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In vivo functional near-infrared spectroscopy measures mood-modulated cerebral responses to a positive emotional stimulus in sheep

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ABSTRACT

The affective state of an animal, which is thought to reflect its welfare, consists of both short-term emotional reactions and long-term general mood. Because this state is generated and processed by the brain, we used non-invasive measurement of such brain activity as a novel indicator variable and investigated the interplay of mood and short-term emotional reactions in animals. We developed a wireless sensor for functional near-infrared spectroscopy (fNIRS), which assesses cortical perfusion changes, and consequently neuronal activity. Mood differences were induced by barren and enriched housing in a total of nine sheep and we observed their brain reaction in response to the positive situation of being groomed. We detected a decrease in cerebral oxyhaemoglobin concentration ($[O_2Hb]$) which persisted during grooming. The localisation of the decrease in the brain did not depend on the site where the stimulus was applied. Also, the intensity of the response did not depend on the intensity of the grooming stimulus and a sham stimulus did not evoke an $[O_2Hb]$ response as seen with a grooming stimulus. Thus, we conclude that the observed haemodynamic brain response was unlikely to reflect pure somato-sensory information. We then found that the amplitude of the $[O_2Hb]$ response was larger if sheep were in a supposedly more negative mood. This contradicts the common assumption that negative mood generally taints reactions to emotional stimuli. Our results also demonstrate the potential of fNIRS for assessing affective states in freely moving animals.

Introduction

The affective states of non-human vertebrate species are relevant in the assessment of welfare (Boissy et al., 2007; Gregory, 2004). Although progress has been made in the measurement of short-term emotional reactions for negative states (Forkman et al., 2007), very few studies have addressed positive states (Broom and Zanella, 2004; Reefmann et al., 2009a,b,c). Indicators for assessing subjective emotional reactions have included behavioural, cardiac and hormonal responses (Boissy and Neindre, 1997). Affective states are associated with structures of the brain such as the limbic system, the anterior cingulate cortex and the prefrontal cortex (Allman et al., 2001; Berridge, 2003; da Costa et al., 2004; Matsunaga et al., 2009; Murphy et al., 2003). Similar to fMRI studies using BOLD signals (e.g. Murphy et al., 2003), the aim of this study was therefore to investigate concentrations of oxy- and deoxyhaemoglobin ($[O_2Hb]$, [HHb]) in the brain as new indicators specific to neuronal activation. We used non-invasive functional near-infrared spectroscopy (fNIRS) in nine freely moving sheep, and chose a repeatable stimulus of positive valence, i.e. being voluntarily groomed by a familiar experimenter. The sheep chosen in our experiment voluntarily approached the experimenter for being groomed and did not avoid her when being groomed thus indicating that they experienced grooming as a positive stimulus. In addition, we have shown in a previous experiment that behavioural and physiological indicators support the notion that grooming is perceived as positive in sheep (Reefmann, et al., 2009c).

In recent experiments on rats (Harding et al., 2004; Mendl et al., 2009) positive or negative mood was induced by housing. Animals in a negative mood state reacted in a clearly more negative manner to ambiguous stimuli (Burman et al., 2008; Harding et al., 2004). This led to the conclusion that short-term emotional reactions are influenced by mood, i.e. animals in a negative mood react more strongly in a negative situation and with greater restraint in a positive situation than animals in a positive mood (Grippo and Johnson, 2009). Such an anhedonia effect is also a well-known phenomenon in humans with depression (Grippo and Johnson, 2009). We thus also addressed the

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question whether cerebral activation in response to the pleasurable stimulus of grooming was modified by housing-induced mood.

Methods

Functional near-infrared spectroscopy

Neuronal activity causes localised changes in cortical oxygenation which can be quantified by near-infrared spectroscopy (NIRS). Based on the absorption and scattering of near-infrared light in tissue, [O_2Hb] and [HHb] can be calculated (Haensse et al., 2005). By using a number of light sources and detectors, these concentrations can be assessed simultaneously at multiple locations (Fig. 1). The approach penetrates about 1 to 2 cm into human brain tissue and can therefore be used to monitor cortical structures of the cerebrum (Gratton et al., 2000). The cerebral cortex of the sheep lies approximately 5 to 9 mm below the scalp surface as seen by computer tomography (CT) of two of our animals. By exposing a subject to an external stimulus, e.g. a sensory input or an emotional situation as in our case, we may deliberately elicit neuronal activity. This activity can be observed as a change in [O_2Hb] and [HHb] and is located in the cortical area responsible for processing the stimulus (functional NIRS, fNIRS) (Villringer and Chance, 1997).

Most available fNIRS systems rely on cable connections for both the power supply and connection of the optical probe to the acquisition device (Wolf et al., 2007). As a prerequisite for functional measurements, animals were not to be sedated, and in order to elicit positive emotions, our experimental design required NIRS measurements on unrestrained subjects allowed to move around freely in a pen. Because of this demanding experimental setup, we designed a miniaturised wireless NIRS sensor specifically for the given purpose (Muehlemann et al., 2008). Its optical probe consists of two detectors and four light sources at two wavelengths each (LED at 760 and 870 nm peak emission wavelength; source–detector distances of 14 and 22 mm, Fig. 1). This configuration of light sources and detectors allowed the recording of the sheep's dorsal cerebral cortex, i.e. the brain areas directly accessible to the fNIRS sensor most likely consisted of the motor and the somatosensory cortices (Vanderwolf and Cooley, 2002). The intensity of the light after transmission through tissue is detected by a silicon PIN photo diode and digitised with a sampling rate of 100 Hz. The data was then transmitted to the host computer for storage and further processing.

Animals

A total of 19 non-reproducing and non-lactating female sheep (10 Lacaune and 9 Swiss White Alpine, all aged between 2.5 and 3 years) were housed as a flock at Agroscope Reckenholz-Tänikon Research Station ART, Tänikon, Switzerland. The individuals selected for the NIRS trials were habituated to wearing the NIRS sensor. During the measurements, the test sheep was led into a trial pen (5.5 m^2) together with another member of the flock so as to avoid stress associated with separation. The NIRS sensor was attached to the top of the test sheep's depilated head, and held in place with a head collar (Fig. 1). We favoured Lacaune sheep over Swiss White Alpine sheep to perform the fNIRS measurement mainly because their scalp wool was less dense, and hence easier to depilate. In addition, previous studies (Reefmann et al., 2009c) indicated that they allowed themselves be groomed more readily and were calmer during handling. All grooming stimuli in both pre-trial and main trial experiments were applied by the same experimenter (N.R.).

Pre-trial experiments

We conducted pre-trials on two Lacaune sheep to refine the experimental protocol, to improve the reliability of the sensor, and to test hardware and software under the experimental conditions. Furthermore, we investigated firstly whether it was possible to detect haemodynamic responses to the grooming stimulus at all, and secondly, whether it was possible to differentiate between haemodynamic responses of emotional processing on the one hand, and mere sensory processing simultaneously elicited by the stimulus, on the other. In other words, we set-up a series of experiments for which we had clear expectations of the signals we would measure if the signals were caused by sensory processing.

We set the light intensities of the sources manually before we initiated the acquisition of the NIRS signals. For each experiment, a single test animal was subjected to repeated measurement blocks consisting of a stimulation interval of 60 s of grooming and a resting interval without grooming of 40 s duration (20 s pre-, 20 s post-stimulation interval). To clearly separate post- and pre-stimulation intervals, only 15 of the 20 s were considered in the evaluation. The measurement blocks were repeated until 10 blocks with satisfactory signal quality, i.e. without severe motion artefacts were obtained for a given test animal (10–12 blocks in each of the experiments described below).

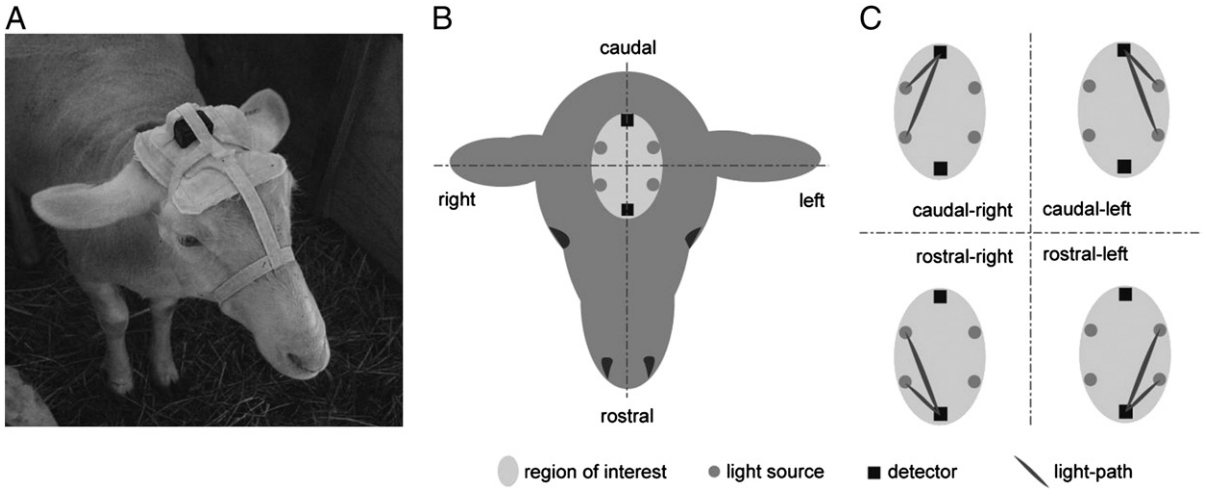

Fig. 1. Position of fNIRS sensor on a sheep's head. A) Lacaune sheep with the wireless sensor attached. The black sensor is partially visible. B) Schematic top view of source and detector positions on the sheep's head. C) By pairing light sources and detectors, eight light-paths are established in total which penetrate localised volumes of the cerebral cortex; the average penetration depth increases with source–detector distance.

The first pre-trial measurement investigated whether a haemodynamic reaction to the grooming stimulus could be seen at all. We conducted a two-phase measurement: in the initial phase, the sheep's ventral neck was groomed (10 blocks). Next, a sham stimulus was applied (10 sham blocks): NIRS was recorded while issuing the audible countdown to indicate the start- and stop time of sham-stimulus intervals, yet no actual tactile stimulation was applied (question 1.A). In a second measurement, grooming during the stimulation interval was interspersed with touching (positioning the flat hand on the wool) during the resting interval. In this way, evidence was obtained as to whether the haemodynamic response of the cortex was evoked purely via sensory input—present during both touching and grooming—or via an emotion-processing effect, presumably only present during grooming (question 1.B).

We performed two more measurements in order to differentiate emotional processing from purely sensory reactions. The first compared the haemodynamic effects of grooming the right side versus grooming the left side of the neck, interspersed with resting intervals. If the response was simply caused by the processing of sensory inputs, one would expect a contralateral cortical reaction and a greatly reduced, if not absent, ipsilateral response (question 2.A). The second measurement compared grooming of the ventral neck and grooming of the belly (interspersed with resting intervals), to determine whether the haemodynamic response was spatially dependent on the stimulus localisation which could be expected, if purely sensory processing was measured (question 2.B).

Two different paradigms were applied to determine the influence of the intensity of grooming on the amplitude of the haemodynamic reaction. In the first of these, intense (with respect to the applied frequency, which was doubled) grooming and light grooming were used as stimuli, alternating with resting intervals (question 3.A). In the second, intense grooming was used as a stimulus while light grooming was performed during the resting interval (question 3.B).

Main trial experiments

The flock was divided into two mixed-breed groups, one for positive-mood (enriched housing) and one for negative-mood (barren housing) treatment. The animals in the positive-mood group (six Lacaune and four Swiss White Alpine sheep) were kept in their familiar environment (5.8 m^2 per animal) in an open building, were treated gently by a familiar caretaker during human–animal interaction, and were fed hay and nutritious pellets. The group as a whole was lured on to a pasture for ½ to 2 h every second or third day. By contrast, the negative-mood group (four Lacaune and five Swiss White Alpine sheep) were kept in an unfamiliar closed building in a restricted space (1.6 m^2 per animal), with the light intensity reduced by 150 lx on average. Their feed consisted of hay offered at irregular times of day. These sheep were treated fairly roughly: their feed was dispensed noisily, and they were chased around the pen. In addition, there was no access to pasture, but sheep were repeatedly led away from the group on their own by holding them tightly by their collar and controlling their steps.

The experimental sheep were used to wearing the NIRS sensor, as well as to being groomed. They were led into the experimental pen. The animal was then repeatedly groomed; each measurement-block consisted of a stimulation interval of 45 s and a resting interval of 60 s duration (30 s pre-, 30 s post-stimulation interval). Only 15 of the 30 s were considered in the evaluation, in order to clearly separate post- and pre-stimulation intervals. This reduced the probability of signal disturbances in the pre-stimulation interval owing to motion artefacts in the post-stimulation interval of the preceding stimulation block.

Signal processing

Low-pass filtering and down-sampling of the raw fNIRS signal improved signal-to-noise ratio (SNR) and decreased computation time in the subsequent processing steps, especially in the final statistical analysis of the data. We found that a sampling rate of 1 Hz for the post-processing of the signal had sufficient bandwidth to detect the changes in [HHb] and [O_2Hb] which develop typically within several seconds, yet still resulted in both reasonable computational demands and satisfactory SNR.

Movement artefacts must be addressed when performing in-vivo NIRS (Izzetoglu et al., 2005). We reduced the signal artefact in three steps: (1) threshold detection on a sliding-window autocorrelation function of the raw signal, (2) logarithmic compression of the artefact intervals, and (3) exclusion of the light paths within stimulation blocks still severely compromised. Light paths in step (3) were excluded if the raw absorption signal overshot the signal range (clipping) or displayed excessive oscillations or peaks with non-physiological temporal dynamics.

To monitor localised perfusion changes in response to neuronal activation, we calculated the changes in [O_2Hb] and [HHb] within the cortical tissue by applying the modified Beer-Lambert law to the recorded light absorption values (Delpy et al., 1988). We applied a differential path-length factor DPF of 7.2 for the light paths with 760 nm sources and one of 4.4 for those with 870 nm sources (Pringle et al., 1999). The median haemoglobin concentrations over the evaluated pre-stimulation intervals were taken as zero baselines of each block, and differences between individuals were taken into account via the statistical analysis.

Statistics—General remarks

In order to incorporate the data of the different light paths of individual animals measured repeatedly across time in the analysis, we applied linear mixed-effects models, implemented in R 2.7.2 to 2.8.1 (R Development Core Team, 2005) using the lme method (Pinheiro and Bates, 2002). We modelled the time course of the [O_2Hb] and [HHb] as natural splines of the continuously coded time variable (Venables and Ripley, 2002) so as to obtain a smooth but unrestricted representation of these concentrations as a function of time. The degree of freedom (*df*) of the splines was optimised to allow for enough flexibility to reflect physiological signal components, but without reflecting an overly detailed and noisy picture. For that purpose, we evaluated the spline models repeatedly with increasing *df* values. The model estimates were based on a pure maximum-likelihood approach, which allowed comparison of the models differing in their fixed effect, i.e. direct comparison of their *df* values (Pinheiro and Bates, 2002). We chose the *df* by increasing it stepwise and checking model quality based on significant model improvements and relatively lower AIC and BIC values.

A full model was then calculated and all non-significant interaction terms were subsequently dropped in a stepwise backward approach based on type-III-like sums of squares while retaining all main effects. Thus, we report only statistically significant interactions and significant main effects not included in interactions. Many models were calculated using this stepwise backward approach, and a high denominator degree of freedom was available for all terms that included time. Because both these aspects increase type-I errors, we considered only effects reaching a *p*-value smaller than 0.001 to be significant.

At 1 Hz, the data was densely spaced along time and some autocorrelation was visible in the residuals of the models. We therefore included an autoregressive process to account for this time-dependency (Pinheiro and Bates, 2002). Although our sample of individual sheep was small, we recorded many more values which allowed us to verify assumptions (Gelman and Hill, 2007). Accordingly, we confirmed assumptions via graphical analysis of residuals to check for normality and heteroscedasticity.

Statistical analysis—Pre-trials

Because few motion artefacts occurred in the pre-trials, [O_2Hb] and [HHb] served as response variables without block averaging. In each model, data from one sheep was quantified. We evaluated the above-mentioned conditions with two models each, one for the [O_2Hb] and one for [HHb], by including the following main fixed effects: (1.A) grooming versus sham (factor with 2 levels) and (1.B) grooming interspersed with touching, with an interest in the effect on the time course of the signal; (2.A) grooming on the right versus the left side of the body (factor with 2 levels), and (2.B) grooming on the belly versus the ventral neck (factor with 2 levels); as well as (3.A) light versus intense grooming (factor with 2 levels), and (3.B) intense grooming interspersed with light grooming, with an interest in the effect on the time course itself.

The additional fixed effects were time (in seconds: natural splines with 27 and 19 *df*'s for [O_2Hb] and [HHb], respectively; due to numerical issues, 17 *df*'s for [HHb] in question 2.B), and three dichotomous variables for lateral localisation, longitudinal localisation, and penetration depth. The random effects were the different light paths nested within the experimental blocks, and an autoregressive process of order 2 was included for the residuals.

Statistical analysis—Main trials

The block averages of the [O_2Hb] and [HHb] for each light path and individual sheep were used as response variables in two linear mixed-effects models in R (R Development Core Team, 2005). We investigated the influence of the fixed effects mood (factor with two levels: negative versus positive mood), time (in seconds: natural spline with 17 and 9 for [O_2Hb] and [HHb], respectively), and three dichotomous variables describing the geometric properties of the light paths as described above. In the full model, we included all possible interactions up to the four-way interaction; all four-way interactions (and some lower-level interactions) could be dropped owing to their non-significance. The random effects were the light paths nested in animal identity, and an autoregressive process of order 3 was included for the residuals.

Nine sheep (four Lacaune and one Swiss White Alpine sheep in the barren, bad mood and four Lacaune in the enriched, good mood treatment) were measured, applying eight different source–detector combinations (light paths, 72 paths in total). Each light path was median-averaged across grooming blocks and consisted of 75 measurement points at 1 Hz, totalling 5400 calculated values for [O_2Hb] and [HHb].

Results

Pre-trials: The effect of grooming and its comparison versus sham

Question 1.A: while the sheep was being groomed on its ventral neck, the [O_2Hb] signal decreased with stimulus onset and continued below baseline over the entire stimulation interval. By contrast, the [O_2Hb]

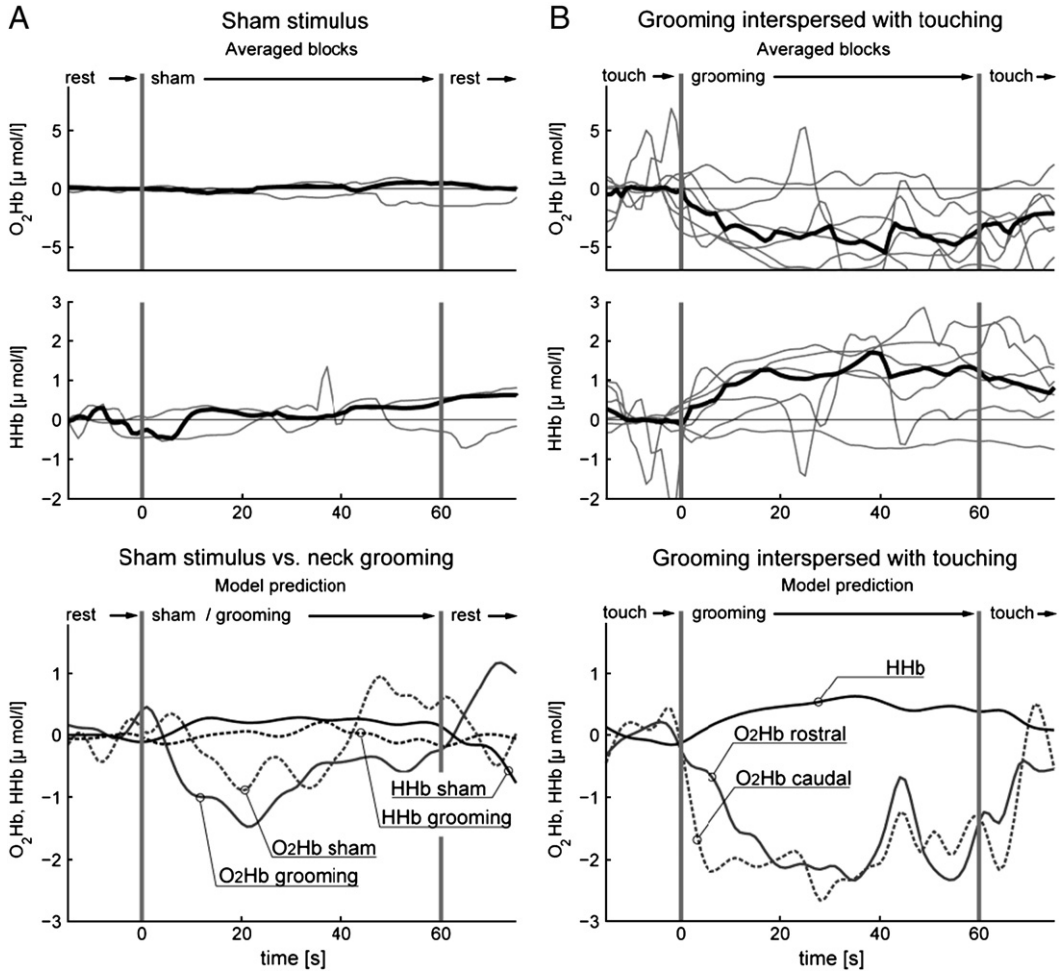

Fig. 2. Effect of grooming (one animal). The top two panels display the changes in concentration of O_2Hb and HHb for individual non-excluded blocks (thin lines), as well as their median (bold lines) for an exemplary single light path (caudal, left hemisphere, long source–detector distance). The bottom panel displays the predicted values of the linear mixed-effects model using splines with all light paths taken into account. A) Sham versus grooming stimulation (question 1A; exemplary long-distance, caudal, left-hemisphere light path of a sham stimulation). B) Neck grooming was applied during stimulation, while the experimenter merely touched the animal during non-stimulation (question 1B; same light path as above).

signal of the sham stimulation decreased with a 7 s delay in respect to stimulus onset and returned to baseline before the stimulation terminated (interaction time × stimulus type: $F_{27,9736} = 3.37$, $p < 0.0001$, one subject, Fig. 2A). Moreover, the [HHb] signals increased more strongly for the sham stimulus than for neck grooming (interaction time × stimulus type: $F_{19,9752} = 2.95$, $p < 0.0001$, Fig. 2A).

Question 1.B: neck-grooming resulted in strong decreases in [O_2Hb] compared to those of the interspersed resting intervals, when the sheep was merely touched. The [O_2Hb] response emerged more rapidly in the caudal than in the rostral region of the cortex (interaction time × longitudinal localisation: $F_{27,4485} = 2.43$, $p = 0.0001$, one subject, Fig. 2B). [HHb] showed a consistent increase at stimulus onset, with no statistically significant differences between the light paths (main effect time: $F_{19,4520} = 6.21$, $p < 0.0001$, Fig. 2B).

Pre-trials: Grooming different body areas

Question 2.A: no statistically significant difference between the haemodynamic responses of the left and the right cortical hemispheres was observed when comparing grooming on the left and right side of the neck (interaction time × lateral localisation × stimulus type before removal from model: $F_{27,4712} = 1.40$, $p = 0.08$, one subject), although the [O_2Hb] response amplitude was generally more pronounced for stimulation on the left side (interaction time × stimulus type: $F_{27,4820} = 4.11$, $p < 0.0001$, Fig. 3A).

Question 2.B: in the comparison of neck-grooming with belly-grooming, changes in [HHb] were not influenced by stimulus type and fell slightly short of our significance criterion (main effect time: $F_{17,4967} = 2.36$, $p = 0.0013$, one subject, Fig. 3B). The [O_2Hb] showed a distinct response for the neck-grooming stimulation blocks, while no clear haemodynamic effect was observed for the belly grooming. In addition, the model identified some modulation of the signal owing to the longitudinal localisation, which was small compared to the differences between neck and belly grooming (interaction time × stimulus type × longitudinal localisation: $F_{27,4876} = 3.31$, $p < 0.0001$, Fig. 3B).

Pre-trials: Varying stimulus intensity

Question 3.A: a significant difference in [O_2Hb] response in terms of stimulus intensity and localisation of haemodynamic response (caudal versus rostral) was found when we compared intense grooming versus light grooming interspersed with a resting interval. The difference between light and intense grooming was small compared to the response amplitude, which was similar for both stimulus modes (interaction time × stimulus type × longitudinal localisation: $F_{27,4075} = 2.52$, $p < 0.0001$, one subject, Fig. 4A). For both stimulus types, [O_2Hb] response was faster in the caudal than in the rostral areas, and the observed drop was quicker for the intense stimulation. The [HHb] response did not display a dependence on

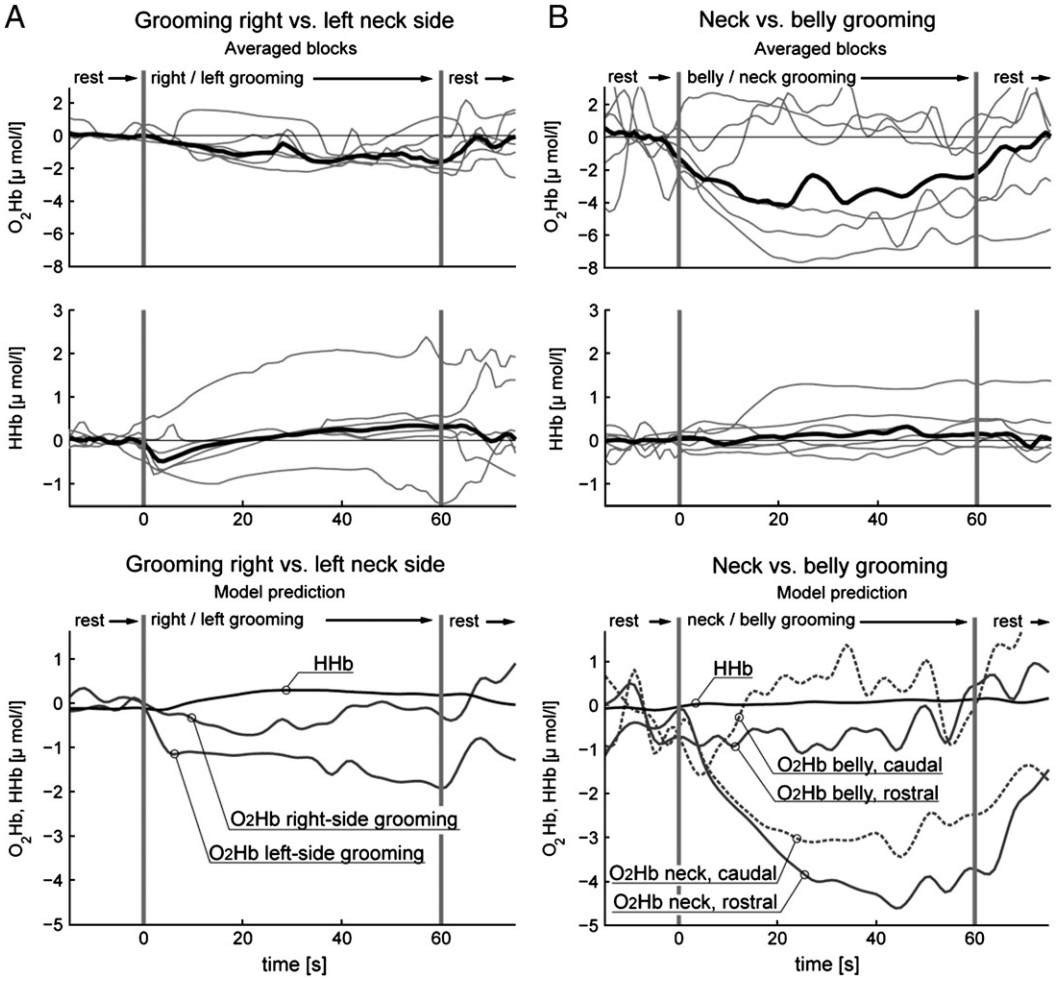

Fig. 3. Effect of localisation (one animal). The top two panels display the changes in concentration of O_2Hb and HHb for individual non-excluded blocks (thin lines), as well as their median (bold lines) for an exemplary single light path (caudal, left hemisphere, long source–detector distance). The bottom panel displays the predicted values of the linear mixed-effects model using splines with all light paths taken into account. A) Grooming of the left versus the right side of the neck (question 2A; exemplary long-distance, caudal, left-hemisphere light path of the stimulation intervals on the left and right sides of the neck). B) Neck- versus belly grooming (question 2B; same light path as above).

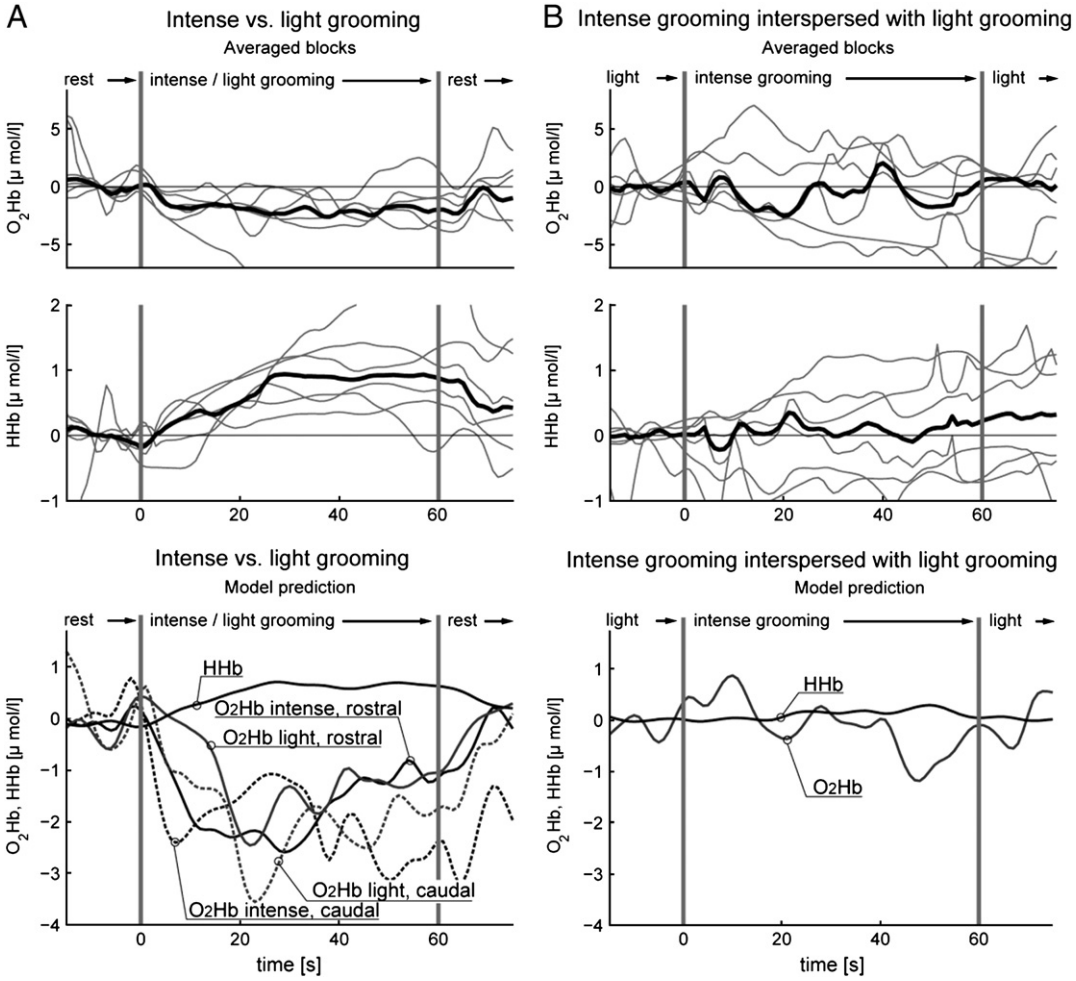

Fig. 4. Effect of intensity (one animal). The top two panels display the changes in concentration of O_2Hb and HHb for individual non-excluded blocks (thin lines), as well as their median (bold lines) for an exemplary single light path (caudal, left hemisphere, long source–detector distance). The bottom panel displays the predicted values of the linear mixed-effects model using splines with all light paths taken into account. A) Intense versus light grooming (question 3A; exemplary long-distance, caudal, left-hemisphere light path for both intense and light grooming). B) Constant grooming with increased intensity during stimulation intervals (question 3B; same light path as above).

stimulus type or localisation, but showed a time course consistent with stimulus onset and end (main effect time: $F_{19,4164} = 5.48$, $p < 0.0001$, Fig. 4A).

Question 3.B: the changes in [O_2Hb] (main effect time: $F_{27,3800} = 4.16$, $p < 0.0001$, one subject, Fig. 4B) and [HHb] (main effect time: $F_{19,3808} = 1.43$, $p = 0.10$, Fig. 4B) measured while switching between grooming of different intensities without interspersed resting intervals were small compared to the measurements with resting intervals between grooming stimulation, and did not depend on the specific light path. In the case of [O_2Hb] response, the observed time course was significant, but unrelated to stimulus onset and end.

Main-trials: Investigating the dependence on mood

The block-averaged [O_2Hb] depended significantly on the subjects' mood and on the longitudinal localisation (caudal vs. rostral) of the response in the cerebral cortex (interaction time × mood × longitudinal localisation: $F_{17,5260} = 3.60$, $p < 0.0001$, nine subjects, Fig. 5). [O_2Hb] decreased in the caudal cortex markedly for the animals in a negative mood, at the same time as it increased in the rostral regions. By contrast, [O_2Hb] decreased less sharply for the animals in a positive mood in both the caudal and rostral cortices, but in a somewhat delayed manner in the rostral cortex. There was no further effect of either lateral localisation ($F_{1,59} = 1.41$, $p = 0.24$) or penetration depth ($F_{1,59} = 1.98$, $p = 0.16$) on [O_2Hb]. The [HHb] showed a stimulus-dependent change over time (time: $F_{9,5319} = 4.74$, $p < 0.0001$, Fig. 5), but did not depend on either mood ($F_{1,7} = 3.04$, $p = 0.12$) or location of measurement (longitudinal localisation: $F_{1,60} = 0.27$, $p = 0.61$, lateral localisation: $F_{1,60} = 0.47$, $p = 0.49$, penetration depth: $F_{1,60} = 0.40$, $p = 0.53$).

Discussion

We observed changes in cortical haemoglobin concentration in response to grooming of the ventral neck. These were temporally consistent with both stimulus onset and end, and comparable to changes observed in humans (Wolf et al., 2002). Given that we observed a decrease in [O_2Hb] and an increase in [HHb], we can rule out the possibility of the observed reactions being exclusive responses of the motor and somatosensory cortices because these areas can be assumed to have been part of the measurement area and if activated an inverse pattern of [O_2Hb] and [HHb] concentrations would have been expected. If somatosensory reactions were responsible for the observed changes, we would expect the location of a response to match body location and intensity of grooming. Despite this, the results showed no change in the laterality of the haemodynamic response when grooming was switched from one side of the animal to the other (question 2.A), no systematic change in location of the response when grooming was switched between the ventral neck and belly (question 2.B), no change in the amplitude of the response between intense and light grooming (question 3.A), and no change whatsoever in response between intense and light grooming without a rest period (question 3.B). These findings suggest that what we were

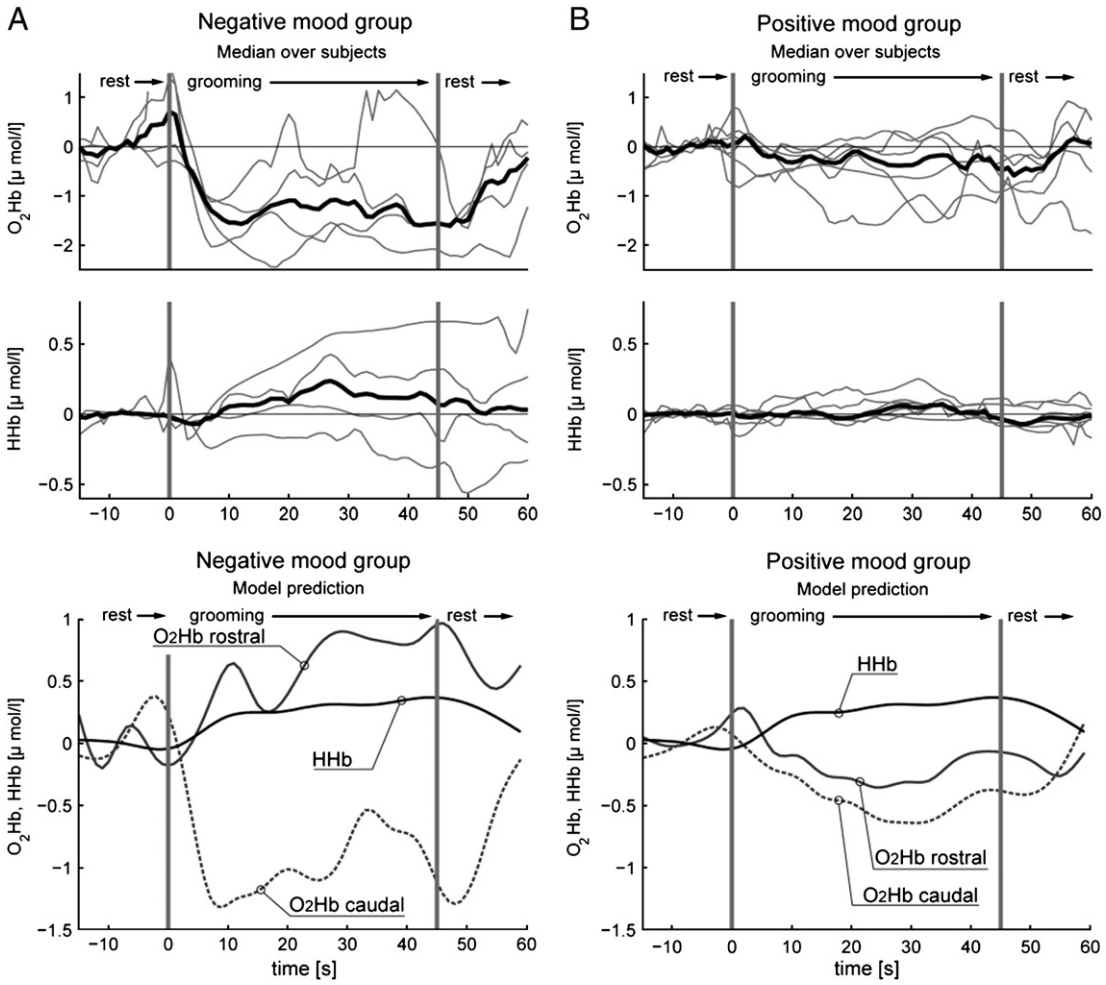

Fig. 5. Haemodynamic reactions to grooming of sheep in a negative (A) and positive (B) mood. The upper two panels show the stimulation block averages of the O_2Hb and HHb changes for an exemplary light path (caudal, left hemisphere, deep penetration) of each individual sheep (thin lines) and the median of these block averages (dark bold lines). The bottom panel displays the predicted values of the linear mixed-effects model using splines based on all animals and all light paths.

observing primarily was not somatosensory or motor cortical reactions, but rather the processing of emotion itself.

As mentioned, we observed a decrease in [O_2Hb] and an increase in [HHb]. In studies on humans, neuronal activation has been linked with an increase in [O_2Hb] and a decrease in [HHb] (Wolf et al., 2002). Two non-exclusive hypotheses may explain our unexpected finding. Firstly, the "vascular steal" hypothesis assumes that blood flow increases in activated cerebral regions and decreases in surrounding regions (Shmuel et al., 2002; Smith et al., 2004), i.e. [HHb] increases and [O_2Hb] decreases. Secondly, a deactivation of brain regions would also be consistent with the inverted response (Sakatani et al., 2006; Wenzel et al., 2000). Given that emotional processes were responsible for the observed haemodynamic responses, though, a shift of [O_2Hb] towards areas of emotional processing may have occurred. Since no effect of light-penetration depth was noted, it would seem that neither the anterior cingulate cortex nor the limbic system, which lie ventrally from the measurement localisation, were responsible for such a shift. It is plausible that the observed delay in rostral as opposed to caudal decrease of [O_2Hb] in the positive-mood group as well as the increase in the rostral area in the negative-mood group may indicate a shift of blood flow from the caudal to the rostral area. A drop in [O_2Hb] would first become apparent in the caudal regions. A strong rostral activation was directly reflected by an increase in [O_2Hb]. Our observations thus imply a shift to rostral emotion-processing systems and a deactivation of the motor cortex. The rostral activation most likely involves the prefrontal cortex (Matsunaga et al., 2009), and thus a cognitive processing of the emotional reaction (Panksepp, 2003).

The amplitudes of the [O_2Hb] changes were higher for sheep in the negative-mood group than for those in the positive-mood group. Assuming that the amplitude correlates with the intensity of the individuals' perception, the emotional impact of the positive stimulus (grooming) was more pronounced in sheep in a negative mood. The positive stimulus contrasted more with the negative mood, and this disparity may explain the stronger brain response. This pattern was corroborated in that sheep in the negative-mood group showed a stronger decrease in the number of ear-posture changes and the proportion of asymmetric ears compared to sheep in the positive-mood group (Reefmann, Muehlemann, Wechsler & Gygax, unpublished results) which also indicate that sheep in the negative mood group experienced grooming as more positive in contrast to the sheep in the positive mood group (Reefmann et al., 2009a). The pattern is unexpected, since rats in a negative mood react with more restraint in operant conditioning procedures (Burman et al., 2008; Harding et al., 2004). It would seem that the interaction of mood and short-term emotions in our study differs from that of the operant approach. The operant approach is more demanding, since an ambiguous stimulus must be cognitively appraised (Panksepp, 2003), while brain responses to emotionally valenced stimuli might be more direct. The approach presented by us here should therefore be extended to situations with negative emotional stimuli to enable us to investigate whether such situations

can be mitigated by inducing a general positive-mood state in animals (Barak, 2006; Fredrickson, 2001).

In our study, we induced different mood states by changes in the housing conditions. In future studies it would be advisable to have an additional and independent test for mood by e.g. investigating the cognitive bias of the animals involved (e.g. Mendl et al., 2009). Nevertheless, we are confident that our changes in housing conditions have induced differential mood states in accordance to previous experiments using a diversity of species (Bateson and Matheson, 2007; Burman et al., 2009; Doyle et al., 2010; Harding et al., 2004; Matheson et al., 2008). Similarly and due to their subjectivity, we can only indirectly assess emotions, i.e. by the use of indicator variables. Whereas such physiological and behavioural variables are quite well established for negative experiences (e.g. Forkman et al., 2007) the study of indicators for positive experiences has just begun (e.g. Boissy et al., 2007; Reefmann et al., 2009a,b,c). It is currently unknown how well these variables indeed reflect emotional experiences or whether they just depend on the activation and deactivation of stress related mechanisms and how closely stress and emotional experiences coincide. We think that the use of brain activity as an indicator for emotional experiences is promising because subjective experiences are generated and processed by the brain and thus the activity in the relevant areas might more closely indicate emotions than other physiological and behavioural indicators. These issues will become clearer if experiments similar to ours will be repeated using other positive and negative stimuli and the generality of the response in different indicator variables can be assessed.

Conclusions

Negative mood seemed to intensify the reaction of sheep to the emotionally positive event of being groomed. This contradicts the common assumption that negative mood generally taints reactions to emotional stimuli (Grippo and Johnson, 2009). This is the first study measuring affective states in the brain of unrestrained and freely moving animals. fNIRS has the potential to non-invasively measure brain activity in response to emotional or other cognitive processes in freely moving subjects.

Statement of compliance with legal requirements

The study was approved and licensed by the cantonal office (Frauenfeld, Thurgau, Switzerland, F2/06). The housing conditions of the sheep were above the Swiss minimum legal standards, even for the 'negative mood' treatment.

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