

23. Gähwiler, S., Bremhorst, A., Tóth, K., & Riemer, S. (2020). Fear expressions of dogs during New Year fireworks: a video analysis. *Scientific reports*, *10*(1), 1-10.

### **The effect of the neurohormone oxytocin on human-directed social behaviour in dogs**

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. We have published a review article (2) based on the available literature in the field and carried out empirical research (4, 5, 7, 10, 13, 14), culminating in the edition of a special issue (12) in the topic.

The first approach used in the project administered the neurohormone oxytocin intranasally to privately owned family dogs and tested its effect on certain forms of social behaviour, namely the processing of human emotional facial expressions and empathy-like behaviour in a contagious yawning task. Dogs have been shown to excel in reading human social cues, including facial cues. 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.

Previous studies have found that human yawning is contagious to dogs, but the results are still controversial. It is also debated whether contagious yawning is a sign of empathy, and the physiological mechanisms behind this phenomenon are also unknown. Our goal was to further investigate if human yawning is contagious to dogs, and how it is affected by intranasally administered oxytocin. We also tested if contagious yawning was related to dogs' empathetic skills measured via an owner-completed questionnaire. Dogs (N = 33) were presented with human yawns (yawning condition – YC) or mouth openings (gaping condition – GC) on two test occasions after intranasal administration of 12 IU oxytocin or placebo. Contagious yawning (defined as a significant increase in the number of yawns in YC as compared to GC) was not found in either of the treatment groups and the number of yawns was not related to the owner-reported emotion contagion of the dogs. However, oxytocin pre-treatment significantly decreased the number of yawns in dogs (in general, but more particularly during the phase when human yawns and gaping were presented). Furthermore, there was a positive relation between signs of stress and anxiety (mouth licking) and the number of yawns (in general, but more particularly during the post phase when human yawns and gaping were not any more presented). We conclude that dogs' yawning observed in this situation may largely be due to situational stress. Thus the difference between the

oxytocin and the placebo group is possibly caused by the decreased social stress and not by oxytocin's hypothesised effect on empathy.

The next methodology focused on single nucleotide polymorphisms in the oxytocin receptor gene, investigating whether genetical variations would co-vary with individual differences in certain social behaviour such as gaze following and dog-owner attachment. 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. We investigated associations between oxytocin receptor gene (OXTR) polymorphisms and social behaviour 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 analyse associations between single nucleotide polymorphisms (SNP) in the OXTR (dogs: -213AG, -94TC, -74CG, rs8679682, children: rs53576, rs1042778, rs2254298) and behaviour. 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 behaviour 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-behaviour 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.

Variations in human infants' attachment behaviour 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 behaviour. 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 behaviour 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' behaviour 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 behaviour 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.

An effort was made to include in our studies the third methodological approach known from the literature: the investigation of peripheral oxytocin levels. Our efforts to reliably measure oxytocin from non-invasively collected samples (e.g. urine) were not successful, despite involving analytical chemists expert in tandem-mass spectroscopy. Thus we ended up with blood-oxytocin measurements through our collaborating partners from the Romanian Academy of Sciences and published a methodological study in which we replicate previous findings of canine serum oxytocin increase following a positive dog–human interaction. We provided a detailed description of both the immunoassay method used as well as the behavioural protocol (including crucial time-parameters). This will serve as a base for further studies that both our group as well as others in the field will conduct.

Additionally we collaborated with the University of Bern to carry out a behavioural study regarding firework-related stress (23), a phenomenon that has serious applied implications and has been suggested to be related to the oxytocin system. A high proportion of pet dogs show fear-related behavioural problems, with noise fears being most prevalent. Nonetheless, few studies have objectively evaluated fear expression in this species. Using owner-provided video recordings, we coded behavioural expressions of pet dogs during a real-life firework situation at New Year’s Eve and compared them to behaviour of the same dogs on a different evening without fireworks (control condition), using Wilcoxon signed ranks tests. A backwards-directed ear position, measured at the base of the ear, was most strongly associated with the fireworks condition (effect size: Cohen’s  $d = 0.69$ ). Durations of locomotion ( $d = 0.54$ ) and panting ( $d = 0.45$ ) were also higher during fireworks than during the control condition. Vocalisations ( $d = 0.40$ ), blinking ( $d = 0.37$ ), and hiding ( $d = 0.37$ ) were increased during fireworks, but this was not significant after sequential Bonferroni correction. This could possibly be attributed to the high inter-individual variability in the frequency of blinking and the majority of subjects not vocalising or hiding at all. Thus, individual differences must be taken into account when aiming to assess an individual’s level of fear, as relevant measures may not be the same for all individuals. Firework exposure was not associated with an elevated rate of other so-called ‘stress signals’, lip licking and yawning.

### **The use of non-invasive polysomnography for the study of the neural mechanisms behind social cognition in dogs**

Prior to the start of the project our research group had published the first ever attempt to apply a non-invasive polysomnography method for dogs in order to measure brain activity (EEG) in a way that is both welfare-compatible and directly comparable to human data. During this project we aimed to use this protocol for studying brain mechanisms underlying social behaviour. To this end several further methodological investigations were carried out (6, 8, 11, 16-18, 22), followed by empirical studies focusing on the main topic of the project (3, 9, 19, 20) and review papers in prestigious journals highlighting the theoretical importance of this novel approach (1, 15).

There is an ongoing need to improve animal models for investigating human behaviour and its biological underpinnings. The domestic dog (*Canis familiaris*) is a promising model in cognitive neuroscience. However, before it can contribute to advances in this field in a comparative, reliable, and valid manner, several methodological issues warrant attention. We reviewed recent non-invasive canine neuroscience studies (including fMRI and EEG methodology), primarily focusing on (i) variability among dogs and between dogs and humans in cranial characteristics, and (ii) generalizability across dog and dog–human studies. We argue not for methodological uniformity but for functional comparability between methods, experimental designs, and neural responses.

We conclude that the dog may become an innovative and unique model in comparative neuroscience, complementing more traditional models.

Within the field of cognitive neuroscience the dog (*Canis familiaris*) is an especially promising non-invasive translational model of sleep research. Studies on the relationship between sleep and cognition in dogs and other canines are only just emerging, but still very scarce. In a second review we provided insight into canine sleep and sleep-related physiological and cognitive/behavioural phenomena. We show that dogs do not only fulfil all behavioural and polygraphic criteria of sleep, but are characterized by sleep homeostasis, diurnal pattern of activity, circadian rhythms, ultradian sleep cycles, socio-ecologically and environmentally shaped wake-sleep structure, sleep-related memory improvement, as well as specific sleep disorders. Developmental patterns of sleep-related physiological indices, as well as parallel trends in age-dependent changes in cognition and sleep were evidenced in dogs.

Methodological investigation during the project included an in-depth analysis of the reliability of sleep structure scoring, as well as the investigation of the first-night effect, as well as the influence of pre-sleep activity and sleeping locations. Attempts were made to analyse (in addition to sleep structure data and EEG spectrum), the so-called sleep spindles, the density of Rapid eye Movements, and Heart rate. Non-invasive polysomnography recording on dogs has been claimed to produce data comparable to those for humans regarding sleep macrostructure, EEG spectra and sleep spindles. While functional parallels have been described relating to both affective (e.g., emotion processing) and cognitive (e.g., memory consolidation) domains, methodologically relevant questions about the reliability of sleep stage scoring still need to be addressed. In Study 1, we analysed the effects of different coders and different numbers of visible EEG channels on the visual scoring of the same polysomnography recordings. The lowest agreement was found between independent coders with different scoring experience using full (3 h-long) recordings of the whole dataset, and the highest agreement within-coder, using only a fraction of the original dataset (randomly selected 100 epochs (i.e.,  $100 \times 20$  s long segments)). The identification of drowsiness was found to be the least reliable, while that of non-REM (rapid eye movement, NREM) was the most reliable. Disagreements resulted in no or only moderate differences in macrostructural and spectral variables. Study 2 targeted the task of automated sleep EEG time series classification. Supervised machine learning (ML) models were used to help the manual annotation process by reliably predicting if the dog was sleeping or awake. Logistic regression models (LogREG), gradient boosted trees (GBT) and convolutional neural networks (CNN) were set up and trained for sleep state prediction from already collected and manually annotated EEG data. The evaluation of the individual models suggests that their combination results in the best performance:  $\sim 0.9$  AUC test scores.

In spite of numerous differences, dogs' comparable sleep pattern, as well as several phenotypic similarities to humans on both the behavioural and neural levels, make this species a most feasible model in many respects. Our aim was to investigate whether the so-called first-night effect, which in humans manifests as a marked macrostructure difference between the first and second sleep occasions, can be observed in family dogs. We used a non-invasive polysomnographic method to monitor and compare the characteristics of dogs' ( $N = 24$ ) 3-hr-long afternoon naps on three occasions at the same location. We analysed how sleep macrostructure variables differed between the first, second and third occasions, considering also the effects of potential confounding variables such as the dogs' age and sleeping habits. Our findings indicate that first-night effect is present in dogs' sleep architecture, although its specifics somewhat deviate from the pattern observed in humans. Sleep macrostructure differences were mostly found between occasions 1 and 3; dogs

slept more, had less wake after the first drowsiness episode, and reached drowsiness sleep earlier on occasion 3. Dogs, which had been reported to sleep rarely not at home, had an earlier non-rapid eye movement sleep, a shorter rapid eye movement sleep latency, and spent more time in rapid eye movement sleep on occasion 3, compared with occasion 1. Extending prior dog sleep data, these results help increase the validity of further sleep electroencephalography investigations in dogs.

Given prior findings with humans and/or dogs, our goal was to assess, in 16 family dogs (1.5–7 years old; 10 males; 10 different breeds) the effects of pre-sleep activity and timing and location of sleep on sleep electrophysiology. All three factors had a main and/or interactive effect on sleep macrostructure. Following an active day, dogs slept more, were more likely to have an earlier drowsiness and NREM, and spent less time in drowsiness and more time in NREM and REM. Activity also had location- and time of day-specific effects. Time of day had main effects; at nighttime, dogs slept more and spent less time in drowsiness and awake after first drowsiness, and more time in NREM and in REM. Location had a main effect; when not at home, REM sleep following a first NREM was less likely. Findings are consistent with and extend prior human and dog data and have implications for the dog as an animal model and for informing future comparative research on sleep.

There have been many comparative studies on dog behaviour; however, several easily measurable and analysable psychophysiological variables that are widely used in humans are still largely unexplored in dogs. One such measure is rapid eye movement density (REMD) during REM sleep. The aim of this study was to test the viability of measuring REMD in dogs and to explore the relationship between the REMD and different variables (sex, age, body size, and REM sleep duration). Fifty family dogs of different breeds and ages (from 6 months to 15 years old) participated in a 3-h non-invasive polysomnography recording, and the data for 31 of them could be analysed. The signal of the electro-oculogram (EOG) was used to detect the rapid eye movements during REM sleep, and REMD was calculated based on these data. The duration of REM sleep had a quadratic effect on REMD. Subjects' REMD increased with age, but only in male dogs with short REM sleep duration. Furthermore, in the case of dogs with short REM sleep, the interaction of body mass and REM sleep duration had a significant effect on REMD. No such effects were found in dogs with long REM duration. These results suggest that relationships may exist between REMD and several different variables.

Non-REM bursts of activity in the sigma range (9–16 Hz) typical of sleep spindles predict learning in dogs, similar to humans and rats. Little is known, however, about the age-related changes in amplitude, density (spindles/minute) and frequency (waves/second) of canine spindles. We investigated a large sample (N = 155) of intact and neutered pet dogs of both sexes, varying in breed and age, searching for spindles in segments of non-REM sleep. We recorded EEG from both a frontal midline electrode (Fz) and a central midline electrode (Cz) in 55.5% of the dogs, in the remaining animals only the Fz electrode was active (bipolar derivation). A similar topography was observed for fast ( $\geq 13$  Hz) spindle occurrence as in humans (fast spindle number, density on Cz > Fz). For fast spindles, density was higher in females, and increased with age. These effects were more pronounced among intact animals and on Fz. Slow spindle density declined and fast spindle frequency increased with age on Cz, while on Fz age-related amplitude decline was observed. The frequency of fast spindles on Fz and slow spindles on Cz was linked to both sex and neutering, suggesting modulation by sexual hormones. Intact females displayed higher frequencies than males and neutered females. Our findings support the argument that sigma bursts in the canine non-REM sleep are analogous to human sleep spindles, and suggest that slow and fast spindles

display different trajectories related to age, of which an increase in frontal fast spindles is unique to dogs.

In both humans and dogs sleep spindle occurrence between acquisition and recall of a specific memory correlate with learning performance. However, it is not known whether sleep spindle characteristics are also linked to performance beyond the span of a day, except in regard to general mental ability in humans. Such a relationship is likely, as both memory and spindle expression decline with age in both species (in dogs specifically the density and amplitude of slow spindles). We investigated if spindle amplitude, density (spindles/minute) and/or frequency (waves/second) correlate with performance on a short-term memory and a reversal-learning task in old dogs (> 7 years), when measurements of behavior and EEG were on average a month apart. Higher frequencies of fast ( $\geq 13$  Hz) spindles on the frontal and central midline electrodes, and of slow spindles ( $\leq 13$  Hz) on the central midline electrode were linked to worse performance on a reversal-learning task. The present findings suggest a role for spindle frequency as a biomarker of cognitive aging across species: Changes in spindle frequency are associated with dementia risk and onset in humans and declining learning performance in the dog.

Although a positive link between sleep spindle occurrence and measures of post-sleep recall (learning success) is often reported for humans and replicated across species, the test–retest reliability of the effect is sometimes questioned. The largest to date study could not confirm the association, however methods for automatic spindle detection diverge in their estimates and vary between studies. Here we report that in dogs using the same detection method across different learning tasks is associated with observing a positive association between sleep spindle density (spindles/minute) and learning success. Our results suggest that reducing measurement error by averaging across measurements of density and learning can increase the visibility of this effect, implying that trait density (estimated through averaged occurrence) is a more reliable predictor of cognitive performance than estimates based on single measures.

Following the above described methodological studies, and some adjustments to our original polysomnography protocol, further studies were carried out investigating the relationship between dogs' emotion processing and brain activity during sleep. One of the studies (19) used emotional face expressions similar to the above described eye-tracking study, while in another study (3, 9) used more naturalistic social interactions were used, similar to those used in 10. Finally, we carried out an experiment regarding the effect of being watched (20.) Dogs have outstanding capabilities to read human emotional expressions, both vocal and facial. In the present study, we manipulated dogs' (N = 15, in a within subject design) sleep structure by specifically disrupting REM versus Non-REM sleep, while maintaining equal sleep efficiency (monitored via non-invasive polysomnography). We found that both the number of awakenings as well as relative Non-REM (but not relative REM) duration influenced dogs' viewing patterns in a task where sad and happy human faces were simultaneously projected with sad or happy human voice playbacks. In accordance with the emotion laterality hypothesis, the interaction between sound valence and Non-REM sleep duration was specific to images projected to the left (regardless of image-sound congruency). These results reveal the first evidence of a causal link between sleep structure and inter-specific emotion-processing in the family dog.

The effects of emotionally valenced events on sleep physiology are well studied in humans and laboratory rodents. However, little is known about these effects in other species, despite the fact that several sleep characteristics differ across species and thus limit the generalizability of such findings. Here we studied the effect of positive and negative social experiences on sleep macrostructure in dogs, a species proven to be a good model of human social cognition. A non-

invasive polysomnography method was used to collect data from pet dogs ( $n = 16$ ) participating in 3-hour-long sleep occasions. Before sleep, dogs were exposed to emotionally positive or negative social interactions (PSI or NSI) in a within-subject design. PSI consisted of petting and ball play, while NSI was a mixture of separation, threatening approach and still face test. Sleep macrostructure was markedly different between pre-treatment conditions, with a shorter sleep latency after NSI and a redistribution of the time spent in the different sleep stages. Dogs' behaviour during pre-treatments was related to the macrostructural difference between the two occasions, and was further modulated by individual variability in personality. This result provides the first direct evidence that emotional stimuli affect subsequent sleep physiology in dogs.

Little is known about the effect of emotional experiences on dogs' subsequent sleep physiology, a set of phenomena heavily interrelated with emotions in the case of humans. The present paper examines heart rate (HR) and heart rate variability (HRV) during dogs' sleep, measures that are influenced by both positive and negative emotions in awake dogs. In Study I, descriptive HR and HRV data is provided on  $N = 12$  dogs about the different sleep stages (wake, drowsiness, non-rapid eye movement (non-REM), REM; scoring based on electroencephalogram (EEG) data). We conclude that wakefulness is characterised by higher HR and lower HRV compared to all sleep stages. Furthermore, drowsiness is characterised by higher HR and lower HRV than non-REM and REM, but only if the electrocardiogram (ECG) samples are taken from the first occurrence of a given sleep stage, not when the longest periods of each sleep stage are analysed. Non-REM and REM sleep were not found to be different from each other in either HR or HRV parameters. In Study II, sleep HR and HRV measures are compared in  $N = 16$  dogs after a positive versus negative social interaction (within-subject design). The positive social interaction consisted of petting and ball play, while the negative social interaction was a mixture of separation, threatening approach and still face test. Results are consistent with the two-dimensional emotion hypothesis in that following the intense positive interaction more elevated HR and decreased HRV is found compared to the mildly negative (lower intensity) interaction. However, although this trend can be observed in all sleep stages except for REM, the results only reach significance in the wake stage. In sum, the present findings suggest that HR and HRV are possible to measure during dogs' sleep, and can potentially be used to study the effect of emotions not only during but also after such interactions.

Ample evidence suggests that dogs possess enhanced skills in reading human visual attention, but it remains to be explored whether they are sensitive to the audience effect in their interactions with humans. The present study aimed to investigate how dogs' behavior is affected by their owners' visual attention while performing a repetitive task (bringing an object back to an unfamiliar experimenter while the owner waited passively). We assumed that if dogs are susceptible to the audience effect, their task persistence and task performance would vary according to their owners' attentiveness. A group of adult pet dogs ( $N = 27$ ) were repeatedly presented with an object retrieval task by the experimenter ( $N = 20$  trials) while owners either ignored their dogs (Inattentive Owner condition) or paid attention to their dogs' actions (Attentive Owner condition). Behavioral observations were complemented with the owner's reports of their relationships with their dogs (assessed by means of an owner-pet attachment questionnaire) and dogs' spectral EEG sleep profile (recorded during 3-h-long daytime sleep). Although dogs, independently of their owners' attentional state, were generally willing to comply with the fetching task, they were faster to approach the toy object and gazed significantly longer at their owners when he/she was paying attention. This finding is reminiscent of peer influence observed in humans. Further, characteristics of relationship insecurity (relationship anxiety and avoidance) were associated with dogs' task

persistence and performance. Dogs of owners with higher relationship anxiety tended to approach the toy object less frequently, and dogs of owners with higher relationship avoidance and anxiety were more hesitant to approach the toy object. We also found that dogs' individual susceptibilities to the audience effect is related to EEG spectral power of both REM and non-REM sleep as well as in pre-sleep (drowsiness) in a trait-like manner. These results, in line with previous findings, support the notion that dogs have a somewhat human-like susceptibility to the audience effect, a trait which might be linked to more complex mechanisms, such as self-presentation or reputation management, helping the two species to become effective social partners.

### **Summary and ongoing work**

We have made a significant advancement in understanding the relationship between dogs' human-like socio-cognitive skills and their regulation by the oxytocin system. However, there are a couple of points we feel incomplete and if possible we would like to ask for a no-cost extension in order to publish research we carried out during the project duly acknowledging the Bial foundation in the papers. We have recently submitted a review paper about the relationship between sleep and emotions in collaboration with our research partners at the Romanian Academy of Sciences (the paper being the result of work carried out by a post doc who's salary was partially covered by the Bial grant). In addition, after an initial rejection of the paper, we are in the process of re-writing and re-submitting the experimental work concerning Question I. in the original proposal (measuring the effect of intranasal oxytocin and different forms of social interaction on social influencability using a food preference task). In addition to these two pending publications which are an integral part of the project, the unfortunate global events of last year made conference organisers to postpone most of the events we were planning to participate at (with a few exceptions where an online conference was held). This year we are planning to take part in these re-scheduled (online) scientific forums and present the results as originally planned. We have not spent the proposed budget (experiments had to be suspended due to local regulations prohibiting the recruitment of volunteers, and conference costs as well as open access publication fees will need to be paid only when the above detailed tasks are carried out). Thus we do not yet ask for the final report payment, but instead would like to properly conclude the tasks undertaken.