al., 2003; Kazlauckas et al., 2005). Consequently, it has been shown that cortisol (CORT) is part of the stress response in mammals, which in turn makes it linked to reduced proclivity to interact with new objects or spaces. Notably, it has been shown that domesticated animals show lower glucocorticoid levels and more frequent exploratory behaviors relative to undomesticated individuals (Hemmer, 1990; Trut et al., 2004).

Hormonal correlates of social play have been investigated mainly in rodent species (Vanderschuren et al., 1997; Pellis and Pellis, 1998; Trezza et al., 2010; Taylor et al., 2012). There are very few studies exploring this link in other domesticated species (Sachs and Harris, 1978; Orgeur, 1995; Nunes et al., 1999). However, Horváth et al. (2008) showed that differences in the way humans interact with dogs in a playful interaction (affiliative vs. disciplinary) affect the cortisol levels of the dogs; an affiliative style decreased cortisol levels whereas a disciplinary one increased the hormone levels.

Strikingly, although social play is considered an important component of social behavior and has been carefully studied in canids (Bekoff, 1995; Bekoff and Allen, 1998; Rooney et al., 2001), the relationship between play behavior and both cortisol and the so-called "social hormone" oxytocin (OT) has only been recently explored. Recent evidence shows that salivary oxytocin in dogs is significantly increased after affiliative human–dog interaction (MacLean et al., 2017). This is relevant since oxytocin may promote socialization by its anxiolytic effects (Lancaster et al., 2017); for instance by promoting social play, especially in novel situations (for a review Kis et al., 2017). Similarly, recent evidence shows the effects of intranasal OT administration on dog behavior (Romero et al., 2014; Kis et al., 2015; Nagasawa et al., 2015; Oliva et al., 2015; Romero et al., 2015).

These studies show a link between intranasal OT administration and affiliative behaviors directed by dogs toward their owners and toward other familiar dogs, suggesting that the administration of intranasal OT increases affiliative behaviors in dogs in a social context.

Relevantly, one study shows that intranasal OT administration increased the amount of play signals dogs gave to both familiar humans and conspecifics (Romero et al., 2015). Together, this evidence shows that the intranasal administration of OT triggered higher levels of affiliation, social orientation/approach, and gazing toward familiar individuals (Romero et al., 2014, 2015; Nagasawa et al., 2015). Collectively these results suggest that, similar to human-based studies, OT might help reveal the mechanisms of cooperation, and might also be essential to the behavioral displays that constitute the basis for the formation of social bonds.

The present study aims to explore the associations among exploratory and play-soliciting behaviors and plasma hormone measurements of cortisol and oxytocin in the domestic dog. To accomplish this, dogs were allowed to freely move about a novel arena for 10 min while being observed and video-recorded by two experimenters. Immediately following the behavioral trial, blood samples were collected and analyzed for cortisol (the predominant glucocorticoid in canids) and oxytocin. We hypothesized that (i) according to the literature showing that levels of cortisol modulate exploratory behavior in mammals, dogs' exploratory behavior would be negatively correlated with cortisol concentrations and (ii) since peripheral oxytocin levels increase in both humans and dogs as a result of physical contact mostly in affiliative contexts (Odendaal and Meintjes, 2003; Handlin et al., 2011; Mitsui et al., 2011; Rehn et al., 2014), oxytocin levels would be positively correlated with a previously defined suite of play-soliciting behaviors in dogs (Bekoff, 1972, 1974).

MATERIALS AND METHODS

Animals and Behavioral Sessions

Purebred or mixed breed Labrador retriever dogs (n = 14, mean age: 4.1 years, 10 male, 4 female, see **Table 1** for details) privately owned by local families served as study subjects. All subjects were naïve to the present study and to both the arena and the experimenters involved. This study was carried out in accordance with the approval of the Bloomington Institutional Animal Care and Use Committee (BIACUC, protocol 12-016). All owners signed a consent form prior to the session and they did not interact in any way with the experimenters or with their dogs after the drop-off. Testing was conducted at a local facility that provides canine training, daycare and veterinary services in Bloomington, IN, United States.

The sessions were carried out on separate days; thus, only one dog was tested on each day. Dogs were brought to the facility by their owners, who then left the facility. Each dog was placed alone in a grooming room kennel for between 10 and 15 min while the experimenters prepared the room. When the room was ready, they were fetched by one of the experimenters (experimenter 1 or E1) who walked them through the door. At the door, E1 took off the dogs' walking collar allowing them to enter a clean, disinfected training room (17 × 15 m) clear of toys and other small objects. This room had concrete floors and walls, no windows but two doors, one at the front and the other one in the back of the room with small windows on top of them. Dogs could behave freely for 10 min. All sessions took place between 0830 and 0930 EST.

TABLE 1 | Demographic data: Sex, weight (kilograms), age (years).

Demographic data			
Dog ID	**Sex**	**Weight**	**Age**
1	F	23.3	12
2	F	45.9	6
3	F	35.6	4
4	F	29.6	3
5	M	30.11	2
6	M	34.9	2.5
7	M	31.9	5
8	M	33.4	6
9	M	28.1	0.7
10	M	43.7	1
11	M	29.1	0.6
12	M	43.8	12
13	M	32.4	1
14	M	34.2	2

Two video cameras simultaneously recorded the dogs' behavior during this time. Experimenter 1 (E1), who entered with the dog, stood then in the middle of the arena not moving from that location, but turning with the dog to face it without interacting with it during the entire session. E1 wore an ear-mounted digital camera (Looxcie 2). Experimenter 2 (E2) sat on a chair in a corner of the room and made no eye contact nor interacted in any manner with the dog. E2 handled a Sony HDR-CX160 video camera to capture the dogs' behavior from different angles. Two active behavioral categories were coded and calculated for the exactly 10 min session: exploratory behavior (relative duration) and play-soliciting behavior (frequency), along with more passive behaviors such as sitting or lying down. These behaviors were treated as mutually exclusive. According to previous studies, exploratory behavior was operationally defined as locomotive behavior usually accompanied by sniffing and distal or close visual inspection in a relaxed manner (Prato-Previde et al., 2003; Rehn et al., 2013; Marshall-Pescini et al., 2017); play-soliciting behavior was coded using behavioral features adapted from the categories used by Bekoff (1972, 1974). We coded the behavior as play soliciting when it fell into any these categories: play bow (shoulders lowered beneath hips, forelimbs on, or near ground), exaggerated approach (approach with higher amplitude stepping or tail wagging than a normal approach), approach/withdrawal (dog approaches then abruptly turns and runs; typically used to solicit chasing behavior), paw intention (contact to person with paw off the ground), and leap-leap (two high-amplitude leaps in which the forelimbs are

TABLE 2 | Cortisol and oxytocin levels (picograms per milliliter) and the frequency and duration for each behavior during 10 min dog spent with experimenters E1 and E1.

			Data		
Dog ID	**CORT (pg/mL)**	**OT (pg/mL)**	**Exploratory behavior duration (min)**	**Play-soliciting behavior (counts)**	**Sit/Stand/Lay < 2 m duration (min)**
1	5855.56	NaN	3.24	1	6.89
2	7631.32	159.9	1.64	0	8.3
3	1462.48	392.1	6.25	23	1.71
4	1267.63	161.1	5.14	15	2.69
5	4148.59	NaN	4.74	28	0.26
6	1304.09	162.1	5.86	15	0.17
7	2340.07	50.9	3.82	1	5.92
8	1099.43	158.5	8.85	7	0
9	2768.5	NaN	9.91	2	0
10	8518.74	114.9	0.95	0	8.7
11	3286.77	287.6	9.39	11	0
12	2706.47	79	4.8	4	5.09
13	1106.17	225.7	9.39	11	0
14	7538.05	162.2	5.15	7	4.11

lifted off the ground, and hit the ground, simultaneously). These behaviors, and proximity were coded and quantified using the ELAN software (Lausberg and Sloetjes, 2009) by two independent coders who were trained to recognize these behaviors but blind to dogs' hormone concentrations. The inter-rater correlations for exploratory and play-soliciting behaviors were 0.88 and 0.91, respectively ($p < 0.001$). Raw data were converted into frequencies and relative duration using custom in-house routines written in the MATLAB environment (The MathWorks, Inc., Natick, MA, United States).

Blood Collection and Hormone Assays

At the end of the session, subjects were led to the veterinary clinic and blood was collected into chilled EDTA-treated tubes within 4 min to assess physiological status. Blood was drawn with a 22-gauge needle from the cephalic vein (1.5cc–3cc drawn). Half the sample was transferred to a second tube and immediately treated with aprotinin (500 KIU/ml) to inhibit protease activity. Samples were centrifuged (4°C, 1500 × *g*, 15 min) and plasma was stored in polypropylene tubes at −80°C until analysis. Cortisol was measured in untreated plasma using an enzyme immunoassay (EIA) kit (901-701; Enzo Life Sciences). Samples were diluted 1:4 and assayed in duplicate according to the manufacturer's instructions. Serial dilution of pooled dog plasma yielded a displacement curve parallel to the cortisol standard curve ($r^2 = 0.98$). Mean intra-assay variability was 3.7% and inter-assay variability was 4.6% ($n = 3$ plates). Aprotinin-treated plasma was assayed for oxytocin using a commercial EIA kit (900-153; Enzo Life Sciences) according to the manufacturer's instructions. Samples were diluted 1:4 and assayed in duplicate on a single plate; three dogs (one female and two males, see **Table 1** for details) were removed from the analysis due to insufficient volume. Serial dilution of pooled dog plasma yielded a displacement curve parallel to the oxytocin standard curve ($r^2 = 0.98$). Recovery of known amounts of oxytocin standard added to a pool of plasma extracts was 100.7 ± 11.7% ($y = 1.04x + 1.1$; $r^2 = 0.99$). Mean intra-assay variability was 3.8%. Data summary is presented in **Table 2**.

Statistical Analyses

To statistically test our main hypotheses – relationship between both exploratory and play-soliciting behaviors and hormone concentrations – we implemented hierarchical multiple regression in order to further explore the influence between physiological (OT, CORT) and the most relevant demographic factors (age, sex) over exploratory and play-soliciting behavior. In view of both physiological measures, we built the first block of predictors using the *forced entry* method. In order to build the whole model, the second block included both physiological and demographic predictor variables (OT, CORT, Age, Sex). Preliminary analyses were performed ensuring no violation of multilinear regression assumptions (Durbin–Watson = 2.345, 1.838; Standardized residuals <1.943). The weight predictor was left out of the model due to high multicollinearity (VIF > 5). Furthermore, collinearity analyses showed that our four predictors were within analysis range, possible multicollinearity was discarded when using four predictors and no other factors were removed (Tolerance >0.561; VIF <1.781).

Considering the number of study subjects and the missing data points in OT measurement, we implemented a complementary analysis. Given the missing data points for OT measurements from three study subjects, we created a simulated dataset using the non-parametric permutation framework implemented in the MATLAB environment. Thus, 100 new datasets were constructed without missing values. The simulated 300 values were drawn from a distribution with similar parameters as the original data (range = 50.9+/−2 std to 392.1+/−2 std; mean = 177.6; median = 161.1, **Figure 1**). The 100 complete simulated datasets were also analyzed using hierarchical multiple regression procedure described above.

All statistical analyses were performed using JASP software version 0.8.5.1 (JASP Team, 2016).

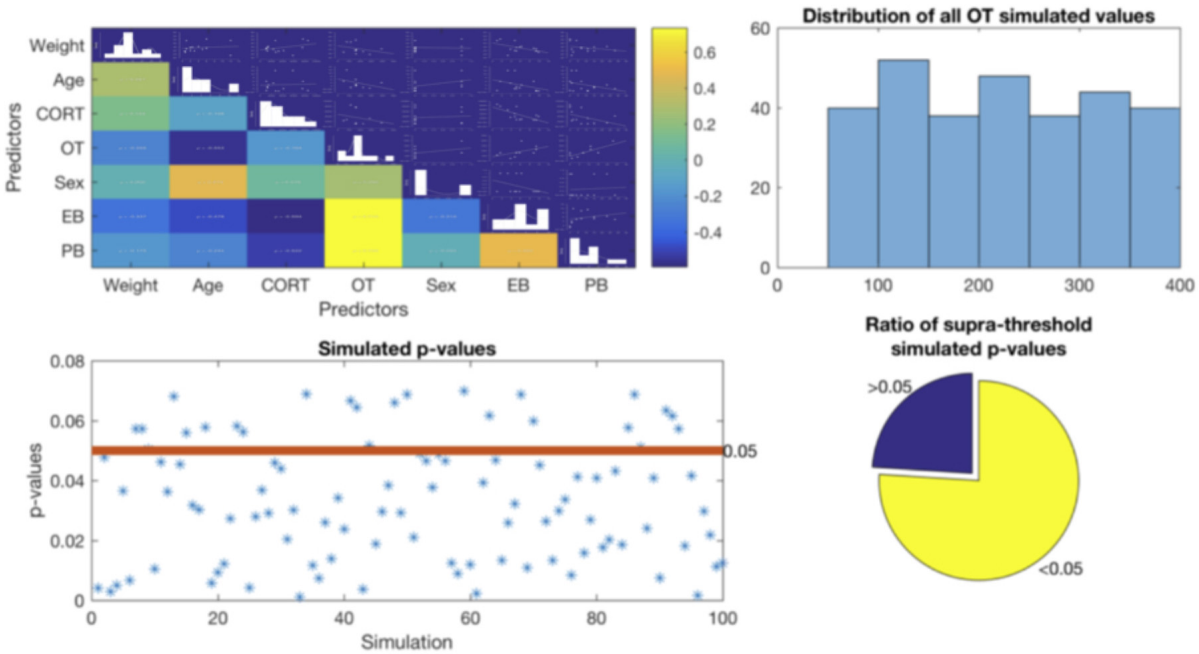

FIGURE 1 | Top Left – Correlation matrix between all variables. Top right – distribution of all OT simulated values showing no tendency. Bottom left – all *p*-values obtained from the simulated data. Red line indicates the considered statistical threshold, less than 30% of these *p*-values were above threshold (bottom right).

RESULTS

Main Analysis: Hierarchical Multiple Regression

Physiological predictors were entered in the first step of hierarchical multiple regression (**Table 3**, model 0: OT, CORT), explaining 59% of exploratory behavior variance [R^2 = 0.586; R^2_{adj} = 0.482, $F(2,8) = 5.653$, $p = 0.029$] and 80% of play-soliciting behavior variance [R^2 = 0.801; R^2_{adj} = 0.752, $F(2,8) = 16.126$, $p = 0.002$]. Both models including only physiological predictors were statistically significant. Observation of Beta (β) coefficients indicates that CORT made a statistically significant contribution for both exploratory behavior (β = −0.601, $p = 0.034$) and play-soliciting behavior (β = −0.458, $p = 0.023$), while OT contributed significantly only to play-soliciting behavior (β = 0.665, $p = 0.003$). Demographic variables were included in order to build the final model in step 2 (**Table 3**, model 1: Sex, Age, OT, CORT). The model as a whole explained 80% of exploratory behavior variance [$R^2 = 0.799$; $R^2_{adj} = 0.665$, $F(4,6) = 5.954$, $p = 0.028$] and 83% of play-soliciting behavior variance [$R^2 = 0.829$; $R^2_{adj} = 0.714$, $F(4,6) = 7.256$, $p = 0.018$]. Including demographic variables significantly improved both models. However, the additional 21% of exploratory behavior variance was not a statistically significant change [R^2 change = 0.213, $F(2,6) = 3.178$, $p = 0.115$]. Similarly, adding demographic variables only increased 3% of play-soliciting behavior explained variance, not yielding statistically significant results [R^2 change = 0.027, $F(2,6) = 0.640$, $p = 0.640$].

Therefore, in the final model, β coefficients indicate that CORT made a significant unique contribution to both exploratory (β = −0.557, $p = 0.032$) and play-soliciting (β = −0.514, $p = 0.032$) behaviors, while OT might have a contribution to play-soliciting behavior, which cannot be untangled with our current dataset (β = 0.525, $p = 0.057$, see "Discussion" section). All other β coefficients were well above statistical threshold ($p > 0.05$). See **Tables 3, 4,** and **Supplementary Table 1** for model ANOVA results and model coefficients.

Supplementary Analysis: Hierarchical Multiple Regression With Simulated Data

As expected, the first level of our simulation results replicated the findings of the main analysis hierarchical model. That is, CORT was negatively correlated with both exploratory and play-soliciting behaviors, while OT was positively correlated only with play-soliciting behavior. Likewise, the simulation further shows that although adding demographic predictors increases the overall model performance, the R^2 change is not statistically significant. Thus, the second level of our simulation partially replicates the main analysis final model. Accordingly, CORT robustly shows a negative correlation with both exploratory and play-soliciting behaviors. However, the simulation is not congruent with our findings regarding OT. Our simulated results show that fewer than 30% of simulations replicate our OT hierarchical multiple regression analysis (β > 0.544 ± 0.065, $p < 0.074 \pm 0.018$). This indicates no statistically significant contribution of OT to play-soliciting behavior. In contrast, the other >70% of simulations suggest a significant OT contribution to play-soliciting behavior (β > 0.661 ± 0.086, $p < 0.027 \pm 0.004$),

TABLE 3 | Hierarchical multiple regression models summary.

Model	*R*	R^2	Adjusted R^2	RMSE	R^2 change	*F* change	df1	df2	*p*	Durbin–Watson
Exploratory behavior model summary										
0	0.765	0.586	0.482	2.052	0.586	5.653	2	8	0.029	
1	0.894	0.799	0.665	1.651	0.213	3.178	2	6	0.115	2.345
Null model 0 includes OT (pg/mL), CORT (pg/mL).										
Play-soliciting behavior model summary										
0	0.895	0.801	0.752	3.638	0.801	16.126	2	8	0.002	
1	0.910	0.829	0.714	3.900	0.027	0.480	2	6	0.640	1.838
Null model 0 includes CORT (pg/mL), OT (pg/mL).										

TABLE 4 | Hierarchical multiple regression model ANOVA.

Model		Sum of squares	df	Mean square	*F*	*p*
Exploratory behavior model ANOVA						
0	Regression	47.61	2	23.805	5.653	0.029
	Residual	33.69	8	4.211		
	Total	81.30	10			
1	Regression	64.94	4	16.234	5.954	0.028
	Residual	16.36	6	2.726		
	Total	81.30	10			
Null model 0 includes OT (pg/mL), CORT (pg/mL).						
Play-soliciting behavior model ANOVA						
0	Regression	426.85	2	213.42	16.126	0.002
	Residual	105.88	8	13.23		
	Total	532.73	10			
1	Regression	441.46	4	110.37	7.256	0.018
	Residual	91.26	6	15.21		
	Total	532.73	10			
Null model 0 includes CORT (pg/mL), OT (pg/mL).						

Figure 1. We will discuss this discrepancy in the following section.

DISCUSSION

The present study provides evidence for a link between behavior of dogs in a novel setting and physiological measures taken immediately after. Thus presenting new evidence about the relationship among cortisol, oxytocin and exploratory and play-soliciting behaviors in freely behaving domestic animals. Along with confirming an expected link between CORT and exploratory and play-soliciting behavior, this is the first study, to our knowledge, presenting data and a simulation associating oxytocin with play-soliciting behavior.

Our main analysis, a two-level hierarchical multiple regression, suggested that reduced CORT predicts an increment of both play-soliciting behavior frequency and exploratory behavior duration. These results are in line with previous studies (e.g., Lupien and McEwen, 1997; Kalivas and Nakamura, 1999), and its robustness is seen on both levels of the main analysis as well as both levels of the simulation. Therefore, our overall results confirm that decreased CORT levels predict both exploratory behavior duration and play-soliciting behavior frequency.

Furthermore, the first level of our analysis suggests that increased levels of OT might be relevant for frequency increments of play-soliciting behavior. However, while demographic factors failed to make statistically significant contributions, when added to the model they suggest that the role of OT in play-soliciting behavior is not conclusive (with a *p*-value of 0.057). Moreover, our supplementary analysis outcome is divided regarding OT's contribution to play-soliciting behavior, as less than 30% of simulations follow the non-conclusive result of the main analysis. In other words, adding demographic factors to the hierarchical multiple regression models decreases the statistical compatibility of the observed OT relationship with increasing play-soliciting behavior frequency. Although there are many reasons to explain the slight OT *p*-value increment (a 0.047 difference between models) we have enough evidence to support the discussion of three factors: (i) low subject sample, (ii) sex of subjects, and (iii) missing

values for OT readings, and we will proceed to address them.

Regarding the low subject sample, we would like to point at the fact that these kind of studies are expensive and complicated to implement: (i) it is difficult to find domestic dogs whose owners are willing to allow drawing blood for data acquisition, (ii) locating facilities willing to host such research is onerous, and (iii) acquiring necessary resources including financial support is always burdensome.

Regarding imbalanced sex ratio, it must be said that it is a function of low subject sample attributable to the challenge of recruiting willing owners. Even though sex, as a relevant predictor, was not consistent enough to have a significant impact on our results, we interpret the result from the full exploratory behavior model ($\beta = -0.5$, $p = 0.059$) as providing evidence that future studies should make all possible efforts to equalize sex ratio in the sample.

Finally, regarding the missing OT values, the results in our second supplementary analysis using 100 simulated datasets suggest that the negative relationship between CORT levels and both exploratory behavior duration and play-soliciting behavior frequency is robust, since it was consistently present in both models (with and without demographic predictors). The simulation further confirms that although adding demographic predictors increase the overall model performance for both behaviors, the R^2 changes are not large enough to become relevant. Furthermore, the positive relationship between OT and play-soliciting behavior remained intact in the first step of all hierarchical models using simulated data (no demographic data included). However, when demographic predictors were added in the second step, more than 70% of our simulated hierarchical models suggested that increased OT levels made a unique and significant contribution to incrementing play-soliciting behavior frequency (**Figure 1**). The discrepancy between the original hierarchical multiple regression analysis and the simulation suggests that the missing OT measurement values might be a more important factor to account for the observed 0.047 *p*-value increment when adding demographic factors into the hierarchical model.

Taken together, our analyses not only support but also expand our first hypothesis, suggesting that reduced CORT levels are linked to both the duration of exploratory behaviors and the frequency of play-soliciting behaviors in freely behaving domestic dogs. We interpret this relationship as a physiological signature of an *openness* to explore for longer times and consequently, to engage in more frequent interactions, such as play-soliciting behaviors. Furthermore, our results suggest a possible relationship between OT and the enactment of play-soliciting behaviors by freely behaving domestic dogs that must be further investigated.

Directions for Future Work

Previous studies have shown anxiolytic-like, stress-reducing effects of oxytocin in mammals (Uvnas-Moberg et al., 1994; Windle et al., 1997). Thus, it is possible that reduced fear relates to approach responses and *perhaps* exploratory behavior in dogs, evidenced by reduced CORT and possible increased OT.

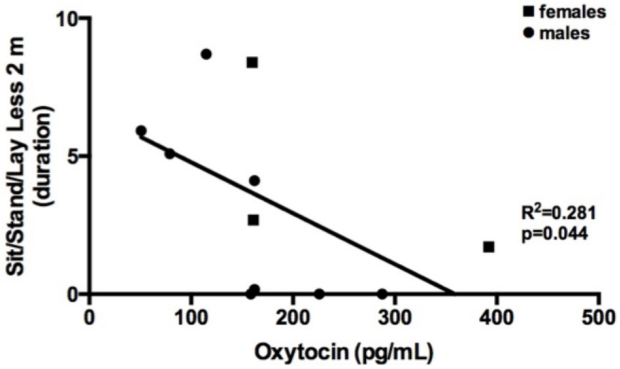

FIGURE 2 | Correlation between Standing, seating, laying less than 2 m away from the experimenters (relative duration) and plasma oxytocin (pg/mL).

The possibly reciprocal and/or regulatory relationship between OT and CORT is still an open question. However, the origin of the physiological relationship between CORT, OT, and exploratory and play-soliciting behaviors might lie in the domesticated nature of *Canis lupus familiaris* (Hemmer, 1990; Trut et al., 2004). In canids, research has shown that both dog and owner oxytocin levels increase after positive social interactions (Miller et al., 2009; Nagasawa et al., 2009; Handlin et al., 2011). In our study the experimenters did not directly interact with dogs, remaining neutral during the session. Furthermore, dogs displaying physical proximity between 0 and 2 m to the experimenters showed overall lower levels of oxytocin concentrations; the relationship between dogs' oxytocin levels and play-soliciting behavior is not explained by physical proximity between the dog and the experimenters (**Figure 2**).

The role of hormones in processes such as exploratory and play-soliciting behavior that enable dogs to deal with novel anthropogenic environments and which may have contributed to the domestication of dogs from wolves (Belyaev et al., 1985; Trut et al., 2004), should be further explored. We believe that our present study contributes with more evidence to the current line of research of causal mechanisms by manipulating these hormones in domestic dogs using methods already considered acceptable for research on human subjects such as hormonal administration (e.g., Romero et al., 2015; Kis et al., 2017; Persson et al., 2017; Temesi et al., 2017 for a critical review).

CONCLUSION

This article provides some evidence of a functional relationship between hormonal measures of CORT and OT and adaptive behavior (play-soliciting and exploration) in freely behaving domestic dogs. We have taken into account several possible

factors in our analyses and interpretations, from the nature and quality of the measurements to demographic factors to statistical robustness. Future studies should consider naturalistic structured and semi-structured experimental approaches linking behavior with (neuro) physiological measures, taking into account demographic factors such as age and relevant interphase factors such as the sex of the dog; and socio-historic factors such as the playfulness of the dog, history of interaction with young humans, among others, to take full account of interaction between humans and animals in comparative studies (Parada and Rossi, 2018).

AUTHOR CONTRIBUTIONS

AR and CA conceptualized the research. AR, CA, GD, and CB designed the experiments. AR and CB collected behavioral and physiological data. AR and RS analyzed the physiological data. FP and AR implemented the statistical analyses. AR, FP, CA, and GD wrote and edited the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyg.2018.01559/full#supplementary-material

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ORIGINAL RESEARCH
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Endogenous Oxytocin, Vasopressin, and Aggression in Domestic Dogs

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Aggressive behavior in dogs poses public health and animal welfare concerns, however the biological mechanisms regulating dog aggression are not well understood. We investigated the relationships between endogenous plasma oxytocin (OT) and vasopressin (AVP)—neuropeptides that have been linked to affiliative and aggressive behavior in other mammalian species—and aggression in domestic dogs. We first validated enzyme-linked immunosorbent assays (ELISAs) for the measurement of free (unbound) and total (free + bound) OT and AVP in dog plasma. In Experiment 1 we evaluated behavioral and neuroendocrine differences between a population of pet dogs with a history of chronic aggression toward conspecifics and a matched control group. Dogs with a history of aggression exhibited more aggressive behavior during simulated encounters with conspecifics, and had lower free, but higher total plasma AVP than matched controls, but there were no group differences for OT. In Experiment 2 we compared OT and AVP concentrations between pet dogs and a population of assistance dogs that have been bred for affiliative and non-aggressive temperaments, and investigated neuroendocrine predictors of individual differences in social behavior within the assistance dog population. Compared to pet dogs, assistance dogs had higher free and total OT, but there were no differences in either measure for AVP. Within the assistance dog population, dogs who behaved more aggressively toward a threatening stranger had higher total AVP than dogs who did not. Collectively these data suggest that endogenous OT and AVP may play critical roles in shaping dog social behavior, including aspects of both affiliation and aggression.

Keywords: oxytocin, vasopressin, aggression, dog, behavior

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INTRODUCTION

Aggressive behavior in dogs is a serious concern for reasons related to both public health and animal welfare. In the United States it is estimated that dogs bite ∼4.5 million Americans annually, with approximately half of these bites directed toward children (Centers for Disease Control and Prevention, 2003; Gilchrist et al., 2008). In addition to this impact on human lives, aggression (toward humans or other dogs) is also one of the most common reasons that dogs are relinquished to animal shelters (Salman et al., 1998), with ∼2 million of these dogs being euthanized every year (Patronek et al., 1996). Despite these widely recognized concerns, we know relatively little about the psychological and biological factors underlying dog aggression.

Aggression is an adaptive—but occasionally pathological—form of social behavior which can be motivated by a wide range of psychological states. Dog aggression may result from cognitive appraisals of the social environment which result in fear, anger, or, predatory motivations, and individuals may differ in their tendency to express or inhibit behavioral responses to these emotions. The biological mechanisms which facilitate aggressive behavior are diverse, and different forms of aggression may be mediated by different neurobiological substrates (Panksepp and Zellner, 2004).

Previous studies on the biology of canine aggression have focused predominantly on the role of androgens and the serotonergic system. Although, testosterone is positively associated with aggression in many species (Archer, 1988), studies of androgens and aggression in dogs have been largely inconclusive. For example, in some studies gonadectomy (which yields decreased androgen production) has been linked to a mild reduction in male dog aggression toward both other dogs and people (Neilson et al., 1997), whereas in others neutered dogs were found to be more aggressive (Guy et al., 2001). Findings on the serotonergic system have been more consistent than those for androgens. Specifically, some dogs with a history of aggression are characterized by low levels of serotonin or serotonin metabolites—in both blood and cerebrospinal fluid (CSF)—and this finding is especially pronounced in lineages prone to aggression (Reisner et al., 1996; Haug, 2008; Rosado et al., 2010; León et al., 2012; Amat et al., 2013). Because of the inhibitory effect of serotonin on aggression, one common intervention for aggressive dogs has been to increase serotonin availability through selective serotonin reuptake inhibitors (SSRIs; Haug, 2008). Although, testosterone and serotonin may both have important roles in regulating aggression, research with other mammalian species indicates that oxytocin and arginine vasopressin also play major roles in the inhibition and facilitation of aggressive behaviors (Carter, 1998; Caldwell et al., 2008; Albers, 2012). However, few studies have investigated the links between these neuropeptides and aggressive behavior in dogs.

Oxytocin (OT) and arginine vasopressin (AVP) are closely related nonapeptides with wide ranging effects on social behavior, cognition, and stress responses (Carter, 1998; Goodson and Bass, 2001; Carter et al., 2008; Donaldson and Young, 2008). Although, the biological effects of OT and AVP can be similar in many cases, in others they are antagonistic (Neumann and Landgraf, 2012). With respect to affective states and social behavior, OT inhibits the sympathoadrenal axis, reduces anxiety, and can promote affiliative behavior. In contrast, AVP increases sympathoadrenal activity, is anxiogenic, and in some cases facilitates aggression (Ferris, 1992; Carter, 1998). However, both peptides can have effects that are sex- and species-specific, and depend on site of action in the brain, as well as characteristics of the receptor (Kelly and Goodson, 2014). Moreover, both peptides are capable of binding to one another's receptors, and the dynamic balance between OT and AVP is hypothesized to mediate a wide spectrum of emotional states and social behaviors (Neumann and Landgraf, 2012).

Recent studies with dogs have highlighted the role of OT in affiliative behavior and positive affective states. For example, dogs exhibit an increase in OT after friendly interaction with a human (Odendaal and Meintjes, 2003; Rehn et al., 2014; Nagasawa et al., 2015; MacLean et al., 2017b), or other pleasurable experiences (Mitsui et al., 2011; Beetz et al., 2012). Recently, polymorphisms in the oxytocin receptor gene (*OXTR*) have been linked to human-directed social behavior in dogs (Kis et al., 2014; Oliva et al., 2016b), and dogs treated with intranasal OT have been documented to exhibit increased affiliative behavior toward both humans and other dogs (Romero et al., 2014; Nagasawa et al., 2015; but see Hernádi et al., 2015). Lastly, OT administration has been documented to enhance some aspects of dog-human communication (Oliva et al., 2015), including cognitive skills that may be convergent between humans and dogs (MacLean and Hare, 2015; MacLean et al., 2017a). Thus, current data suggest that OT both facilitates and responds to some types of affiliative and cooperative social interaction in dogs.

Although, no studies have investigated the role of AVP in dog aggression, data from other mammalian species suggest that AVP plays an important role in regulating aggression toward unfamiliar individuals. For example, early studies on AVP and aggression revealed that microinjection of AVP into the hypothalamus of golden hamsters led to increased aggression toward unfamiliar conspecifics, whereas hamsters receiving an AVP antagonist displayed a dose-dependent decrease in biting and latency to attack unfamiliar individuals (Ferris and Potegal, 1988; Ferris, 1992; Ferris et al., 1997, 2006; Albers, 2012). Although, these findings have been replicated in several other species (Bester-Meredith et al., 2005; Gobrogge et al., 2007), other experiments reveal that AVP can both facilitate or inhibit aggression, depending on the site of action in the brain or sex-specific factors (Kelly and Goodson, 2014; reviewed in Albers, 2015), and AVP may be critical for some forms of affiliative behavior (Carter et al., 1995). In contrast to these rodent studies which have addressed localized functions of AVP, human studies have measured AVP in cerebrospinal fluid (CSF) or the periphery to assess potential links between overall circulating levels of AVP and social behavior. With respect to aggression, Coccaro et al. (1998) measured AVP in human CSF and found positive associations between AVP and a life history of aggression, and studies administering intranasal AVP in men led to decreased perceptions of friendliness in unfamiliar faces (Thompson et al., 2006).

Taken together, these findings suggest that OT may play a larger role in affiliative social behavior, anxiolysis, and the inhibition of aggression, whereas AVP—though also critical to bond formation and parental behavior—may play a larger role in anxiogenesis and aggression. To investigate the links between OT, AVP, and aggressive behavior in dogs we conducted two studies in which dogs were individually exposed to various stimuli: (1) three-dimensional dog models, (2) video images of other dogs, and/or (3) a threatening human, and we recorded the resulting aggressive responses. Free (unbound) and total (free + bound) plasma OT and AVP concentrations were determined and used as predictors of behavior in these contexts. We first conducted a series of methodological studies to validate sample preparation protocols for the measurement of OT and AVP in dog plasma (SOM). In Experiment 1, we compared the behavior and OT/AVP

concentrations of two group of dogs: a "case group"—dogs recruited because of their known history of aggression toward unfamiliar conspecifics—and a "control group"—dogs with no previous history of aggression toward conspecifics, who were matched to cases on the basis of breed, sex, and age. These dogs were exposed to life-like three-dimensional dog models, as well as video-projected stimuli featuring dogs engaged in a variety of non-aggressive behaviors. In Experiment 2 we compared the hormone concentrations of a population of assistance dogs—who have been selectively bred for affiliative and non-aggressive behavior—and the companion dogs tested in Experiment 1. We also tested the assistance dogs with the video stimuli used in Experiment 1, as well as in a temperament evaluation during which dogs were exposed to a life-like three-dimensional dog model, and an unfamiliar human who approached the dog in a threatening manner.

GENERAL METHODS FOR EXPERIMENTS 1–2

Oxytocin samples were assayed using commercially available enzyme-linked immunosorbent assay (ELISA) kits from Arbor Assays (K048) and Cayman Chemical (500440). The Arbor Assays kit was used for all analyses with the exception of the measurement of free OT with the assistance dog population in Experiment 2. This change was implemented because free OT concentrations measured with the Arbor Assays kit in Experiment 1 were near the lower limit of detection, and subsequent analyses in our lab revealed that free OT in dog plasma was detectable in a better region of the standard curve with the Cayman Chemical kit. All vasopressin samples were assayed using a commercially available ELISA kit from Enzo Life Sciences (ADI-900-017A).

Recent data suggest that OT binds strongly to plasma proteins which may prevent its detection in plasma (Martin and Carter, 2013; Martin, 2014; Brandtzaeg et al., 2016). Given its structural similarity to OT and the presence of a disulfide bridge, it is likely that AVP exhibits similar binding patterns. We have recently shown that a reduction/alkylation and protein precipitation (R/A PPT) procedure—which liberates bound OT from plasma proteins—allows for the detection of much higher concentrations of OT, and have validated this approach with dog plasma analyzed by ELISA (Brandtzaeg et al., 2016). Due to the protein precipitation step, this process also eliminates the matrix interferences commonly observed when working with neat plasma. Here we used this approach for both OT and AVP (see SOM).

For the measurement of free OT and AVP, all samples were processed using solid phase extraction (SPE) as described in the Supplemental Materials (SOM). We expected that samples processed using solid phase extraction (SPE) should capture "free" peptide concentrations, reflecting acute activity at the time of the study, whereas samples prepared via R/A PPT should represent total OT and AVP concentrations, and provide a biomarker of longer-term individual differences (free and bound concentrations; Brandtzaeg et al., 2016).

EXPERIMENT 1

Experiment 1 was a case-control study in which dogs with a history of aggression (hereafter cases) toward unfamiliar dogs while walking on leash ("leash aggression") were compared to a matched control group (hereafter "controls") with no history of aggression. We opted to study leash aggression specifically, because we aimed to investigate aggressive behavior in a highly controlled context, in which all dogs could be kept a fixed distance from a controlled stimulus used to elicit aggressive responses. All testing took place at the Veterinary Health and Wellness Center at North Carolina State University's College of Veterinary Medicine.

Method

Subjects

Cases were recruited through dog-related email list-serves and area dog trainers specializing in cases of leash aggression. Recruitment materials solicited owners with dogs who routinely snarl, growl, or lunge at unfamiliar dogs while on leash. Individuals expressing an interest in the study participated in an initial phone screening to verify that their dog exhibited a chronic pattern of aggression toward other unfamiliar dogs, and met the inclusionary criteria described below. When cases met the inclusionary criteria, matched controls (based on breed, sex, and age) were recruited from a database of pet owners maintained by the Duke Canine Cognition Center. Owners of control dogs participated in an initial screening to verify that their dog was not aggressive toward unfamiliar conspecifics, and that dogs met the basic inclusionary criteria. In some cases, dog owners canceled their appointments after one member of a case-control dyad had been tested or scheduled, in which case we retained data from the unmatched subject and assigned this dog to the nearest case-control grouping based on sex, age, and breed. In cases when an exact breed match was not possible, we attempted to match subjects based on breed group.

All owners were offered a free veterinary exam, complete blood count (CBC), and serum biochemistry panel for their dog in exchange for participation. To be eligible for participation subjects were required to be between 1 and 9 years of age, 4.5–70 kg in weight, spayed, or neutered, and to have an up-to-date rabies vaccination. Dogs were excluded from participation if they had chronic illnesses, a history of aggression toward humans or familiar dogs within the household, abnormal results for the CBC or chemistry panel, or had received psychoactive medications within the past 30 days. In total, 46 dogs began the study, but three subjects were subsequently excluded either because blood could not be collected during the veterinary exam ($N = 2$), or due to abnormalities in the CBC results ($N = 1$). Subject demographics for matched cases and controls are shown in **Table 1**. All dog owners signed informed-consent documents prior to participation and testing procedures adhered to regulations set forth by the Institutional Animal Care and Use Committee at North Carolina State University (Protocol#: 14-184-O).

TABLE 1 | Subject demographics for Experiment 1.

Breed	Group	Case/Control	Sex	Age (years)
German shepherd	A	Case	F	6
German shepherd	A	Control	F	9
Beagle mix	B	Case	F	7
Beagle	B	Control	F	4
American staffordshire terrier mix	C	Case	M	4.5
American staffordshire terrier mix	C	Control	M	4
Weimaraner	D	Case	M	6
Golden retriever	D	Control	M	7
Golden retriever	E	Case	F	3
Golden retriever	E	Control	F	4
Potcake dog	F	Case	M	2
Labradoodle	F	Control	M	4
American staffordshire terrier	G	Case	F	2
American staffordshire terrier mix	G	Control	F	3
Lhasa apso mix	H	Case	M	8
Cavalier king charles spaniel	H	Control	M	7
German shepherd	I	Case	M	4
German shepherd	I	Control	M	4
Hound mix	J	Case	M	6
American staffordshire terrier mix	J	Case	M	5
Treeing walker coonhound	J	Control	M	6
Corgi mix	K	Case	F	3
Corgi mix	K	Control	F	6
Australian cattle dog mix	L	Case	F	3
Border collie mix	L	Control	F	3
Boxer	M	Case	M	5
Boxer	M	Control	M	3
Poodle (standard)	N	Case	M	5
Mastiff mix	N	Case	M	4.5
Poodle (standard)	N	Control	M	6
Terrier mix	O	Case	M	6
Border collie mix	O	Control	M	6
Golden retriever	P	Case	F	4
Golden retriever	P	Control	F	3
Greyhound	Q	Case	M	4
Greyhound	Q	Control	M	2.5
Labrador retriever	R	Case	M	8
Labrador retriever	R	Control	M	9
Australian cattle dog	S	Case	M	5
Australian cattle dog	S	Control	M	8
Australian shepherd mix	T	Case	M	7
Australian cattle dog	T	Control	M	4

Apparatus and Stimuli

A schematic of the testing room is shown in **Figure 1**. The test room was divided into two sections by a wall of filing cabinets (1.22 m high) with a 1.7 m gap in this wall to allow subjects to view stimuli presented in the other half of the room. An opaque curtain was hung across this gap so that subjects could see through to the other side of the room only during periods of stimulus presentation. Owners sat in a chair 2.5 m from the dividing wall with a rubber mat adjacent to this chair where dogs were positioned at the start of the study. All dogs wore a 1.25 m leash attached to a neck collar to allow them to move freely within a fixed radius from their starting position during the test. Video

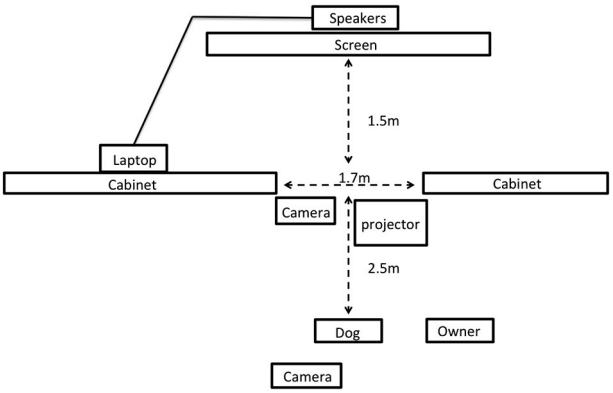

FIGURE 1 | Schematic of the testing room for Experiment 2 (not to scale).

stimuli were projected with an NEC video projector (model VT695; only present during video trials) on a 1 × 1.5 m white poster board that was positioned 1.5 m behind the opening in the room divider. Audio speakers were positioned behind this poster board so that sounds were presented from directly behind the area where visual stimuli were presented. All trials were filmed with two high definition camcorders, one positioned at the back of the room, which captured the stimulus presentations and dog (from the rear), and the other from the gap in the center of the room, which captured the dog's behavior (oriented toward the dog's face). This second camera was the primary angle used for all behavioral coding.

Three-dimensional stimuli consisted of life-like dog models of three different sizes (small—Jack Russell Terrier; medium—Shetland Sheepdog; large—Old English Sheepdog) and three inanimate objects (control stimuli) of comparable size (yellow box, black trash bag filled with paper, inflated blue yoga ball). Prior to the presentation of each stimulus we played a brief (∼2 s) auditory stimulus to attract the dog's attention to the area in which the visual stimulus would be presented. For dog models these stimuli were barking sounds appropriate for the specific model's body size, and for control stimuli these sounds were arbitrary sound effects. Video stimuli (each 15 s long) consisted of footage from DOGTV (a company which produces digital content optimized for dog vision). Dog clips included images of a dog being walked on leash, a dog resting in the grass, and two dogs playing. Control clips included images of water running across rocks, and panoramas of a forest canopy or flowers. Audio content was added to these clips such that all control clips had soft instrumental music, and dog clips included the sound of the dog panting, or of dog play vocalizations. The audio for dog clips was digitally edited so that the sounds and visual images were synchronous.

Procedure

Veterinary Exam and Background Information

After scheduling their research visit, all participants were provided with an electronic link to complete the Canine Behavioral and Research Questionnaire (C-BARQ), a validated and reliable instrument that measures various aspects of canine

behavior, and has been used in previous studies of canine aggression (Duffy et al., 2008; van den Berg et al., 2010). Upon arrival, clients were escorted to a quiet consultation room where a veterinary technician collected background information about the dog's behavioral and medical history, and clients signed informed consent documentation. Dogs were then taken to a nearby exam room and the attending veterinarian performed a physical examination and collected the first blood sample to be used for a CBC, chemistry panel, and analysis of free and total OT and AVP. At the conclusion of the exam, dogs and their owners were brought to the test room where the primary experimenter (E1) explained the procedure and provided instructions for handling the dog during the test. Owners were asked to hold the dog's leash against a fixed point on the arm of their chair, and to refrain from interacting with their dog during the test trials, regardless of the dog's behavior.

Three-Dimensional Stimuli

At the start of each trial, the curtain was closed preventing dogs from viewing activity on the experimenter's side of the room. E1 positioned himself behind the curtain with the stimulus, and a second experimenter (E2) played the audio clip to attract the dog's attention. E2 then opened the curtain with a pulley, and dogs observed E1 and the stimulus for 15 s at which point the curtain was closed. For dog stimuli, E1 held a leash that was looped around the model dog's neck, and gently petted the dog. For control stimuli, E1 performed comparable motions (e.g., touching or patting the box and bag, or rotating and lightly bouncing the ball). E1 looked at the stimulus throughout the trial and avoided eye contact with the subject. Six trials were conducted and the stimulus type (dog model, control object) alternated between trials. We used two fixed orders for the stimulus presentation (order 2 was the reverse of order 1) and the stimulus order was counterbalanced within groups (case and control) and consistent within matched case-control pairs. Examples of the procedure and subject responses are shown in Movie S1.

Video Stimuli

At the conclusion of the final three-dimensional stimulus the curtain was left open, E1 left the testing area and started the video. Like the three-dimensional stimuli, the order of video stimuli alternated between clips featuring dogs, and control clips (nature scenes), and we used two orders of stimuli presentation that were counterbalanced as described above. Each clip was separated by 10 s during which the screen was black. At the conclusion of the final video stimulus dogs were taken to a nearby exam room and a second blood sample was collected to assess changes in free OT and AVP after exposure to the test stimuli.

Scoring and Analysis

From video we coded each trial for the duration of barking and growling, as well as the number of times that the dog lunged at the stimulus, or raised her upper lip in an aggressive display. Raised hackles (erectile hairs along the back of the dog) could not be coded due to heterogeneity in coat type in this diverse sample, which precluded comparable measures across subjects. All trials were coded by two independent observers blind to the hypotheses and inter-rater agreement was excellent for all measures (bark: $R = 0.95$; growl: $R = 0.98$; lunge: $R = 0.95$, raise lip: $R = 0.89$). Preliminary analyses revealed much stronger reactions to the three dimensional stimuli than the video stimuli, with very few dogs exhibiting aggressive responses to the latter (**Table 2**). Therefore, our analyses of behavior during the test were restricted to trials incorporating the three-dimensional stimuli. Prior to analysis, each subjects' scores were averaged across the three test trials involving dog stimuli (i.e., excluding trials with control objects) and all data were standardized by conversion to z-scores (within each behavioral category). Because a raised-lip display was observed in only one dog, this variable was dropped from analysis. For the purpose of generating a composite index of aggressive behavior, we conducted a principal components analysis with the z-scores for barking, growling and lunging. The first principal component was loaded positively by all three variables, accounted for 54% of variance, and scores for this component were used as the primary measure of aggression (hereafter "composite aggression score").

TABLE 2 | Behavioral differences between the test (dog) and control (non-dog) conditions with three-dimensional and video-projected stimuli.

	Behavior	*t*	*p*
3D stimuli	Bark	−2.42	0.02
	Growl	−2.38	0.02
	Lunge	−3.08	<0.01
Video stimuli	Bark	−1.03	0.31
	Growl	−1.51	0.14
	Lunge	–	–

Dogs behaved more aggressively toward the conspecific models when presented with three-dimensional, but not video stimuli (video stimuli were not sufficient to invoke aggressive responses). Lunging was not observed in response to the video.

Hormonal predictors of case-control status were assessed using conditional logistic regression (Gail et al., 1981). Unless otherwise noted, comparisons of behavior between the case and control groups were implemented using linear or generalized linear mixed models with the pair identifier for each matched-pair as a random effect. For hormonal analyses we included sex, age, body mass, and assay plate as covariates, and for behavioral analyses we included sex, age, and body mass as covariates. In all cases, individual model predictors were evaluated in a model-comparison framework using a likelihood ratio test to determine the change in likelihood when individual variables were added to the model. Hormone data were log transformed to better meet the assumptions of parametric analysis, and subsequently converted to *z*-scores to facilitate the interpretation of regression coefficients. All analyses were conducted in the R language and environment for statistical computing (R Core Team, 2017).

Sample Collection and Hormone Analysis

All blood samples were collected into vacutainers containing K3 EDTA, centrifuged for 20 min at 3,000 RPMs, and the separated plasma was divided into 1 mL aliquots and frozen at −80°C

until assay. Samples from matched cases and controls were run on the same plate. We analyzed free OT and AVP in samples collected both before and after the experiment in order to assess short-term changes in peptide release. However, pre and post samples were highly correlated (OT: $R = 0.69$; AVP: $R = 0.67$) with no significant changes across time in either group (SOM). Therefore, we report the results only from the pre-test samples below, and additional analyses involving post-test samples, and changes across time are reported in the SOM. Total OT and AVP were measured only in pre-test samples as this measure was intended to provide a longer-term and more stable measure of individual differences.

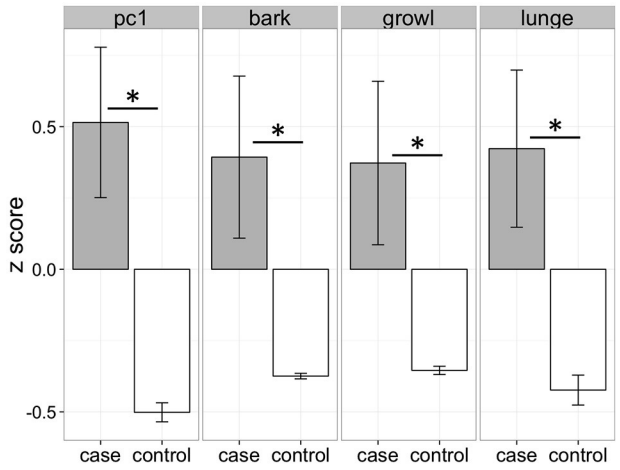

FIGURE 2 | Behavioral differences between cases and controls when confronted with lifelike three-dimensional dog models. Cases and controls differed significantly for all measures (pc1: $\chi^2 = 13.75$, df = 1, $p < 0.01$; bark: $\chi^2 = 8.74$, df = 1, $p < 0.01$; growl: $\chi^2 = 6.27$, df = 1, $p = 0.01$; lunge: $\chi^2 = 7.80$, df = 1, $p < 0.01$). PC1 represents the scores from the first principal component in a principal components analysis including barking, growling, and lunging. *$p < 0.05$.

Results

Behavior

Overall, dogs exhibited significantly more time barking and growling, and lunged at the stimulus significantly more frequently when three-dimensional dogs were presented in comparison to three-dimensional control objects (paired *t*-tests, **Table 2**). In contrast, video stimuli provoked very few responses in either condition, and behavior did not vary depending on whether the video featured another dog, or control content (**Table 2**). Therefore, analyses of behavior during the experiment focus exclusively on the trials incorporating three-dimensional stimuli.

Cases and controls differed significantly in their responses to the dog stimuli with cases exhibiting more barking, growling, and lunging than controls, as well as higher composite aggression scores (**Figure 2**). These behavioral differences were specific to test trials (in which dog models were presented) and the behavior of the groups did not differ during control trials (bark: $\chi^2 = 1.30$, df = 1, $p = 0.25$; growl: $\chi^2 = 1.34$, df = 1, $p = 0.25$; lunge: $\chi^2 = 0.04$, df = 1, $p = 0.84$).

Free Oxytocin and Vasopressin

A conditional logistic regression revealed that cases had significantly lower free AVP levels prior to the test than controls (**Figure 3**; **Table 3**), but that free OT concentrations did not differ

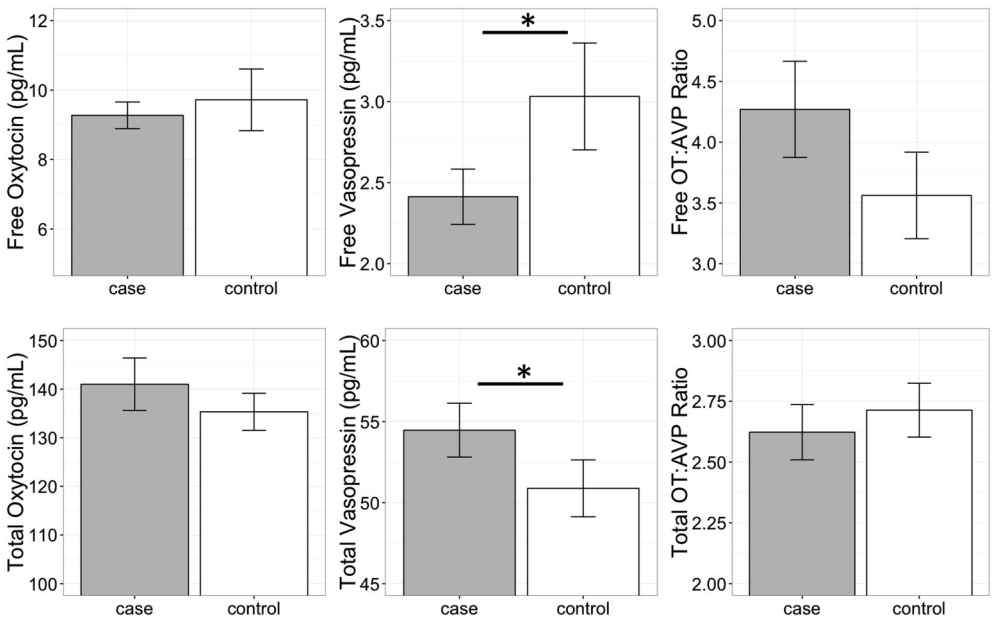

FIGURE 3 | Free and total plasma oxytocin (OT) and vasopressin (AVP) concentrations, as well as the OT:AVP ratio in dogs with a history of aggression (cases) and matched controls (no history of aggression). Cases had significantly lower free plasma AVP, and significantly higher total plasma AVP. *$p < 0.05$.

TABLE 3 | Results from conditional logistic regression models in Experiment 1.

Hormone measure	Predictor	β	Odds ratio	SE (β)	χ^2	*p*
Total	Oxytocin	0.05	1.05	0.46	0.01	0.91
	Vasopressin	1.52	4.57	0.80	5.42	**0.02**
	Age (years)	−0.16	0.85	0.35	0.21	0.65
	Weight (kg)	−0.15	0.86	0.09	3.61	0.06
Free	Oxytocin	0.04	1.04	0.57	0.01	0.94
	Vasopressin	−2.50	0.08	1.08	10.20	**0.00**
	Age (years)	−1.20	0.29	0.65	6.27	**0.01**
	Weight (kg)	−0.22	0.80	0.11	5.86	**0.02**

Odds ratios reflect the odds of membership in the aggressive relative to control groups (values >1 indicate positive associations between the predictor and aggressive group membership whereas values <1 indicate negative associations). Bolded values show significant associations.

between groups (**Figure 3**; **Table 3**). Specifically, a one standard deviation increase in free AVP was associated with a 0.08 odds ratio of being in the case relative to the control group. Cases tended to have higher free OT:AVP ratios, but this difference was not significant (**Figure 3**; **Table 3**). Using the C-BARQ as a measure of aggression outside the experimental context, we observed similar findings. Specifically, dogs reported to be more aggressive toward other dogs were characterized by significantly lower levels of free plasma AVP (SOM).

Total Oxytocin and Vasopressin

In contrast to the analysis of free peptide concentrations, a conditional logistic regression revealed that cases had significantly higher total AVP than controls (**Figure 3**; **Table 3**). A one standard deviation increase in AVP was associated with an odds ratio of 4.5 for being in the case relative to the control group. Neither total OT, nor the total OT:AVP ratio differed between groups (**Figure 3**; **Table 3**). Lastly, using total peptide concentrations as predictors of C-BARQ scores, total AVP was positively associated with higher levels of dog-directed aggression (SOM).

Discussion

Cases and controls differed significantly in their reactions to the three-dimensional models, with cases exhibiting higher levels of aggression than controls. Because similar behavioral differences were not observed when subjects were presented with control stimuli, this suggests that aggressive responses were both specific and social in nature, and presumably triggered by the verisimilitude of the models. However, it is noteworthy that not all cases reacted aggressively toward the dog models (SOM), despite the fact that these dogs were recruited specifically due to owner-reports of chronic aggression toward unfamiliar conspecifics. Therefore, while model dogs may provide a useful tool for research purposes, response to these inanimate models is unlikely to correlate perfectly with real-world behavior (Shabelansky et al., 2015).

AVP (but not OT) concentrations prior to the test differed significantly between cases and controls. However, the direction of this difference depended on whether only the free portion, or total peptide concentrations were considered, an issue we revisit below. Specifically, cases were characterized by lower levels of free AVP, but higher levels of total AVP. In contrast, neither OT measure differed significantly between cases and controls. This finding lends further support to the idea that while structurally and functionally related, AVP may be more strongly associated with aggression than OT.

The differences between cases and controls in this study may reflect atypical characteristics of the case population, the control population, or both. Specifically, whereas cases were recruited due to a history of aggression toward other dogs, we required that controls had not exhibited notable aggression toward conspecifics, possibly creating a contrast between dogs at extreme ends of a behavioral continuum. To address whether dogs in the control group were characterized by abnormally low levels of fear or aggression toward humans or other dogs, we compared C-BARQ scores for these dogs to the general distributions for pet dogs reported in Hsu and Serpell (2003). Average C-BARQ scores for control subjects in Experiment 1 fell within the interquartile range for large samples of pet dogs, suggesting that control subjects were not atypical with respect to these behavioral traits.

Lastly, it is important to note that although this experiment employed a case-control design, there were some limits in our ability to match subjects based on breed and age (sex was successfully matched for all groups). Specifically, in some cases where exact breed matches were not possible, we matched subjects based on breed group and morphological characteristics (e.g., body mass). Similarly, age could not be matched exactly in many cases, but on average, paired cases and controls did not differ in age by more than 1.5 years. Therefore, although we obtained reasonable groupings of cases and controls, future studies may benefit by more exact matching on these parameters.

EXPERIMENT 2

Experiment 2 evaluated hormonal predictors of variance in social behavior in a population of candidate assistance dogs tested at Canine Companions for Independence (CCI) in Santa Rosa, CA. Additionally, we compared endogenous OT and AVP levels between this population and the population of pet dogs studied in Experiment 1. Because this assistance dog population has been under active selection for friendly and non-aggressive temperaments for >40 years, we expected that if OT and AVP play critical roles in shaping these traits, then this population may exhibit unique neuroendocrine characteristics relative to a population of pet dogs.

Method

Subjects

Thirty candidate assistance dogs from CCI participated in Experiment 2. Demographic information for all subjects is shown in **Table 4**.

Procedure

All assistance dogs participated in an initial temperament test (designed and implemented by CCI) which included two social events (threatening stranger and unfamiliar dog)

TABLE 4 | Subject demographics for Experiment 2.

Breed	Sex	Age	Intact
Lab—Golden cross	M	1.8	No
Lab—Golden cross	F	1.6	Yes
Lab—Golden cross	M	1.6	No
Lab—Golden cross	F	1.6	Yes
Lab—Golden cross	M	1.6	No
Lab—Golden cross	F	1.6	Yes
Lab—Golden cross	F	1.6	Yes
Lab—Golden cross	M	1.6	No
Lab—Golden cross	M	1.6	No
Lab—Golden cross	F	1.6	Yes
Lab—Golden cross	M	1.6	Yes
Lab—Golden cross	F	1.5	Yes
Lab—Golden cross	M	1.8	No
Labrador retriever	M	1.7	Yes
Lab—Golden cross	F	1.5	Yes
Golden retriever	M	1.5	Yes
Lab—Golden cross	F	1.7	Yes
Golden retriever	F	1.5	Yes
Lab—Golden cross	M	1.7	No
Labrador retriever	F	1.5	Yes
Lab—Golden cross	M	1.7	Yes
Lab—Golden cross	F	1.7	Yes
Labrador retriever	M	1.5	Yes
Labrador retriever	F	1.5	Yes
Labrador retriever	F	1.5	Yes
Lab—Golden cross	F	1.5	Yes
Lab—Golden cross	M	1.7	No
Lab—Golden cross	F	1.7	Yes
Lab—Golden cross	M	1.6	No
Labrador retriever	M	1.4	Yes

which were video recorded for the purpose of this study. The larger temperament test was administered by walking dogs along a pre-determined path, which included a variety of potentially distracting, startling, or threatening stimuli. Immediately following this temperament test, dogs were also tested with the video stimuli used in Experiment 1. In addition to these behavioral tests, puppy-raisers completed C-BARQ evaluations for the majority of these subjects based on the dog's behavior at 1 year of age (the C-BARQ could not be obtained for some dogs who were raised in a prison puppy raising program). Blood draws were performed on all dogs as part of a routine veterinary examination 1 day prior to the behavioral test.

Threatening Stranger (TS)

In the threatening stranger test the handler walked the dog toward a man (TS) sitting on a bench who was wearing a hooded jacket and holding a cane. As the dog approached, the TS stood up, banged his cane on the ground, shouted toward the dog in a threatening tone, and walked 2 m toward the dog. During the TS's approach the handler remained stationary with the dog on leash for ∼10 s to observe the dog's reaction. The handler then encouraged the dog to approach the man by walking forward while holding the leash. Once the dog was within arm's reach of the TS, the TS removed his hood, set down his cane, kneeled, and greeted the dog in a friendly manner.

Unfamiliar Dog

In the unfamiliar dog trial subjects confronted a three-dimensional life-like dog model (Old English sheepdog, identical to that from Experiment 1) as they walked (on leash, handled by a trainer) along a sidewalk. This sidewalk wrapped around the exterior of a building such that the model was first became visible at a distance of ∼10 m. Trainers first led the dogs to a point 6 m from the model before turning around and returning to a distance of 10 m from the model. Trainers and dogs then made two additional approaches to distances of 3 and 0.6 m from the model, each time turning around and walking ∼5 steps away from the model before the next approach. On fourth and final approach dogs were allowed to freely inspect the model before advancing to the next item in the temperament test.

Video Stimuli

The video stimuli and procedure were identical to Experiment 1.

Scoring and Analysis

All trials were scored from video by two independent observers.

Video Stimuli

Barking, growling, snarling, and lunging were coded as in Experiment 1. However, only barking and growling were observed, and only in a single dog, thus behavioral responses to the video stimuli were excluded from subsequent analysis.

Threatening Stranger

From video we coded whether dogs barked, growled, snarled, or lunged at the threatening stranger (TS). We also classified the dog's initial reaction to the TS into one of the following four categories: (1) Dog resisted the handler and was not easily coaxed toward the TS, (2) Dog resisted the handler but was easily coaxed toward the TS, (3) Dog moved toward TS confidently alongside the handler, (4) Dog moved toward TS confidently in front of the handler. Lastly, we used the following classifications for dogs' reactions to the TS after he removed his hood and greeted the dog (recovery): (1) Dog did not recover from threat and continued to avoid TS after his change in demeanor, (2) Dog remained skittish but was willing to greet and be touched by the TS, (3) Dog was eager to greet TS and showed no signs of fear or hesitation. Inter-rater agreement was excellent for all measures (bark: kappa = 1; growl: kappa = 1; snarl: kappa = 1, initial reaction: $R = 0.97$, recovery: $R = 0.91$), but lunging was not observed so was dropped from analysis. For the purpose of analysis, barking, growling, and snarling were combined into a single composite aggression score. Because each variable was coded as a binary measure (1: present, 0: absent), the composite aggression score was calculated as the sum of barking, growling and snarling for each dog (range 0–3).

Unfamiliar Dog

From video we coded whether dogs barked, growled, or snarled at the unfamiliar dog. We also classified the dog's approach toward the unfamiliar dog (UD) into one of the following four

TABLE 5 | Results from statistical models comparing hormone concentrations between a population of pet, and candidate assistance dogs.

Dependent measure	Predictor	β	χ^2	*p*
Free oxytocin	Population	−0.56	4.49	**0.03**
	Sex	−0.37	2.18	0.14
Total oxytocin	Population	−0.87	14.39	**<0.01**
	Sex	−0.03	0.02	0.89
Free OT/AVP ratio	Population	−1.41	3.78	**0.05**
	Sex	0.17	0.56	0.45
Free vasopressin	Population	0.34	2.01	0.16
	Sex	−0.19	0.63	0.43
Total vasopressin	Population	−0.30	1.53	0.22
	Sex	0.29	1.42	0.23
Total OT/AVP ratio	Population	−0.32	5.33	**0.02**
	Sex	−0.10	0.56	0.45

Bolded values indicate significant associations.

categories: (1) Dog resisted handler when approaching UD and was not easily coaxed toward UD (2) Dog resisted handler when approaching UD but was easily coaxed toward UD (3) Dog approached UD confidently but was easily redirected away from UD by handler (4) Dog approached UD confidently and was not easily redirected away from UD by handler. There was only one instance of barking and growling, and no instances of snarling, and these variables were therefore dropped prior to analysis. Inter-rater agreement for the approach measure was good ($R = 0.88$).

C-BARQ

As in Experiment 1, we assessed the association between plasma peptide levels and C-BARQ scores for measures related to human- and dog-directed fear and aggression.

Statistical Analysis

Due to limited variability and high skew in the behavioral measures, all behavioral measures were discretized into two quantile groups corresponding to low and high scores on each measure using the Hmisc package (Harrell, 2015) in the R Environment for Statistical Computing (R Core Team, 2017). Associations with plasma peptide levels were tested by fitting generalized linear models predicting behavior as a function of OT, AVP, and sex. Individual model predictors were evaluated in a model-comparison framework using a likelihood ratio test to determine the change in likelihood when individual variables were added to the model. For population comparisons we included sex as a covariate in the analyses. Hormone data were log transformed prior to analysis to better meet the assumptions of parametric analyses, and subsequently standardized to facilitate interpretation of regression coefficients.

Sample Collection and Hormone Analysis

Samples were collected and processed as described in Experiment 1. Assistance dog samples were initially analyzed for free OT and AVP using the same methods from Experiment 1 to allow direct comparison of free OT and AVP concentrations between assistance and pet dogs. The majority of assistance dog samples ($N = 19$) were run on the same plates as pet dog samples allowing a direct comparison between these groups. Eleven assistance dog samples were run on a plate containing no pet dog samples, so to control for inter-assay variance we excluded these samples from the population comparison for free OT and AVP. For comparison within the assistance dog group, all samples were re-run on the same plate to allow the most direct comparisons within this population. For comparisons of free OT and AVP within the assistance dog population, we adopted a modified extraction protocol which yielded improved OT and AVP recovery (SOM). OT samples for this analysis were also measured using a different ELISA kit, which permitted detection in a better region of the kit's standard curve (see SOM for validation). For comparison of total OT and AVP all pet and assistance dog samples were initially run on the same plates allowing all individuals to be included in the population comparison. As above, all assistance dog samples were then rerun on the same plate allowing the most direct comparisons within this population.

Results

Population Comparison

Comparison of pet and candidate assistance dog samples revealed a population difference for both free and total OT (**Table 5**) but not for either measure of AVP (**Table 5**). Specifically, the candidate assistance dogs—who were systematically bred for calm temperaments and friendly demeanors—had higher free and total plasma OT than pet dogs (**Figure 4**). Additionally, assistance dogs had higher free and total OT:AVP ratios then pet dogs (**Figure 4**; **Table 5**).

Threatening Stranger and Unfamiliar Dog

Hormonal associations with behavior in the unfamiliar dog and threatening stranger test are summarized in **Table 6**. Although, dogs varied in their willingness to approach the unfamiliar dog and threatening stranger, there were few cases of aggressive behaviors. In the threatening stranger test, only a minority of subjects (∼20%) barked, growled, or snarled at the stranger, and there were no significant associations between these behaviors and free OT, AVP, or the OT:AVP ratio (**Table 6**). There were no associations between the free hormone measures and initial reactions to the threatening stranger (**Table 6**), however, dogs who remained skittish or were hesitant to greet the stranger after he changed demeanor had higher free OT:AVP ratios (low AVP relative to OT). Analysis of total peptide concentrations revealed that dogs who behaved aggressively toward the threatening stranger had significantly higher total AVP than dogs who did not, mirroring the patterns observed in Experiment 1 (**Table 6**). Dogs who behaved aggressively in this context also exhibited significantly lower OT:AVP ratios than dogs who did not (**Table 6**). Lastly, in the unfamiliar dog test, dogs who exhibited the most confident approaches toward the model dog were characterized by higher free OT:AVP ratios than dogs who were more reluctant to approach, and there were no other significant associations (**Table 6**).

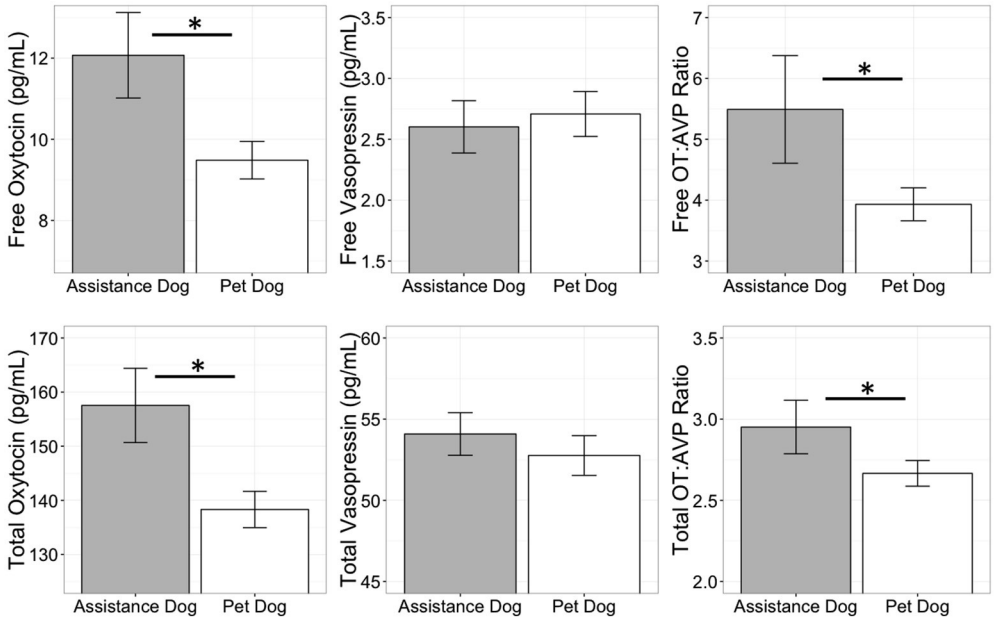

FIGURE 4 | Comparison of free and total plasma oxytocin (OT) and vasopressin (AVP) concentrations, as well as the OT:AVP ratio in pet and assistance dogs. *$p < 0.05$.

C-BARQ

Within the assistance dog population there was minimal variation on all C-BARQ measures with the exception of dog-directed fear, which was negatively associated with the free OT:AVP ratio (SOM).

Discussion

The results of Experiment 2 are consistent with, and build on the findings from Experiment 1. Although, there was minimal aggressive behavior in this sample, dogs who behaved aggressively toward the threatening stranger had higher total AVP concentrations, and higher total AVP relative to OT, corroborating the patterns observed in Experiment 1. We also observed a population difference in OT, but not AVP concentrations between the pet dog population from Experiment 1, and the population of candidate assistance dogs. Specifically, candidate assistance dogs had higher free and total plasma OT than pet dogs, as well as a higher ratio of OT to AVP on both measures. Given that this population has been actively selected for calm, affiliative, and non-aggressive temperaments for more than 40 years, one possibility is that this phenotype has been achieved in part due to upregulation of the oxytocinergic system. This possibility is supported by data from dog OT administration studies, in which exogenous OT has been shown to increase affiliative behavior (Romero et al., 2014; Nagasawa et al., 2015; but see Hernádi et al., 2015), and promote calm emotional states by decreasing heart rate and increasing heart-rate variability (Kovács et al., 2016). However, this population difference should be interpreted cautiously as several factors varied between the assistance and pet dog populations. Specifically, whereas the pet dog population was heterogeneous with respect to breed, age and body size, the assistance dog population consisted entirely of young retriever dogs. Given potential for breed differences in OT genetics (Bence et al., 2016) future investigations of possible differences between pet and selectively bred working populations will benefit from designs controlling for these parameters. Similarly, many individuals in the assistance dog sample were intact at the time of testing, whereas all pet dogs were sterilized. Given that OT and AVP can be affected by androgens or estrogens, it is possible that these factors may have also contributed to the observed population difference. However, within the assistance dog population, measures of both free and total OT were higher on average, among sterilized than intact dogs, making it unlikely that sterilization status accounts for the observed population difference in OT concentrations.

GENERAL DISCUSSION

Aggression is a complex and multifaceted construct that is likely to have an equally complex etiology (Blanchard et al., 2003). Aggressive behaviors may be influenced by a wide range of cognitive, genetic, epigenetic, and environmental factors, as well as an interrelated network of hormonal and physiological processes. Here we report the first systematic investigation of the relationships between endogenous oxytocin (OT), vasopressin (AVP), and aggression in domestic dogs. Our main findings were associations between plasma AVP concentrations and aggressive behavior toward both conspecifics and humans, but the direction of these relationships varied depending on whether we considered only the free fraction, or total AVP (free and bound). In Experiment 1, pet dogs with a history of chronic aggression toward conspecifics had lower free AVP, but higher total AVP than a group of controls matched on breed, sex, and age. In Experiment 2, candidate assistance dogs

TABLE 6 | Associations between free and total oxytocin and vasopressin and behavior during the unfamiliar dog and threatening stranger test.

	Free peptide concentration								Total peptide concentration							
	AVP			OT			OT:AVP Ratio		AVP			OT			OT:AVP Ratio	
	β	χ^2	*p*	β	χ^2	*p*	β	χ^2	*p*	β	χ^2	*p*	β	χ^2	*p*	χ^2	*p*
Unfamiliar Dog: Approach	−0.52	1.25	0.26	0.78	2.49	0.11	0.78	4.56	**0.03**	−0.83	2.73	0.1	−0.39	0.81	0.37	0.01	0.91
Threatening Stranger: Aggression	−0.79	2.22	0.14	0.21	0.15	0.70	0.5	1.83	0.18	1.49	6.74	**0.01**	−0.41	0.55	0.46	4.38	**0.04**
Threatening Stranger: Initial Reaction	0.58	1.41	0.23	−0.45	0.84	0.36	−0.49	1.32	0.25	0.28	0.38	0.54	−0.63	1.62	0.2	2.05	0.15
Threatening Stranger: Recovery	0.64	2.3	0.13	−0.51	1.32	0.25	−0.64	3.89	**0.05**	0.35	0.67	0.41	−0.61	2.11	0.15	3.22	0.07

Significant associations are bolded.

who behaved more aggressively toward a threatening (human) stranger had higher total AVP than dogs who exhibited less aggression in this context. Therefore, across studies we observed a consistent positive association between total AVP concentrations and aggressive behavior, with some evidence for an opposite association with free AVP.

As biomarkers, the significance of free vs. total peptide concentrations remains poorly understood. One possibility is that total OT/AVP may provide the most powerful approach for comparing basal individual differences, indexing long-term peptidergic activity. In contrast, free OT/AVP may be a more useful metric for assessing acute responses to stimuli. Therefore, higher total plasma AVP in dogs prone to aggression may reflect general overactivity in the vasopressinergic system, despite the fact that these dogs had lower levels of free AVP before the behavioral test. This possibility is consistent with data implicating AVP as a primary activator of the hypothalamic-pituitary-adrenal (HPA) axis (Scott and Dinan, 1998; Aguilera and Rabadan-Diehl, 2000), which has long been known to facilitate "fight or flight" behavior (Cannon, 1932; Kruk et al., 2004). The positive association between AVP and aggression is also consistent with previous studies in humans, which have revealed a positive correlation between AVP in cerebrospinal fluid and a life history of aggression, as well as animal studies documenting that centrally administered AVP facilitates aggression toward conspecifics, whereas AVP antagonists diminish this response (Ferris, 1992; Ferris et al., 2006).

However, a wealth of data suggests that the functions of AVP and OT may be highly dependent on species and sex-specific factors, as well as the site of action in the brain. For example, studies that have measured local AVP release, or manipulated the AVP system through receptor agonism/antagonism reveal that AVP can both facilitate and inhibit aggression (Veenema et al., 2010; Kelly and Goodson, 2014; De Boer et al., 2015). Similarly, AVP concentrations and the density of AVP tracts in various regions of the brain have also been associated with aggression, but the nature of these relationships has been variable between species (Compaan et al., 1993; Everts et al., 1997; De Boer et al., 2015).

Studies of peripheral peptide concentrations are further complicated by a lack of knowledge regarding the correspondence between central and peripheral peptide release. Although, some studies have reported positive correlations between OT and AVP concentrations in cerebrospinal fluid (CSF) and free OT/AVP in plasma, others have failed to detect this relationship (Kagerbauer et al., 2013; Carson et al., 2014, 2015). Studies of peptide release patterns have been similarly mixed with evidence for both correlated, and independent central and peripheral release (Neumann and Landgraf, 2012). Lastly, the ultimate effects of these peptides depend not only on their concentrations, but also in the distribution and state of their receptors, which vary not only between individuals and species, but also within individuals as a result of other hormonal and epigenetic processes (Carter and Porges, 2013).

Although, we found no direct associations between OT and aggression, the population of assistance dogs—which has been systematically selected for affiliative and non-aggressive

temperaments—had higher free and total OT than the pet dog population. Given that OT has been positively associated with affiliative social behaviors, these findings are consistent with the possibility that the behavioral targets of selection in the assistance dog population are supported by OT-related mechanisms. However, the assistance and pet dog populations were not matched on critical parameters such as age, breed, or sterilization status, and future research will benefit by controlling for these factors.

At the behavioral level, these studies reveal that realistic three-dimensional models are a useful tool for evaluating dog aggression. Dogs recruited for a history of aggression toward other dogs were much more likely to behave aggressively toward these models than matched controls, and across studies, the three-dimensional models elicited stronger reactions than video-projected stimuli. Importantly, responses to these models were not generalized reactions to novel objects, as dogs reacted much less frequently to comparably-sized control stimuli. Therefore, while unlikely to provide a perfect proxy for real-world behavior (Shabelansky et al., 2015), working with life-like models provides a highly-feasible, safe, and ethical option for assessing aggression, and may also be useful for systematic desensitization with dogs prone to aggressive behavior. In contrast, although several studies have successfully used video-projected stimuli with dogs (e.g., Pongrácz et al., 2003), our video stimuli uniformly elicited minimal response. Importantly, these video stimuli were projected to life-like dimensions, and presented with a refresh rate suitable for the flicker-fusion frequency of dog vision (Coile et al., 1989). Thus, while dogs were likely capable of perceiving the video, three-dimensional cues may be required for successfully simulating an encounter with a conspecific.

Collectively, our findings provide preliminary evidence that AVP may be an important mediator of canine aggression and call for further research in this area. Because previous studies have focused primarily on the role of serotonin and testosterone in dog aggression—both of which contribute to pathways that also include OT and AVP (Delville et al., 1996; Dölen et al., 2013)—future research will benefit by addressing the complex interactions between these systems (Weisman and Feldman, 2013). Similarly, several exploratory studies have begun to investigate the effects of intranasal peptide administration in dogs, which in some cases has led to notable changes in social behavior and cognition (Romero et al., 2014; Hernádi et al., 2015; Oliva et al., 2015, 2016a). Therefore, OT/AVP administration provides another non-invasive possibility for investigating how OT and AVP may modulate aggressive behavior in dogs. Notably, previous studies using this approach have found no evidence that intranasal OT decreases aggressive behavior in dogs (Hernádi et al., 2015), which is consistent with our findings that plasma OT levels were unrelated to individual differences in aggression.

Given that we found evidence for both positive and negative associations between AVP and aggression, we have no strong predictions regarding how AVP administration may influence aggressive behavior. Historically, AVP has been noted to have effects antagonistic to those of OT, increasing anxiety, depression, and stress responses (Benarroch, 2013). However, several studies reveal that AVP administration can have effects very similar to those of OT, decreasing heart rate (Hicks et al., 2014), reducing anxiety (Appenrodt et al., 1998), and increasing sociability (Ramos et al., 2014). Notably, many of the prosocial effects of OT, AVP, and MDMA ("Ecstasy") are believed to act through AVP networks in the brain (Ramos et al., 2013), and blocking AVP, but not OT receptors inhibits many of the effects of both OT and AVP, suggesting that the actions of both peptides are dependent on an AVP receptor-dependent mechanism (Hicks et al., 2014). Therefore, as with OT, the effects of AVP administration are likely to be complex, and moderated by a diverse range of biological factors (Bartz et al., 2011).

Ultimately, dog aggression is a normal and adaptive social behavior, but expressed in the wrong contexts, or to an extreme extent, its consequences jeopardize the welfare of both humans and dogs in our society. It is likely that dog aggression can be motivated by diverse psychological states, including fear and anger. These emotional processes may be facilitated by, or produce effects on, OT and AVP signaling in the brain. Thus, it is important to consider dog aggression at multiple levels of analysis, addressing both the cognitive processes (e.g., appraisal, learning, inhibition), and underlying physiological mechanisms, which mediate these behaviors. The studies presented here suggest that OT and AVP may play important roles in these socioemotional processes, and set the stage for future work evaluating whether treatments and interventions for aggression can be improved by considering the roles of these neuropeptides. Ultimately, we hope that these investigations will lead to increased knowledge of the biology of social behavior, promote human and animal welfare, and help to preserve the unique and long-standing relationship between humans and dogs.

AUTHOR CONTRIBUTIONS

EM, LG, MG, BS, and CC: Designed the experiments. EM, LG, MG, and BS: Conducted the experiments. EM, LG, MG, BS, WM, and CC: Wrote the article.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fpsyg.2017.01613/full#supplementary-material

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