The effect of oxytocin on human-directed social behaviour in dogs *(Canis familiaris)*

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Short title: Oxytocin and social behaviour in dogs

Abstract: The oxytocin system has recently received increasing attention due to its effect on complex human behaviours. In parallel to this, over the past couple of decades, the human-analogue social behaviour of dogs has been intensively studied. Combining these two lines of research (e.g. studying the relationship between dog social behaviour and the oxytocin system) is a promising new research area. The present paper reviews the existing literature on how oxytocin is related to different aspects of human-directed social behaviour in dogs.

Keywords: dog; oxytocin; social behaviour; dog–human relationship

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1. Introduction

Oxytocin – which undoubtedly plays a central role in the expression of the high levels of sociality that are essential to contemporary human behaviour (Carter, 2014) – is in evolutionary terms a remarkably conservative nonapeptide, that appears to play a particularly prominent role in the modulation of social life across mammalian taxa (Yamasue et al., 2012). However, despite the initial consensus on the prosocial effects of oxytocin, different explanations have been proposed for how these effects are mediated and the differences between viewpoints are often implicit rather than clearly delineated (Campbell, 2010). Much of the debate focuses on methodological issues about which are the low level (e.g. cellular) mechanisms behind the oxytocin effects and how results of different studies can be compared (Quintana et al., 2014). Our current knowledge of the behavioural effects of oxytocin in humans is mainly based on three mostly independent approaches: (i) correlational studies measuring oxytocin in the periphery (urine, saliva, blood) or in the Cerebro-Spinal Fluid, (ii) gene × behaviour association studies involving receptor (*OXTR*) polymorphisms, and (iii) experimental studies manipulating (both the peripheral and central) levels of oxytocin using intravenous or intranasal administration (for an evaluation of these approaches regarding their informative value in terms of the underlying central nervous mechanisms see: Heinrichs *et al.* 2009).

The number of published papers on dogs' (social) cognition is rapidly growing (Bensky et al., 2013) due to the fact that dogs have been proven to display human-analogue social skills (Hare and Tomasello, 2005; Miklósi and Topál, 2013). Thus, not surprisingly, researchers have also begun to study the relationship between the oxytocin system and social behaviour in the dog.

2. The effect of dog−human social interaction on peripheral oxytocin levels

The first studies aiming to unravel the relationship between oxytocin and human-directed social behaviour in dogs tested the effect of positive social interaction on peripheral oxytocin levels. It was found (Odendaal 1999, 2000; Odendaal & Meintjes 2003 − data of same subjects published with slighly different focus) that dog−human social interaction increases both dogs' and humans' blood oxytocin level (as measured by high-performance liquid chromatography technique − HPLC) compared to baseline (before interaction). Other physiological changes included increased levels of beta-endorphin, prolactin, phenylacetic acid and dopamine, as well as decreased heart rate. The social interaction in these studies consisted of a maximum 30 minutes session (the intervention was finished when a stable drop of at least 5-10 % in blood pressure was experienced) including softly talking to the dog, gently stroking the dog with long smooth strokes, low-key playing and scratching the body and ears of the dog. Participants (N=18) were both owners with their private dogs and non-owners with dogs from the animal facility of the University of Pretoria. These results were conceptually replicated (Handlin et al., 2011) on N=10 female volunteers and their own male Labrador dogs. At the end of a 3-minute-long interaction, which consisted of petting and stroking different parts of the dog's body and talking to it, an increase in blood oxytocin level was found (using immunoassay technique). Stroking the abdominal area for 15 minutes without social reinforcement such as vocal encouragement and eye contact by experimenters who knew the dogs well, but were neither their owners nor caregivers was also found to increase peripheral oxytocin levels as measured (using radioimmunoassay technique) from urine samples 1 hour after the initiation of stimulus (Mitsui et al., 2011). In this experiment (N=9) dogs from different breeds were tested and stroking was found to increase oxytocin levels compared to baseline similarly to other reinforcing treatments such as eating and exercising, but not drinking water. Reunion after separation from a familiar person was also found to increase blood oxytocin levels in (N=12) laboratory-kept beagle dogs (measured

with immunoassay) compared to a pre-separation baseline phase (Rehn et al., 2014). Furthermore if the familiar person made both physical and verbal contact with the dogs upon reunion oxytocin levels remained higher than baseline in the post-reunion phase as well. But this effect was not found when the dog-human interaction did not involve physical contact (verbal contact only or when ignoring the dog). Recent (unpublished) research (MacLean et al. 2017) has shown that salivary OXT levels also increase similarly to blood oxytocin levels after 10 minutes of free-form friendly interaction with a human experimenter (N=19), but not after a control treatment (dog rested quietly in the same environment, without human interaction; N=19).

These results (and others not measuring dog oxytocin levels directly) have led to the supposition that the oxytocin system plays a crucial role in dog−human interactions and serves as a potential underlying mechanism behind animal assisted therapy (Beetz et al., 2012; Pop et al., 2014). Others (Rehn and Keeling, 2016), however, have pointed out that further studies are needed in the field of dog−human relationships, potentially at the individual dog level (rather than talking about the 'average' dog), and incorporating both the owner's overall caregiving strategy and a dyadic approach. While all the above mentioned studies have been conducted on relatively low sample sizes, and have striking methodological differences (e.g. length and specifics of the interaction) and confounds (e.g. using one's own dog or another dog), taken together these findings present strong evidence that generally positive interactions with a human increase oxytocin levels in dogs. This is in line with results from other species including humans (Feldman et al., 2010; Gordon et al., 2010), rhesus macaques (Maestripieri et al., 2009; Winslow et al., 2003) and prairie voles (Kenkel et al., 2012). The relationship between positive social interaction and oxytocin increase in dogs can serve as a starting point for future research into both individual differences and different types of interactions. It has been found for example, that from the ostensive cues (eye-contact, dog-

100 directed talk, calling the dogs name) that humans naturally use in positive social interactions
101 (Topál et al., 2014), dogs are most sensitive to eye contact, while less sensitive to hearing
102 their own name as opposed to a random name (Kaminski et al., 2012). It can thus be supposed
103 that the elements used in combination during positive dog−human social interactions (eye-
104 contact, petting, dog-directed talk, naming) are not uniformly important in modulating dogs'
105 oxytocin response. It is also likely that the relationship between the dog and the interacting
106 human modulates changes in oxytocin level. Dogs have been shown to behave differently
107 towards humans depending on their familiarity and social relatedness (Horn et al., 2013;
108 Kerepesi et al., 2014), and for example in chimpanzee the differences in relationship have
109 been found to modulate changes in the peripheral oxytocin level after positive interactions
110 (Crockford et al., 2013).

111 Investigation of individual differences has been attempted by a questionnaire-based study
112 (Handlin et al., 2012) during which blood samples were collected from N=10 male Labrador
113 dogs (same subjects as in Handlin et al., 2011) and mean oxytocin levels were measured
114 (immunoassay) during a 60-minute period including a 3-minute social contact with the owner
115 at the beginning. It was found that dogs' mean oxytocin levels were related to items indicating
116 the intensity of the dog–owner relationship (as measured by the Monash Dog Owner
117 Relationship Scale). The study also demonstrated a positive correlation with the frequency of
118 owners kissing their dogs and the perceived bond with the dog, and a negative correlation
119 with the frequency of giving food treats to their dog. Higher oxytocin levels in the dogs were
120 also associated with the owners having a perception of their dogs being less difficult to look
121 after and less thought of as making a mess. These results can be due to both differences in
122 baseline oxytocin levels as a function of the above psychological characteristics as well as a
123 differential reaction to social interaction with the owner depending on their relationship. A
124 more recent study (Pekkin et al., 2016) has also found a relationship between behavioural

scales of a validated questionnaire and dogs' oxytocin levels. Specifically *General fearfulness*, *Noise fear frequency* and *Reactivity index* (derived from a set of questions about fearful reactions towards loud noises) were positively related to baseline urinary oxytocin to creatinine ratio (measured with an enzyme-linked immunosorbent assay kit) in N=23 dogs suffering from noise phobia (the original study sample consisted of N=28 dogs from 14 breeds, where Lagotto Romagnolo, N=7, and Staffordshire Bullterrier, N=6, were the most frequent breeds). These results show that, at least in this specific sample of noise phobic dogs, urinary oxytocin is not as good an indicator of positive welfare states as suggested before (Mitsui et al., 2011). These low-sample size studies, while still preliminary, suggest that focusing on individual differences in dog behaviour and their relation to oxytocin levels is a valid approach that needs attention in the future. In addition to the questionnaire survey Pekkin et al. (2016) also conducted a behavioural test where the effect of a deep pressure vest (10-12 mmHg) versus a light pressure vest (2-3 mmHg) and control (no vest) treatment was assessed in a simulated firework test during three two-minute intervals (pre-noise, noise, recovery) in a within subject design. Urine samples were collected at least one week prior to the test, one at baseline and one after wearing the deep vest for 30 minutes. The two urine samples did not differ regarding oxytocin to creatinine ratios and there was a strong positive inter-correlation. The authors also report that there were no correlations between urinary oxytocin and salivary cortisol levels; we should note, however, that saliva samples were collected on the days of the behavioural test (total of four samples before and after each test occasion), thus although no difference was found in cortisol level between treatments or test days, as the oxytocin and cortisol samples were collected on different days, the lack of correlation is not surprising). On the other hand, urinary oxytocin levels correlated positively with time spent near the owner during the recovery interval in case of the deep vest treatment. The authors also reported a behavioural effect that they labelled as "vest-induced increase of

owner-seeking". This was an interval × treatment interaction showing that dogs in the Control, but not in the Light vest and Deep vest treatment, spent less time near their owner during the noise interval compared to the pre-noise or recovery intervals. The authors speculate that this effect might be modulated by oxytocin levels, although they admit not being able to show an effect of the vest on the oxytocin level.

A more recent study (Romero et al., 2014) found that in an experimental situation where owners of the dogs were instructed to sit quietly and not to actively interact with their dogs, neither the time subjects spent in close proximity to their owners nor the affiliation subjects provided to or interchanged with them was related to the oxytocin increase ratio (posttest / pretest OXT levels). In a similar experimental situation (Nagasawa et al., 2015), where the owner was instructed to remain seated in a chair, but was otherwise free to interact with his/her dog during 30 minutes, it was found that dogs (N=8) that gazed longer to their owner showed a higher oxytocin change ratio (as measured from urine samples collected right before and 30 minutes after the interaction) compared to dogs (N=22) that gazed shorter to their owner. The duration of dog-to-owner gaze significantly explained the oxytocin change ratio in dogs and the oxytocin change ratio in owners correlated significantly with that of dogs. Furthermore in case of hand-raised wolves (N=11), who did not gaze at their 'owners' (animal management professionals) and thus gazed significantly less than even dogs in the short gaze group the duration of wolf-to-owner gaze did not correlate with the oxytocin change ratio in either owners or wolves. These results have prompted the authors and others (Maclean and Hare, 2015) to speculate about an oxytocin-gaze positive loop and the coevolution of human-dog bonds. Others (Fiset and Plourde, 2015), however, have suggested that any conclusions about the coevolutionary process are premature and should be presented with great care. It has been argued for example (Kekecs et al., 2016) that there are several confounding differences between the dog and wolf arm of the experiment such as owner sex

(82% female for dogs and 55% female for wolves) that seriously limit the interpretation of the results. Furthermore there was a significant difference in the baseline oxytocin values of the dog and wolf owners (thus the apparent difference between dogs and wolves may be simply due to a ceiling effect), and the rearing and socialization of animals was also different.

Sex	Age	Breeds	Dog's social background	Method	Behavioral protocol	Results	Reference
7 males (3 castrated); 11 females (5 spayed)	6.4 years old (between 2-11 years old)	9 Beagles, 2 Border Collies, 1 Bull Dog, 1 Cocker Spaniel, 1 Dachshund, 3 Labradors, 1 Staffordshire bull terrier	Known to have placid temperaments and used to human contact (some belonged to the human participants and some were provided to non-owners)	High Performance Liquid Chromatography	Maximum 30 minutes dog-human positive interaction (social gestures only; e.g. talking softly, gently stroking, low-key playing, ears and body scratching)	Increased OT concentration, from 0.1 to 0.5 ng/L ($p < 0.01$)	Odendaal 1999, 2000; Odendaal and Meintjes 2003
10 male	4.7 ± 2.6 years old	10 Labrador Retrievers	With their owners (all owners were females)	Immunoassay	3 minutes of interaction: stroking, petting and talking to the dogs, followed by 57 minutes of ignoring the dog	Increased OT levels 3 minutes after the start of the interaction ($p = 0.027$)	Handlin et al., 2011
5 males (4 castrated); 4 female (3 spayed)	3.17 ± 2.1 years old	1 Flat-coated Retriever, 1 German shepherd, 1 Golden Retriever, 1 Jack Russell Terrier, 1 Labrador Retriever, 1 Miniature Schnauzer, 1 Shiba 2 Standard Poodles	5 were house dogs and 4 were local laboratory dogs	Radioimmunoassay	Eating food, exercising (15 min) and stroking abdominal area (15 min) without social reinforcement (e.g. no vocal encouragement or eye contact) by an experimenter knowing the dog, but it is not the owner or the caregiver	Eating and exercising increased urinary OT ($p< 0.05$), as well as stroking ($p < 0.01$), while no urinary OT change was observed after drinking water ($p=0.31$)	Mitsui et al., 2011
All female (N=12)	20 ± 0.2 months	12 Beaggle	Laboratory kept dogs	Immunoassay	Before, during (4 min) and after the return of a very familiar person. The 4 minutes reunion included 3 groups: physical and verbal contact (PV), verbal contact (V) and ignoring the dog (C). Sampling after 1:30 min (stage 1), 3:45 min (stage 2) and after 120 s of relaxation (stage 3).	Increased OT levels in the PV group during reunion ($p= 0.02$) and relaxation ($p=0.03$). Also, increased OT levels in the reunion session for V ($p=0.06$) and C ($p=0.01$) groups, but no modifications in the relaxation phase for both V and C groups.	Rehn et al., 2014
Same as Handlin et al., 2011	Same as Handlin et al., 2011	Same as Handlin et al., 2011	Same as Handlin et al., 2011	Immunoassay	Same as Handlin et al., 2011. During the period of ignoring the dog, the owners completed	MDORS scores were correlating significantly with dog's blood OT levels. Correlations ($r=0.9$, $p = 0.01$) between owner's OT levels	Handlin et al., 2012

					MDORS scale for dog-owner relationship	at 5 minutes vs. dog's OT levels at 60 min. Increased frequency of owners kissing the dogs was associated with dogs' OT levels (r=0.7, p = 0.02).	
10 males (5 castrated); 18 female (14 spayed)	5.9 years old (between 2-11) years old	14 breeds, where Lagotto Romagnolo (n = 7) and Staffordshire Bullterriers (n = 6) were the most frequent	With owners	Enzyme linked immunoassay kit	Studying the effects of a LIGHT (2-3 mmHg) and DEEP (10-12 mmHg) vests on noise (70- 73 db. recorded fireworks noise) phobia and on urine OT levels.	Pressure vests did not affect urine OT levels. Time spent near the owner when wearing DEEP vest positively correlated to urine OT levels.	Pekkin et al., 2016
8 males; 8 female	6.1 ± 0.7 years old	9 Standard Poodles, 4 Labrador retrievers, 1 German shepherd, 1 Shetland sheep dog 1 Border Collie.	Companion dogs living with their owners	Radioimmunoassay	Dogs sitting on a blanket near the owner, which stands quietly on a chair. After that, an additional partner dog entered the room with a second blanket being placed near the chair (Note – these were separate results from just one male and 4 female from the initial group).	OT increase ratio from urine was not correlated with the time subjects spent in the proximity of the owner or of the other dog. Also, no significant associations were reported between OT and the affiliation processes that subjects exhibited towards each other (e.g. given and received).	Romero et al., 2014
15 males (13 castrated); 15 female (11 spayed) + 11 wolves	4.7 ± 2.7 years old	5 Golden Retrievers, 3 Labrador Retrievers, 3 Miniature Schnauzers, 3 Standard Poodles, 2 Miniature Dachshunds, 2 Shiba-inu, 2 Toy Poodles, 1 Border Collie, 1 Boxer, 1 FlatCoated Retriever, 1 German Shepherd, 1 Jack Russell	Companion dogs living with their owners + wolves – with their 'owners' being animal management professionals	radioimmunoassay	30 minutes interaction with the owner sitting and the dog being allowed to move freely in the room (e.g. free interaction with the dog, except giving food or toys). Recording were made for "dog/wolf to owner gaze", "owner talking to the dog/wolf" and "owner touching the dog/wolf"	OT change ratio from the dog's urine correlated with OT form the owner. The duration of dog to owner gaze was associated with OT change ratio in both dogs and owner. Only owners which received long time gaze from their dogs exhibited a significant increase in OT concentration and also the highest OT change ratio. In wolves (which overall showed reduced gazing) the duration of gaze was not correlated with OT change ratio of both owners and wolves.	Nagasawa et al., 2015

		Terrier, 1 Miniature Bull Terrier, 1 Papillon, 1 Shetland, 1 Sheepdog, 2 mongrels + 11 wolves					

Table 1. Summary of published studies measuring endogenous oxytocin levels in dogs in relation to behaviour.

3. Associations between the polymorphisms in oxytocin receptor gene and social behaviour

Another line of research has focused on genetic polymorphisms related to the key component of the oxytocin system, the oxytocin receptor gene (*OXTR*) and its association with dog behaviour. It had already been shown that the similarity between the human and the dog *OXTR* gene is high (Marx et al., 2011), although a 5-aminoacid-long sequence is missing from the dog *OXTR* that is present in humans and the *in silico* addition of this sequence to the dog *OXTR* results in a considerable protein structure change. The dog *OXTR* protein is 384 amino acid long (compared to the 389 amino acid long human protein) and compared to the human protein contains different amino acids at 26 locations (out of these 8 amino acids have similar chemical properties such as polarity, acidity). Single nucleotide polymorphisms (SNPs) were identified by direct sequencing of the protein coding segment and the flanking regulatory un-translated regions in both different dog breeds and wolves on N=3 individuals per group (Bence et al., 2013). Five novel (-213AG, -94TC, -74CG, -50CG, 19208AG) and three known (rs22927829, rs8679682, rs8679684) SNPs were found and genotyped in larger populations involving German Shepherds (N=71), Border Collies (N=104), Labrador- and Golden Retrievers (N=64), Beagles (N=29) and wolves (N=44). The results confirmed that the identified SNPs are polymorphic not only in the dog breeds, but also in wolves, however allele frequencies differed among the investigated breeds as well as between dogs and wolves. These results have recently been confirmed and extended (Bence et al., 2017) by investigating a large population involving nearly 700 dogs from 10 different breeds (Beagle, N=70; Border Collie, N=144; German Shepherd, N=128; Golden Retriever, N=43; Groenendael, N=22; Hungarian Vizsla, N=32; Labrador Retriever, N=49; Malinois, N=40; Siberian Husky, N=138; Tervueren, N=23). Difference in allele frequencies among dog breeds was significant in the case of all eight SNPs investigated. Furthermore, in the case of the −50C/G and

rs8679682 SNPs there was greater difference in allele frequencies between dogs and wolves (N=42; 34 Eurasian gray, 6 North American timber, 2 Alaskan) than among dog breeds. These two SNPs were thus further investigated in 6 golden jackals *(Canis aureus)*, 8 dingos *(Canis lupus dingo)* and 45 Asian street dogs (pariah dog). Regarding SNP −50C/G, the allele C was found to be more frequent in all populations investigated (including golden jackals) with the only exception of wolves. SNP rs8679682 was monomorphic for allele C both in wolves and golden jackals, while in dogs (including the free-ranging Australian dingo and the Asian street dog pariah) allele T was dominating. These results prompted the authors to conclude that *OXTR* could indeed have been a target gene during domestication and selection for human preferred aspects of temperament and social behaviour.

Kis et al. (Kis et al., 2014a) were the first to use the candidate gene approach in dog oxytocin research. Behaviour in every-day social situations was assessed in association with SNPs in the regulatory regions (5′ and 3′ UTR) of the *OXTR* (polymorphisms: rs8679684, −213AG, 19208AG – the latter two formerly named −212AG and 19131 respectively) in German Shepherds from Hungary (N=104) and in Border Collies from Hungary and Belgium (N=103). Results show that these SNPs are related in both breeds to proximity seeking, and to friendliness towards strangers, although in the latter case an opposite trend was found for the two breeds. This strongly suggests that genetic and cellular mechanisms other than the *OXTR* might play a role in the regulation of the friendliness behaviour. Recent accounts in the human literature have cautioned about the individual (e.g. motivation or anxiety of the subjects) and conditional (e.g. contextual) differences in the effects of oxytocin on social behaviour (Bartz et al., 2011), and these canine results somewhat parallel these ideas in that the influence of an *OXTR* SNP was found to be conditional to a breed effect. A further study (Kubinyi et al., 2013) also found that two of these polymorphisms (19208AG, rs8679682) were related to greeting behaviour of racing Siberian Huskies (N=96). Both allele frequencies

and the direction of the observed gene × behaviour associations were strikingly similar to those found in Border Collies: Siberian Huskies with the GG genotype approached an unfamiliar person in a non-aggressive way more frequently in the greeting test and Border Collies with GG genotypes achieved higher scores on a friendliness scale. Note, however, that in case of the Border Collie study (Kis et al., 2014a) the friendliness scale was composed of the dogs' behaviour in reaction to a threatening stranger and to a passive stranger when facing a problem situation, while the greeting test was part of the proximity seeking scale that was not associated with 19131AG, but with -213AG (not tested in the Siberian Husky study due to Hardy-Weinberg disequilibrium). The fact that the effect of *OXTR* polymorphisms might differ across breeds is further supported by the finding (Ottenheimer et al., 2016) that no effect of the rs8679684 and 19208AG SNPs on owner-assessed personality (Monash Canine Personality Questionnaire) was found on a sample of N=97 dogs from several breeds and mix-breeds. The general association between the *OXTR* and human-directed social behaviours was, however, conceptually replicated on a sample of Border Collies from Austria (N=170) using similar everyday social situations (Turcsán et al., 2014) (unpublished conference paper). Confirming previous results associations with dogs' proximity seeking and friendliness were found. Furthermore a previously not investigated polymorphism in the second exon (rs8679682) was associated with dogs' separation behaviour, their tendency to look at humans in problem situations, their obedience to simple commands and their reaction to a threatening stranger. It was also found that the response of Border Collies to ambiguous social stimuli is related to polymorphisms in the *OXTR* (Park et al., 2014) (unpublished conference paper). Eighteen behavioural variables were recorded in 7 tests on N=86−98 dogs (varying across tests) and the following behavioural variables were associated with *OXTR* SNPs (rs8679682, -94TC and -74CG): frequency of looking at a helper after seeing a motionless person on all four, latency of breaking eye-contact with a neutral person, number of correct choices

following a non-communicative gaze cue, and latency of approaching the non-indicated food location. Importantly a difference in gene × behaviour association patterns was found between the Border Collie populations of the two countries (Austria and Hungary) involved in the study. Such conceptual replications are crucial in behavioural genetic studies, as despite the large sample sizes customary in the field low effect sizes characterise these gene × behaviour studies, and most effects would not remain statistically significant after correction for multiple comparison. Although we do not, at this point, have any information about the intermediary (cellular and molecular) mechanisms directly involved in the regulation of the behaviours by *OXTR* polymorphisms, the indirect gene × behaviour connection seems to be robust. However, while these data show an important mechanism regulating social behaviour in dogs, the effect of a single SNP is very small, thus the current state-of-the-art is not sufficient to offer any practical advice for dog breeders or for the selection of puppies suitable for a given owner or for specific work purposes.

More recent studies have built on the OXTR × behaviour connection to move towards the applied direction for example by investigating *OXTR* as a candidate gene in Golden Retrievers with separation anxiety (van Rooy et al., 2016). The potential relationship between the oxytocin system and canine separation anxiety was originally proposed in a review article by Thielke and Udell (Thielke and Udell, 2016), although the authors of this publication made their suggestion in the context of treating separation anxiety with intranasal oxytocin administration. Van Rooy and colleagues (van Rooy et al., 2016) have investigated in a case−control design (N=42 dogs: 24 affected by separation anxiety based on an owner-report questionnaire and 18 non-affected controls) if separation anxiety is associated with 45 SNPs within 500 kilobases of *OXTR* (Illumina HD 170,000 SNP array), but they found no such evidence. One *OXTR* haplotype (CCA, SNP 9503004) had a raw p-value of association less than 0.05 with a 0.72 versus 0.50 frequencies for case and control respectively, but this p-

283 value did not remain significant after permutation. There is, however, unpublished evidence
284 that an *OXTR* SNP (-213AG) is related to Border Collie dogs' (N=135) attachment towards
285 their owners as measured in a Strange Situation Test (Kovács et al., 2017) (submitted
286 manuscript). Furthermore preliminary evidence was found on a subsample of these dogs
287 (N=65) that polymorphisms in the owners' OXTR (also in interaction with the dogs' OXTR
288 polymorphisms) are related to dog−owner attachment. Another study (Oliva et al., 2016b) has
289 looked at microsatellites at various distances from the *OXTR* in buccal samples of N=75 pet-
290 and blood samples of N=94 shelter dogs of various breeds and mixed breeds as well as in
291 buccal samples of N=12 human-reared wolves (mix of the subspecies: Arctos, Occidetalis
292 and/or Nubilus). Results showed that out of the eight primers investigated the two closest to
293 *OXTR* (located at 9.36 million base pairs within the genome), located at 9.11 million and 9.66
294 million base pairs respectively were significantly associated with species (dog versus wolf).
295 The authors have also looked at the performance of their pet dog subjects in an object choice
296 task with 20 trials of momentary distal pointing after both oxytocin and placebo pre-
297 treatment. There was no significant association between performance (good performers that
298 scored ≥ 18/20 versus poor performers that scored ≤ 12/20) and any of the primers. There was
299 also no significant association between oxytocin response (high oxytocin responders that
300 improved their performance by 3-7 points between sessions versus poor responders whose
301 performance remained the same or declined between sessions) and any of the primers.
302 Taken together these studies present evidence that there is an indirect link between *OXTR*
303 polymorphisms and social behaviour in dogs that, despite being weak, is present in several
304 breeds and across several situations. However, these effect are not uniform across breeds (e.g.
305 the same SNP might have different effects in different breeds), and thus might not manifest in
306 mixed-breed populations and/or in some specific domains (e.g. pointing following). While
307 gene × behaviour association studies offer a potentially powerful approach for mapping causal

genes with modest effects, genome-wide association studies (GWAS) might be a more suitable way to study the genetic background of complex social behaviours (Hirschhorn and Daly, 2005). In dogs no GWAS has been conducted so far, with behaviour genetic research focusing on candidate genes such as the OXTR or DRD4 and TH (Hejjas et al., 2007; Hori, 2013; Wan et al., 2013). Future research thus needs to focus on genome-wide association studies in order to reveal the potentially complex genetic background of human-directed social behaviour in dogs.

Apart from polymorphisms in *OXTR*, environment-induced epigenetic changes might also modulate the oxytocin system and thus influence dogs' behaviour. Recently two methylation sites have been identified in the canine genom by analysing buccal DNA samples from male Golden Retriever, Border Collie and Siberian Husky dogs as well as North American timber wolves (N=8 individuals from all four populations): OXTR_17, a 246 bp long amplicon with 6 CpGs located at 20:9358073–9358318; and OXTR_34, a 148 bp long amplicon with 4 CpGs located at 20:9357391–9357538 (Banlaki et al., 2017). Methylation ratio has been found to differ among the dog breeds investigated as well as between dogs and wolves. Another study from the same group analysed buccal samples of nine dogs (from six different breeds and three mixed breeds) and three timber wolves (Cimarelli et al., 2017) and identified four CpG sites in the OXTR that showed at least 10% variation in their methylation levels among the subjects (located -727, -751, -1371, and -1383 bp relative to transcription start site; transcript variant NM_001198659.1). These were further analysed on a sample of 217 Boder collies (135 females and 95 males) looking for possible association with the dogs' reaction to a threatening stranger (hiding behind the owner, approaching the experimenter in an appeasing vs. aggressive way, remaining passive or retreating at the end of the test). Results indicate that the sex of the dogs was associated with the methylation level in two sites, furthermore different behavioural associations were found in males and females. While most

of the methylation level differences between differently behaving dogs were relatively weak (no longer significant after correcting for multiple testing) these pioneering studies offer an interesting new line of investigation on the epigenetic regulation of behaviour. Future studies should disentangle between inherited or environmentally influenced epigenetic patterns, as well as to investigate the interactions between the methylation levels and the polymorphisms of *OXTR* (e.g. one of the known canine *OXTR* SNPs, –213AG, is located in a CpG island and thus may alter the methylation pattern of the promoter region).

Investigation of tissues other than buccal samples (e.g. different regions of the brain) and the correspondence of the two are needed for both epigenetic and gene expression analyses. While the former is still lacking, the latter was performed (Bence et al., 2017) on post mortem brain samples from three male beagle dogs. Quantitative real-time PCR was used to assess expressional differences of OXTR mRNA between three regions of the dog brain: prefrontal cortex, amygdala and hippocampus. Expression levels were lowest in the prefrontal cortex and highest in the hippocampus. As compared to that observed in the prefrontal cortex, mRNA levels in the amygdala and the hippocampus showed a 2.8- and 16.4-fold increase, respectively.

Sex	Age	Breeds	Dog's social background	Method	Behavioral protocol	Results	Reference
German Shepherd male/female: 58/46 Border Collie male/female: 46/57	German Shepherd mean age±SD: 3.88±2.55 years Border Collie mean age ±SD: 4.28±2.74. All > 1 year.	German Shepherds (N=104) Border Collies (N=103)	Pet dogs. None of the subjects were closely related, i.e. littermate and parent-offspring relationships were excluded.	SNP: rs8679684, −213AG, 19131AG	Greeting by an unfamiliar experimenter while the owner stands motionless next to the dog and holds the leash Separation from the owner (the dog is tethered to a tree on a leash, with the experimenter approaching and greeting it) Problem solving: food in a cage. Threatening stranger Hiding: experimenter releases the leashed dog to go after hiding owner	Carrying the G allele for −213AG was associated with lower proximity seeking in both breeds (German Shepherds: p=0.021, Border Collies: p=0.025). For the rs8679684 polymorphism German Shepherds carrying the A allele, as opposed to the T allele, achieved higher scores on the Friendliness scale (p=0.012), while in Border Collies individuals carrying the A allele were less friendly (p=0.033). The same result holds true for the 19208AG polymorphism (due to linkage disequilibrium), German Shepherds: p=0.008, Border Collies: p=0.013.	Kis et al 2014a
NA 88.66% of dogs were gonadectomized	5.97 (±4.02) years. All > 1 year.	47 purebred and 50 mixed Retriever mix (26), Labrador retriever (10), and golden retriever (1); Working mix (5), miniature schnauzer (4), poodle (3), and German shepherd dog (1); Scent mix (9) and beagle (2); Shetland sheepdog (3), border collie (2), old English sheepdog (1), Australian shepherd (1), and herding mix (1); Boxer (2), Great Dane (2), mastiff-like mix (2), Bernese mountain dog (1), and pit bull (1); Shih tzu (2), papillon (2), and American Eskimo (1); American cocker spaniel (1), wirehaired German pointer (1), Welsh springer spaniel (1), and spaniel mix (1); Spitz mix (2), Eurasier (1), and Siberian husky (1); Silky terrier (1) and terrier mix (1); Greyhound (1)	Pet dogs. Owners had lived with their dog for a minimum of 6 months.	SNP: rs8679684, 19131AG	Monash Canine Personality Questionnaire measuring extraversion, motivation, training focus, amicability, and neuroticism	No associations between OXTR polymorphisms and questionnaire scales.	Ottenheimer et al., 2016
No male/fe	Mean age: 6.2 years	Golden Retrievers	Separation anxiety	45 SNPs	Separation Related Behaviour Score extracted	No difference between cases (separation anxiety) and controls regarding OXTR genotype.	van Rooy et al.,

male ratio provided.	(mean age cases: 4.8 years, mean age controls: 6.1 years)		(N=24), non-affected controls (N=18)	within 500 kilobases of *OXTR*	from the Australian Canine Behavior Survey (based largely on the validated Canine Behavioral Assessment and Research Questionnaire, C-BARQ)		2016
33 males, 42 females	All > 1 year.	No restriction was put on breed	Pet dogs	Microsatellites at various distances from the *OXTR* gene	Object choice task with 20 trials of momentary distal pointing after both oxytocin (24 IU) and placebo pre-treatment	No association between performance and any of the primers.	Oliva et al., 2016b
135 females (45 neutered) and 95 males (32 neutered)	mean age ± SD = 48.07 ± 42.43 months	Border Collies	All kept as pets in Vienna (Austria) and surroundings	DNA methylation analysis, OXTR promoter region (pyrosequencing)	Threatening Approach test: the owner stood motionless behind the dog and held the leash while the experimenter walked toward the dog slowly with the upper body bent toward the dog and staring in the eyes of the dog.	Weak gender-specific associations with methylation level. Female dogs had higher methylation levels than males in position −1371, males had higher methylation levels than females in position −727. Males who hid behind the owner had higher methylation levels in site −751 than those who did not hide behind the owner (significant after correcting for multiple testing). 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 (no longer significant when correcting for multiple testing). Females who approached the experimenter in an appeasing way tended to have higher levels of methylation in site −1383 than those who did not show any sign of appeasement (no longer significant after correcting for multiple testing). (Contrary to this) males who approached the experimenter in an appeasing manner which tended to have lower methylation levels in site −1383 than those who did not (no longer significant after correcting for multiple testing). 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	Cimarelli et al 2017

						(significant after correcting for multiple testing)	

Table 2 . Summary of published studies on dog *OXTR* in relation to behaviour.

4. The effect of exogenous oxytocin administration on dogs' social behaviour

The third approach, experimental manipulation of oxytocin levels, has also been used in dogs. Intravenous administration was validated (Mitsui et al., 2011) by measuring oxytocin (OXT) levels in both blood and urine samples following the injection of 4 times 0.25 ml OXT during the course of 15 minutes. Plasma OXT concentration reached a maximum immediately after the fourth injection (15 minutes after baseline) and then rapidly returned to baseline levels. The peak urinary OXT concentration occurred 1 hour after baseline (45 minutes after the fourth injection) and returned to baseline levels slowly (by the end of the 2^{nd} hour after baseline). A more widely used alternative to study the effect of oxytocin on behaviour is intranasal (instead of intravenous) oxytocin administration (IN-OXT). It was found (Romero et al., 2014) that 15 minutes after administering 40 IU oxytocin intranasally blood OXT levels were elevated compared to both baseline and placebo condition in N=5 dogs. (OXT concentrations were also higher 90 minutes after OXT administration compared to placebo condition, but the difference did not reach statistical significance − p=.080.) Urinary oxytocin concentration of the same subjects was also higher 90 minutes after OXT administration (compared to both baseline and placebo condition) and the increase in urinary OXT levels correlated with the accumulate value of plasma OXT. Similar (unpublished) results were obtained (Temesi et al., 2017) (submitted manuscript) on N=4 laboratory-bred beagle dogs receiving 12 IU IN-OXT; serum oxytocin reached its maximum 15 minutes after the treatment, and the peak urinary oxytocin concentration occurred between 45 and 60 minutes after OXT administration. No such changes were observed in the N=2 placebo-treated beagle dogs. In the Romero et al. (Romero et al., 2014) study heart-rate variability was also measured during the 5-minute intervals before and after the IN-OXT administration and it was found that high frequency (HF; computed by spectral analysis for NN intervals in the range of 0.15 − 1.00 Hz) decreased right after spray administration independent of treatment

received, and during the subsequent 5 minutes it did not significantly vary in the placebo group, while it showed a significant increase in the oxytocin group. Another study (Kis et al., 2014b) found similar results on N=10 pet dogs by showing in a within-subject design that 40 minutes after intranasal administration of 12 IU oxytocin heart rate (HR) decreased and heart rate variability (HRV; standard deviation of RR intervals) increased compared to the placebo group (as measured during a 1-minute-long interval). The same measurement was repeated (Kovács et al., 2016a) on N=39 dogs and apart from confirming that 12 IU oxytocin decreased HR and increased HRV 40 minutes after IN-OXT treatment, it was also found that changes in HR and HRV were related to behaviour in the subsequent experiment (biological motion perception task, see later).

The first study to assess the effect of IN-OXT treatment on dog behaviour (Romero et al., 2014) measured affiliative behaviour during a 60-minute period immediately following the administration of 40 IU oxytocin. The owners of the dogs were instructed to sit quietly in an experimental room and not to actively interact with their dogs, thus any behaviour directed from the dogs to their owners was either ignored or only briefly reciprocated (e.g., dogs received a gentle brief touch or push back when they tried to lick their owner's face). Dogs administered with OXT initiated affiliation (defined as sniffing, licking, gentle touching with the nose or paw, play bouts, and body contact excluding tails) toward their owners more often compared to placebo treatment. Furthermore following OXT administration dogs showed an increased social orientation to their owners (defined as staring, looking at owner or no clear gaze direction but head frontally oriented to owner). Effects of IN-OXT treatment on social behaviour towards conspecifics were also documented in this study as well as in a follow-up analysis (Romero et al., 2015). Other studies have focused on the applied aspects of IN-OXT research. It was found (Kis et al., 2015) that dogs (N=64) that received 12 IU IN-OXT showed an increased positive expectation bias in the cognitive bias paradigm 40 minutes post

treatment compared to placebo groups, and this effect was more pronounced in a communicative compared to a non-communicative context. Moreover in a threatening approach test (N=36) IN-OXT pretreated dogs (12 IU) looked back more at the experimenter standing behind them (40 minutes post treatment), that can be interpreted as social referencing in a mildly stressful situation (Hernádi et al., 2015). However, in the same experiment dogs after having received IN-OXT showed a less friendly first reaction compared to the PL group when the owner was approaching, although IN-OXT and PL pre-treated dogs showed the same reaction to an unfamiliar experimenter approaching. The fact that oxytocin only influenced dogs' first reaction to the owner, but not to the experimenter might suggest that the effect of oxytocin is specific and/or more pronounced towards socially more relevant partners. Individual differences in aggression (measured via questionnaire) also modulated dogs' first reaction in interaction with pre-treatment (OXT vs. PL) and the identity of the approaching human (owner vs. experimenter). This is in line with human studies that indicate a modulating role of baseline aggression on the effect of oxytocin (Alcorn et al., 2014).

The canine analogues of human communicative skills were also investigated. It was found (Oliva et al., 2015) that the administration of 24 IU IN-OXT enhanced the appropriate use of human (momentary distal) pointing and gazing 45 minutes post treatment in dogs (N=62) in a two-way choice task where subjects had to locate hidden food based on human social cues. A further interesting finding of this study is that dogs' enhanced pointing-following performance was maintained for the second test session 5-15 days after IN-OXT administration, that is dogs that received the placebo treatment for the second session (after having received IN-OXT for the first session) performed better compared to dogs that received placebo treatment during the first session; no order effect (first vs. second session) was found for the oxytocin treated dogs. A follow-up analysis (Oliva et al., 2016a) has further shown that while dogs' ability to follow both human pointing and gazing is predicted by

owner reported questionnaire measures (anxious attachment in owners, and contagion of human emotions respectively), no such correlation can be found for their performance following IN-OXT treatment. The finding that dogs' performance in following human momentary distal pointing is enhanced by IN-OXT treatment was conceptually replicated with a slightly different methodology (Macchitella et al., 2016). Subjects (N=14, including puppies as young as 4-month-old) received 2 IU/kg IN-OXT (with their weight ranging from 5 kg to 40 kg) or placebo in a within subject design (with 1–22 days between sessions) and participated in a pointing following task at their homes or at a veterinary centre 15 minutes post treatment. While their results confirmed that dogs chose the baited cup significantly more often in the oxytocin compared to the placebo condition (with dogs in the placebo condition also performing above chance), no effect of subjects' age, test location or order of treatment was found (although the relatively low sample size might not allow for such comparisons). Also, gazing behaviour in female (N=15; 2 gonadally intact, 13 spayed) but not male (N=15; 2 gonadally intact, 13 castrated) dogs was found to increase during a 60-minute period immediately after IN-OXT (40 IU) treatment (Nagasawa et al., 2015). In this experimental situation the owner and two unfamiliar people were seated in a room and human behaviour toward dogs was restricted (they were forbidden to talk to each other or to touch the dog voluntarily). These results, together with the finding that no significant oxytocin change ratio was found in dogs when interaction with humans was limited, is interpreted as further evidence for an oxytocin-gaze positive feedback loop (see above) although no explanation exists for the sex differences found in this but not their previous study.

The effect of IN-OXT (12 IU) on basic mechanisms of social cognition was also investigated using the biological motion paradigm (Kovács et al., 2016a). Dogs (N=39; 40 minutes post treatment) were presented with a moving human point-light figure and its inverted and scrambled version. Results showed that while placebo-pretreated dogs showed a

453 spontaneous preference for the biological motion pattern as expected, there was no such
454 preference after IN-OXT. Using an eye-tracking paradigm unpublished evidence was found in
455 a within-subject design that 40 IU IN-OXT increased the number of fixations laboratory-kept
456 beagle dogs (N=42) made at the eyes of smiling human faces, and it diminished dogs'
457 tendency to revisit the eyes of angry faces more often 45 minutes post treatment (Somppi et
458 al., 2016). A parallel unpublished eye-tracking study (Hernádi et al., 2017) also found that IN-
459 OXT (8 IU for dogs under 18 kg of weight and 12 IU for dogs over 18 kg) has an effect on
460 (N=38) pet dogs' viewing patterns of (male) human emotional faces 35-45 minutes post
461 treatment. However, the results of this study are slightly different as it was found that
462 oxytocin decreases dogs' preferential looking to the eye region of human faces regardless of
463 the displayed emotional expression.
464 While the above studies have predominantly used a mixed sample of several dog breeds
465 and crosses or alternatively focused on one single breed, a recent study (Kovács et al., 2016b)
466 provides evidence that IN-OXT treatment has differential effects on different dog breeds.
467 Border Collies (N=19) and Siberian Huskies (N=19) were tested in situations measuring
468 social responsiveness, and apart from pretreatment (OXT/PL) and breed effects interactions
469 among these two factors were also found in case of several behavioural variables. For
470 example Border Collies, but not Siberian Huskies, looked more at the experimenter after
471 oxytocin administration in the 'Unreachable food' situation; and oxytocin-pretreated Border
472 Collies looked longer at the experimenter's eyes compared to oxytocin-pretreated Siberian
473 Huskies in the 'Tolerance of prolonged eye contact' test, while there was no difference
474 between the two breeds after placebo treatment. These results are not surprising as due to the
475 differential selection of the two breeds (cooperative versus independent workers) they are
476 genetically distinct, and as they are kept for different purposes and thus have different
477 experiences epigenetic changes in the oxytocin system might occur during ontogeny.

Although the sample size is limited, this study also provides the first preliminary evidence that a polymorphism in the *OXTR* gene (−213AG) interacts with the effect of IN-OXT treatment on behaviour in both Border Collies and Siberian Huskies.

Sex	Age	Breeds	Dog's social background	Method	Behavioral protocol	Results	Reference
All males (N=6)	5.29 ± 0.95 years old	Labrador Retreivers	5 dogs from a training center and 1 housedog	Intravenously 4 times every 5 minutes (0.25 ml * 4 = 500 pg/ml in total)	Dogs were kept quiet in their cages after administration	Highest concentration of urinary OT (radioimmunoassay) appeared 60 min after administration and returned to baseline slowly (around 3 hours). Plasma OT concentration reached the maximum immediately after the 4th injection (e.g. after 15 min) and then rapidly (around 1 h) decreased to baseline concentration.	Mitsui et al., 2011
8 males; 8 female	6.1 ± 0.7 years old	9 Standard Poodles, 4 Labrador retrievers, 1 German shepherd, 1 Shetland sheep dog 1 Border Collie.	Pet dogs living with their owners	Intranasal 40 IU in 100 μL solution	Dogs sitting on a blanket near the owner, which stands quietly on a chair. After that, an additional partner dog entered the room with a second blanket being placed near the chair (Note – for blood and urine samples, 5 separate dogs were used).	OT increased social positive behaviours to human and canine partners during 1 h (e.g. increased social orientation and affiliation to owner or approach/affiliation behaviour towards dog partner: as for example in time spent in proximity-1m or reciprocated behaviour in less then 5 sec: sniffing, licking, gentle touching). +Increased blood OT levels (radioimmunoassay) 15 min after administration. +Increased urinary OT concentration 90 min after administration.	Romero et al., 2014
20 males; 19 female	4.46 ± 2.51 years old (all older then 1 year)	18 purebred, from 14 different breeds; 21 mongrels 8 small size (≤ 9 kg), 23 medium size (10-25 kg), 8 large size (> 25 kg)	Pet dogs living with their owners	Intranasal 12 IU (3 puffs)	Specific biological motion perception task. +Owners completed the Neuroticism and Agreeableness scale adapted for dogs	OT decreased heart rate (HR) ($p < 0.05$) and increased hears rate variability (HRV) as measured 40 minutes after intranasal puffs. OT administration affected biological motion perception task, which correlated with both HR/HRV and aspects of dog personality (as in Neuroticism and Agreeableness scale).	Kovacs et al., 2016a
8 males; 8 female	6.1 ± 0.7 years old	9 Standard Poodles, 4 Labrador retrievers, 1 German shepherd, 1 Shetland sheep dog 1 Border Collie.	Pet dogs living with their owners	Intranasal 40 IU in 100 μL solution	Dogs sitting on a blanket near the owner, which stands quietly on a chair. After that, an additional partner dog entered the room with a second blanket being placed near the chair.	OT resulted in dogs initiating play sessions more often and for longer time, as well as increased dog's play motivation.	Romero et al., 2015
28 males; 36 female	4.44 ± 2.67 years old (all more then 1 year)	42 pure breeds from 20 different breeds; 22 mongrels 21 small size (≤ 9 kg), 33 medium size (10-25	Pet dogs living with owners and capable of being motivated with dry food by their	Single intranasal dose of 12 IU	Cognitive bias paradigm (ambivalent situations): learn to discriminate between a bowl with food and an empty one. After OT administration, dogs	OT increased positive expectation bias, 40 min after treatment, with this effects being more pronounced in the communicative context. Note: In a separate test on 10 dogs (3♂and 7♀, 4.33 ± 2.69 mean	Kis et al., 2015

		kg), 10 large size (> 25 kg)	owners		were presented with a bowl located halfway between the aforementioned 2 locations, in a communicative (eye contact, addressing, gently touching) or non-communicative way (placed food behind a curtain, with no communication cues).	age) the 12 IU intranasal administration decreased HR (p=0.002) and increased HRV (p = 0.002).	
20 males (12 intact and 8 neutered) ; 16 female (6 intact and 10 spayed)	4.7 ± 2.6 years old (all of them older then 1 year)	20 purebreds from 14 different breeds: Belgian Shepherd, Black Russian Terrier, Border Collie, Boxer, Bulldog, Central Asian Shepherd Dog, Golden Retriever, Norwitch Terrier, Nova Scotia Duck Tolling Retriever, Schnauzer, Shipperke, Scottish Terrier, Siberian Husky, Stafforsihre Terrier and 16 mongrels	Pet dogs living with their owners	Intranasal 12 IU (3 puffs)	Threatening Approach Test performed either by a female experimenter or the owner. Test consists of unfamiliar female experimenter approaching while owner is sitting silently 0.5 m near the dog. Secondly, experimenter and human are switching roles/places.	OT resulted in a less friendly first reaction to the owner approaching, as compared to placebo, 40 minutes after treatment. OT also resulted in dogs looking back more frequently at the human (owner or experimenter, depending on the phase of the study) standing besides them.	Hernadi et al., 2015
31 males; 31 female	NA (all dogs over 12 months)	NA	Pet dogs living with their owners	Intranasal 24 IU (50 μg) diluted in 5 ml of saline, with half dose in each nostril	Object choice task (OCT): treat hidden in opaque bowl vs. empty opaque bowl, in 2 different testing sessions, 5-15 days apart. Hungry dogs that day had to use experimenter cues to find treat: momentary distal point cue (raise arm and point towards the correct bawl) or gaze cues (gazed towards the correct bawl).	OT increased the dog's scoring by using momentary distal point cues, 45 min after administration. This effect was maintained over 5-15 days to the second session, even in the absence of OT. No treatment effect was found for the gazing cues. There was a gender effect on the behavioural efficacy of OT, which exerted significant effects in female, but no influence on males.	Oliva et al., 2015
33 males; 42 female	NA (all dogs over 12 months)	NA	Pet dogs living with their owners	Intranasal 24 IU	Object choice task (OCT) (please see above study). In addition, owners completed Pet Attachment Questionnaire(for anxious and avoidant attachment),	OT increased dog's performance in OCT. However, the administration of OT affected the initial correlation between OCT performance and owners questionnaire regarding the attachments scores.	Oliva et al., 2016

					Perceptions of Dog Intelligence and Cognitive Skills survey and Monash Dog Owner Relationship Scale.		
11 males; 3 female	46 ± 12.9 months (ranging from 4 to 156 months)	11 breed: 3 German shepherd, 3 Miniature schnauzer, 1 Labrador retriever, 1 English setter, 1 Jack Russell terrier, 1 Miniature pinscher, 1 West-Highland terrier and 3 mix-breed	with owners (but 8 dogs were tested in the lab, while the 6 were tested at home)	Intranasal 2 IU/kg	Variation of OCT with treat hidden in one of the similar cups. 2 different sessions (one OT, one placebo), with an average of 10.1 ± 1.4 days between them (range from 1 to 22 days). Momentary distal point (2 s, 50 cm from the cup)	OT increased dog's performance in OCT, 15 minutes post-treatment.	Macchitella et al., 2016
15 males (13 castrated) ; 15 female (13 castrated)	5.65 ± 0.8 years old	7 Standard Poodles, 6 Golden Retrievers, 6 Labrador Retrievers, 2 Beagles, 2 Border Collies, 2 Schnauzers, 1 Bernese Mountain, 1 Boxer, 1 German Shepherd, 1 Shetland Sheepdog, 1 Siberian Husky	Pet dogs living with their owners	Intransal (40 IU/100 µl)	Owner and 2 others unfamiliar persons seated in a chair and avoiding to talk or touch the dog voluntarily, in a 30 min interaction test	OT increased the gazing time just in female dogs. Urinary OT was also increased in the owners of the female dogs which received OT. Limited interaction with human resulted in no modification of urinary concentration of OT, as well as no significant OT change ratio in these dogs (radioimmunoassay).	Nagasawa et al., 2015
Border Collies: 9 males, 10 females Siberian Huskies: 8 males, 11 females	Border Collies: 3.5 ± 2 years old Siberian Huskies: 4.8 ± 1.9 years old	19 Border Collies 19 Siberian Huskies	Pet dogs living with their owners	Single intranasal dose of 12 IU (3 puffs)	3 behavioral tasks used for the social responsiveness: 1.Unreachable food task- how dogs are changing their communication towards a potential human helper when could not reach food. 2. Potentially dangerous object task: explore dog's behaviour to look towards human when faced a potentially dangerous object or situation. 3. Tolerance of prolonged eye contact with an unfamiliar human.	OT administration resulted in different behavioral manifestations in the 2 breeds, as the Border Collies were more susceptible to social-like manifestations, when compared to Siberian Huskies: looked more at the experimenter in the unreachable food task, gaze more at the owner in the potentially dangerous object test and had an increased tolerance to prolonged eye contact with the experimenter.	Kovacs et al., 2016b

Table 3. . Summary of published studies measuring behavioural changes in dogs after exogenous oxytocin administration

5. Conclusions and future directions

The above outlined studies all point to the direction that oxytocin plays a complex role in regulating human−dog relationships. This is evidenced by the impact of genetic polymorphisms in the oxytocin system (*OXTR* gene), and exogenous oxytocin on different aspect of social behaviour towards humans, as well as by the finding that behavioural treatment with human social stimuli influence peripheral oxytocin levels. However, several methodological discrepancies exist between the published studies (especially in case of the IN-OXT research) due to the fact, that different research groups started to simultaneously and independently work on this topic, that might make future work and comparison of results difficult. Thus a consensus should be reached in the following points: i) dose and delivery method of IN-OXT treatment, ii) duration of waiting period between IN-OXT treatment and test as well as standardization of activity during waiting period, iii) measurement protocol of urinary and blood oxytocin levels.

Studies have administered different doses of IN-OXT (typically ranging from 12 IU to 40 IU; but some subjects received 8 IU, while others up to 80 IU based on body weight) and waiting periods after the treatment also vary (from no waiting period to 45 minutes). As to date there are no (pre-registered) replications of IN-OXT studies the effect of these methodological discrepancies is not yet known. However, there are some general differences across the results reported in dog IN-OXT studies that are indicative of the existence of such effects. For example some studies report a strong effect of repeated testing (carry-over effect; e.g. Kovács et al., 2016a; Oliva et al., 2015), but others do not (e.g. Hernádi et al., 2015; Macchitella et al., 2016). Also, sex differences in the effect of oxytocin have been documented in some studies (e.g. Kovács et al., 2016a; Nagasawa et al., 2015), but not in others (e.g. Kis et al., 2015). While there is no methodological consensus in human IN-OXT research either, several further confounding factors have been identified that are potentially

relevant for dogs as well (Guastella et al., 2013). For example nasal anatomy has been found to influence absorption upon nasal spray administration, thus an easily administered technique to characterise anatomical differences in the nasal cavity might be necessary. In addition to active compounds (e.g. OXT) each nasal spray formulation includes different ingredients that can be described by chemical characteristics that all impact on absorption capacity (such as molecular weight, polarity, pH, chemical modification, lipophilicity, chemical form, polymorphism, pKa, solubility, dissolution rate, mucosal irritancy, osmolarity, and particle size), thus it might be useful to report all formula ingredients in scientific manuscripts. In addition head position, breathing as well as bottle design, insertion depth and administration angle also have an influence.

Standardization in the measurement of peripheral OXT is also necessary in order to compare results from different studies. A difference of four magnitudes has been reported in mean baseline blood oxytocin levels of dogs with 0.1 ng/L (Odendaal and Meintjes, 2003) versus 155.8 pmol/L (Handlin et al., 2011) respectively[1]. High individual variation (and the presence of a few outlier values) might of course cause substantial differences in mean values, but a difference of this magnitude is more likely to be caused by the populations under study being considerably different and/or the methodologies used (HPLC versus immunoassay) yielding vastly different results. Some (McCullough et al., 2013) have already cautioned that commercially available (immunoassay) methods frequently used to measure peripheral oxytocin have been proven to lack reliability when used on unextracted samples of human fluids as they tag molecules in addition to OXT. This leads to plasma oxytocin measures without extraction yielding 100-fold higher values than those found in extracted plasma with the correlation between the two values being practically none (Spearman's rho = −0.10, p = 0.54; Szeto et al., 2012). Some authors report validity measures for their technique used, e.g.

[1] Oxytocin is a 1k Dalton protein with a molar weight of 1007.18734 g/mol, thus *ng* and *pmol* are the same magnitude.

for one Oxytocin ELISA kit (Enzo Life Sciences, Lausen, Switzerland) inter- and intra-assay coefficients of variations were 9.9% and 12.5%, respectively (Pekkin et al., 2016); standardization and comparability of such measures will contribute to research transparency.

Despite the above methodological concerns the results of dog oxytocin research have proved that this area continues to be promising and it holds the potential to contribute to our understanding of human-analogue social skills in dogs. Also, many interesting research questions remain open. Future studies should for example clarify the generalizability of results by investigating e.g. breed effects. Some studies have already shown that different dog breeds differ in allele frequency for certain *OXTR* polymorphisms (Bence et al., 2016), and that these polymorphisms, as well as IN-OXT treatment can have differential effects in case of different breeds (Kis et al., 2014a; Kovács et al., 2016b).

Apart from breeds, further individual differences likely interact with the oxytocin system to influence behaviour, as it has been shown for aggressiveness (Hernádi et al., 2015) and sex of the subjects (Kovács et al., 2016a; Nagasawa et al., 2015), thus future studies should potentially examine such effects. The underlying mechanisms of individual differences in responsiveness to IN-OXT treatment (including differences in the oxytocin receptor gene, or in baseline oxytocin level, etc) should be explored in order to tackle the possibility to use this treatment for problem behaviours.

In general the three, now separate research lines (i.e. measuring endogenous oxytocin levels; studying *OXTR* polymorphisms; investigating the effects of exogenously administered oxytocin) should be combined in the future in order to gain a full picture of how the oxytocin system influences social behaviour. Exonic polymorphisms in the oxytocin receptor gene likely have an influence through alternative splicing, and SNPs in the regulatory (UTR) regions can also influence gene expression, as well as the amount of the protein expressed by altering miRNA binding. Thus *OXTR* polymorphisms likely modify the effect of exogenous

OXT treatment (as preliminary evidence also indicates in Kovács et al., 2016b). Peripheral OXT measurements could ideally quantify both baseline oxytocin levels, and oxytocin level changes in response to IN-OXT treatment. These should be complemented by investigating dose- and repeated (chronic) treatment effects. Using such an integrated approach could shed light on how individual differences in combination with the general effect of oxytocin interact to influence human-directed social behaviour in dogs.

The recent advances of the field, such as genome-wide association studies and direct investigation of epigenetic modifications of the oxytocin system (e.g. methylation of *OXTR*) should also be employed. The latter approach would be especially valuable in order to find out how early social experience influences behaviour (gene × environment interactions). While evidence has already been found that dogs behaving differently have different methylation levels (Cimarelli et al., 2017), it is yet to be examined how these different methylation levels emerge as a result of environmental influence, as for example no effect of owner interaction style on methylation levels was found.

Another way of combining the three main methods of oxytocin research in the future is to integrate the examination of *OXTR* polymorphisms into studying the effect of social interactions on oxytocin levels. It would be further interesting to see how behavioural treatments influencing oxytocin levels modify subsequent behaviour of the subjects, and how this is modulated by the magnitude of change in oxytocin levels. This could be paralleled by IN-OXT administration in order to test if the increase in endogenous and exogenous oxytocin levels would have the same behavioural effects. Validation of the effects of behavioural treatments on the oxytocin system can have great therapeutic significance because this may open a possibility for treating problem behaviours, or improving symptoms of social deficits in a manner more natural and non-invasive than IN-OXT treatment (i.e. behavioural treatment involves much less discomfort, it can be administered repeatedly for an extended period of

time and the risk of side effects is minimal, if any). Given the recent advances in the field of canine neuro-behavioural research (Bunford et al., 2017), behavioural measurements could be complemented in the future by non-invasively acquiring brain activity data (EEG, fMRI) in response to either IN-OXT treatment or positive social interaction while keeping track of changes in OXT level.

Together these results will further validate the dog as a model of human social cognition, not only at the behavioural but also at the physiological and neurohormonal levels. This will provide us with a useful animal model to study the mechanisms of complex human behaviours for pharmaceutical purposes. Furthermore it will contribute to our understanding of how convergent evolution has shaped social cognition in dogs while adapting to the human environment.

Conflict of interest

All authors declare that there is no conflict of interest.

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