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BRAIN FUNCTIONAL NEAR INFRARED SPECTROSCOPY IN HUMAN INFANTS

CEREBRAL CORTICAL HAEMODYNAMICS COUPLED TO NEURONAL ACTIVATION IN RESPONSE TO SENSORY STIMULATION

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Thesis for doctoral degree (Ph.D)

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Cover: "**The Infrared Helix**". The image was captured by the NASA's Spitzer Space Telescope (telescope's infrared array camera), California Institute of Technology.

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The human newborn infant is able to perceive a myriad of different sensory stimuli. The "hard problem" is now to define how and to what extent newborn infants mature their ability to perceive and become aware of the surrounding world after birth. How much can be retained in their brain and influence future developmental junctures.

This thesis is dedicated to Jan Winberg, Professor Emeritus of Paediatrics at the Karolinska Institute, whom I had the fortune and honour to work with at the beginning of my PhD studies.

ABSTRACT

The assessment of cortical activation in the neonatal brain is crucial in the study of brain development, as it provides precious information for how the newborn infant processes external or internal stimuli. Thus far functional studies of neonates aimed to assess cortical responses to certain external stimuli are very few, due to the lack of suitable techniques to monitor brain activity of the newborn. Near Infrared Spectroscopy (NIRS) has been found to be suitable for functional studies of the infant brain. By this method haemodynamic changes coupled to cortical activity can be monitored.

The overall aim of the research is to assess how the brain is processing sensory stimuli (pleasant and unpleasant) in infants using a non-invasive technique such as NIRS.

Studies of smell perception (studies 1 & 2)

Olfaction was mainly used as the paradigm in these studies. Smelling is essential for neonatal behavioural adaptation in many mammals, including humans.

Methods **Study 1** Twenty-three healthy, full-term newborn infants were included in the study at a postnatal age between 6 hours and 192 hours. As odorant sources we used (i) the own mother's colostrum; (ii) vanilla essence; (iii) distilled water as a negative control. The NIRS optodes were placed over left orbito-frontal gyrus of the frontal lobe. **Study 2** Twenty preterm newborn infants in stable condition at testing were studied. As odorant sources a disinfectant solution containing benzalconio chlorate (0.25%), ethylic alcohol (66.29%), excipients such as lemon oil, acetone, iso-propilic alcohol, camphor and a detergent containing dipropylene glycol methyl ether, water and mineral essences were used. The NIRS optodes were placed bilaterally over left orbito-frontal gyrus of the frontal lobe.

Main findings and conclusion **Study 1** The main finding of this study was that the NIRS technique can be used in the neonatal period to record activity in the orbito-frontal cortex - as mirrored by changes in blood circulation - during exposure to biologically meaningful as well as artificial odors, colostrum and vanilla, respectively. The magnitude of the response in the illuminated region during colostrum exposure was inversely related to postnatal age. **Study 2** This study demonstrated that the odors of solutions commonly used in NICUs might elicit a decrease in blood oxygenation in an area likely to include the orbito-frontal olfactory area. These haemodynamic changes are likely to be the result of a dynamic, physiological regulation of regional CBF based upon the olfactory- and trigeminus-related areas of the brain.

Study of pain perception (study 3)

Supraspinal pain processing of pain in neonates and preemies is still poorly understood.

Methods Forty preterm neonates at 28–36 weeks of gestation and mean postnatal age of 30.7 h were studied following standardized tactile (skin disinfection) and painful (venipuncture) stimuli. Changes in regional cerebral haemodynamics were monitored by near infrared spectroscopy (NIRS) over both somatosensory cortices in 29 newborns, and over the contralateral somatosensory and occipital areas in 11 newborns.

Main findings and conclusion Painful and tactile stimuli elicit specific haemodynamic responses in the somatosensory cortex, implying conscious sensory perception in preterm neonates. Somatosensory cortical activation occurs bilaterally following unilateral stimulation and these changes are more pronounced in male neonates and preterm neonates at lower gestational ages.

Study of auditory perception (study 4)

The aim of the study was to assess differences in activation pattern in response to auditory stimuli before and after the induction of anaesthesia with sevoflurane.

Methods The "Water music", by Handel, was presented to 7 infants aged between 18 and 22 months. NIRS was recorded in different conditions: baseline with no music when the child was asleep, during the music with the child sleeping before anaesthesia, and during the music when the child was in deep anaesthesia.

Main findings and conclusion We observed pronounced bilateral [HbO_2] increase during sleeping, similar to that previously reported in waken subjects and suggesting that the infant perceives the auditory stimulus and likely processes it. When the infant is anaesthetised and many neuronal circuits are not functioning, the auditory stimulus can still be perceived as suggested by the increase of HbO_2 in one hemisphere, but processing might be altered.

Overall conclusion

These studies and other studies that have been carried out in parallel by other groups demonstrate that NIRS is a suitable technique to assess cortical activation in response to varying forms of sensory stimulation in human infants. The technique is likely to play an important role in providing new insights into the ontogeny of cortical function, as well as possibly providing a sensitive means for the early detection of perinatal cortical impairment.

Keywords: Near infrared spectroscopy, newborn, infant, brain, sensory stimulation, cortical activation.

ORIGINAL PAPERS

This thesis is based on the following original papaers that will be referred to by their Roman numerals:

I. Bartocci Marco, Winberg Jan, Ruggiero Carmelina, Bergqvist L Lena, Serra Giovanni, Lagercrantz Hugo. Activation of olfactory cortex in newborn infants after odor stimulation: a functional near-infrared spectroscopy study. Pediatric Research, 2000, 48:18-23.

II. Bartocci Marco, Winberg Jan, Papendieck Gesa, Mustica Teresa, Serra Giovanni, Lagercrantz Hugo. Cerebral haemodynamic response to unpleasant odors in the preterm newborn measured by near-infrared spectroscopy. Pediatric Research, 2001, 50: 324-30.

III. Marco Bartocci, Lena L. Bergqvist, Hugo Lagercrantz, K.J.S. Anand. Pain activates cortical areas in the preterm newborn brain. Pain, 2006, 122: 109-117.

IV. Marco Bartocci, Dick Sjögren, Fredrik Ullén, Hugo Lagercrantz. Auditory awareness of music during sleep and anaesthesia. A near infrared spectroscopy study in infants. Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

a.u.	arbitrary units
BOLD	blood oxygen level dependent
CBF	cerebral blood flow
CBFV	cerebral blood flow velocity
CBV	cerebral blood volume
CMR	cerebral metabolic rate
Cyt_{ox}	cytochrome oxidase
DPF	differential path length factor
$etCO_2$	end tidal volume CO_2
fMRI	functional magnetic resonance imaging
Hb H	deoxygenated haemoglobin
Hb O_2	oxygenated haemoglobin
Hb tot	total haemoglobin (= HbO_2 + HbH)
HR	heart rate
NICU	neonatal intensive care unit
NIR	near infrared
NIRS	near infrared spectroscopy
PET	positron emission tomography
r-CBF	regional cerebral blood flow
RR	respiratory rate
saO_2	peripheral oxygen saturation

1 INTRODUCTION

The main goal of modern neonatology is to promote brain wellbeing, by guaranteeing its growth and development and avoiding neurological sequelae. To predict as early as possible the neuro-developmental outcome of the sick baby would be important in focusing the limited rehabilitation resources optimally, as well as in supporting parents of at-risk newborns. The new frontiers of technology allow the physician to address the problem not merely from a pure diagnostic angle, limited to establishing the presence or not of certain damage but also from a functional and more dynamic viewpoint (figure 1.1). This kind of approach has developed enormously during the last decade and is based on an apparently simple, but in reality still unclear developmental process of cause-effect. This is what has been named as "functional approach", meaning the assessment of brain response, and more extensively of the whole nervous system, to the exposure to a stimulus, either sensory or motor or cognitive. Brain functional studies, which until recently have mainly been reserved for adults, are becoming an emerging area of paediatric and neonatal research.

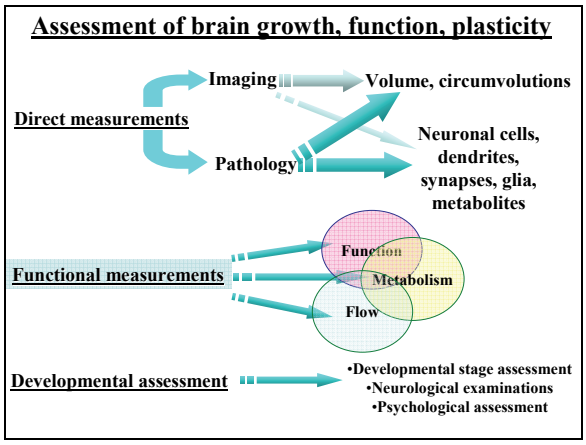
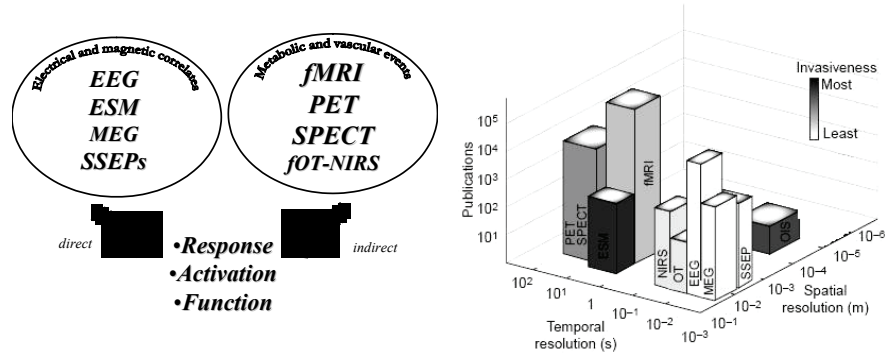


Figure 1.1 *Different diagnostic and experimental approaches to brain function, plasticity and maturation*

One of the main complications in functional studies of the developing brain is that the degree of response has to be related and interpreted according to the degree of maturation (sensorial as well as anatomical and cognitive), which is often not exactly known. From a constructionist point of view it is the very sensorial experience able to affect and modulate brain development. This experience may already start *in utero* according to the latest scientific reports (Wadhwa 2005; Wadhwa et al. 2001), as well as to ancient popular beliefs. This type of assessment is even more difficult when considering the brain of infants born prematurely or with some kind of damage (Aylward et al. 2005).

To explore the complexity of brain cortical responses to a given stimulation many techniques are currently available (figure 1.2). Positron emission tomography (PET) and functional magnetic resonance imaging (fMRI) are surely the most known. Besides these there are several emerging optical methods, among which Near Infrared Spectroscopy (NIRS) is the one that we have used in our investigations.



a) b)

Figure 1.2 *a) Different approaches to cortical activation. b) Comparison of different functional human brain mapping techniques. Spatial resolution, temporal resolution and number of published peer-reviewed articles are indicated. EEG, electroencephalography; ESM, electrical stimulation mapping; fMRI, functional magnetic resonance imaging; MEG, magnetoencephalography; OT, optical techniques; NIRS, near infrared spectroscopy; PET, positron emission tomography; SPECT, single photon emission computed tomography; SSEPs, sensory evoked potentials Modified from (Pouratian et al. 2003).*

This thesis will present data from functional studies of newborns and young infants who have been monitored using NIRS during exposure to a given sensory stimulation.

The overall aim of these studies was to give new insights into brain activation-related haemodynamic changes by using a non-invasive technique such as NIRS and to correlate these findings to sensory and behavioural responses of the maturing brain.

2 OPTICAL METHODS FOR FUNCTIONAL STUDIES

When entering biological tissues photons are exposed to different interactions with all the tissue components. At the heart of the optical methods absorption and scattering are the most important processes that have to be taken into account (Cohen et al. 1970; Cohen et al. 1968; Hill and Keynes 1949), although there are other types of interactions which will not be discussed in this thesis. During absorption tissues can transform radiant energy into different forms of energy, inducing for example fluorescence or phosphorescence. Scattering is the random change in direction of the particles (photons) due to collision with particles of the medium traversed (tissue). Scattering may be at *unchanged frequency* if the medium is stationary or accompanied by a *Doppler shift* due to interaction with moving particles of the tissue (e.g. blood cells) (Villringer and Chance 1997).

2.1 THE INTRINSIC OPTICAL SIGNAL

Light which has interacted with brain tissue contains information about the brain's functional state (Grinvald et al. 1986a; Jöbsis 1977). This information can be obtained by assessing and interpreting two different phenomena which derive from two different types of optical signal following functional brain activation. The first, which in this thesis will be only briefly mentioned, is the *fast signal* (Gratton et al. 1995; Wolf et al. 2002a). It appears in the range of milliseconds. It is presumably due to changes in the scattering properties of the neuronal membrane which occur together with the electrical depolarization, cell swelling and increased heat production. The *fast signal* is an intrinsic optical signal and allows mapping of functional organization with an excellent spatial resolution (DeSoto et al. 2001; Gratton et al. 1995; Gratton and Fabiani 2001; Gratton et al. 1997a; Gratton et al. 1997b; Rinne et al. 1999; Steinbrink et al. 2000; Stepnoski et al. 1991; Tasaki 1999; Toronov et al. 2000) (figure 2.1).

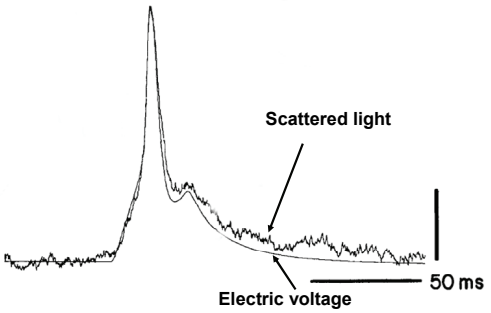


Figure 2.1 *Fast neuronal signal. Light scattering "follows" electric voltage changes during neuronal activity.(Stepnoski et al. 1991)*

Light scattering changes associated with neural activity are at the basis of the intrinsic signal, which has been recently called "event-related optical signal" (EROS). EROS is grounded on the fact that the light scattering properties of neural tissue change when the tissue is active (Gratton and Fabiani 2001). This phenomenon has been

known for more than 50 years (Hill and Keynes 1949), and has been used to study the behaviour of large numbers of individual neurons in parallel. The intrinsic optical properties of the brain tissue have been impressively demonstrated by studying the reflected light of exposed brain cortex. By measuring the optical intrinsic signals in vivo, high resolution functional information about the architecture of the cat and mouse visual cortex has been obtained (Grinvald et al. 1991; Grinvald et al. 1986a; Grinvald et al. 1986b; Mrsic-Flogel et al. 2003; Schuett et al. 2002) and visualisation of the functional anatomy of the rat whiskers barel cortex has been achieved (Grinvald et al. 1986a; Masino et al. 1993; Narayan et al. 1994).

2.2 THE HAEMODYNAMIC SIGNAL

The haemodynamic signal is based on the absorption of NIR light by haemoglobin that has different absorbance spectra depending on its oxygenation status. Thus it is of crucial importance to understand the mechanisms underlying the relationship between neuronal activity and cerebral haemodynamic changes in order to interpret the data collected when using optical techniques that rely on this kind of signal.

2.2.1 Coupling

The complex relationship between blood flow, metabolism and neuronal activity is not yet clear, although during the last decade there has been a remarkable improvement in the knowledge of this area. This panorama is even more complicated when exploring the newborn brain, and especially the premature. In this case it is difficult to separate the functional ramifications of the brain insult (e.g. IVH, PVL) that often affect the preterm brain from the developmental significance of the alteration in neurogenesis, migration, myelination cell death, synaptogenesis and vascular growth, which may even occur in the absence of major insults (2002; Weindling 2002; Weindling and Kissack 2001). Despite that the relationship between cerebral blood flow and energy metabolism in the newborn has been studied quite extensively, most of the information is reported during either resting conditions or pathological circumstances (Boylan et al. 2000; Greisen 2005; 1997; Jayasinghe et al. 2003; Kissack et al. 2005; Pryds et al. 2005). There is thus little information in the literature regarding the mechanisms underlying haemodynamic changes associated with cortical activity in the newborn term and preterm brain.

For more than a decade it has been known that blood vessels are linked to neuronal activity through the so-called mechanism of neurovascular coupling, also known as functional hyperemia (Harder et al. 1998). Neurovascular coupling is strictly correlated to so-called neurometabolic coupling, and together guarantee to maintain an appropriate energy flow into the neural tissue during conditions of increased neuronal activity. One of the first metabolic consequences coupled to neuronal activation is trigger-increased glucose consumption and glucose demand. This metabolic response is amplified by an increased transport of glucose through the blood-brain barrier – neurobarrier coupling (figure 2.2) (Leybaert 2005; Silver and Erecinska 1994). Neurovascular coupling or functional hyperemia hence serves to supply blood and nutrients to the local needs of a given area of the brain that is active by regulating blood flow (Harder et al. 1998; Iadecola 1992; Kuschinsky 1997; Lou et al. 1987; Villringer and Dirnagl 1995; Wahl and Schilling 1993).

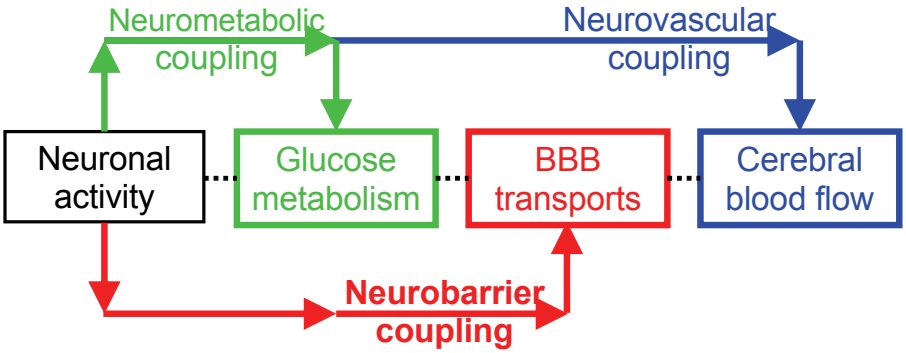


Figure 2.2 *Neuronal activity can trigger various responses. BBB, blood-brain barrier. (From Leybaert, 2005, with permission)*

From studies of adults it emerges that despite arterioles and small arteries containing <5% of the blood volume in the brain parenchyma, they control most of the resistance and therefore blood flow at a local level. Changes in blood volume and flow in parallel with neuronal activation should be mostly attributed to the activity and changes of these vessels (Villringer and Dirnagl 1995). Cerebral activation by sensory stimulation has been demonstrated to be accompanied by adjustments of vascular resistance and blood flow in the activated region. Studies by the group of Winn and co-workers have also revealed that pial arterioles supplying the somatosensory cortex dilate in response to contralateral nerve stimulation, although the underlying mechanism of such vasodilation remains unclear (Ngai et al. 1988; Ngai and Winn 2002). A main role in arterial vasodilation seems to be played by vasoactive metabolites released during cortical synaptic activity including adenosine, H, K, and nitric oxide (Berne et al. 1981; Dietrich et al. 1996; Fujii et al. 1992; Winn et al. 1981). At birth the sensitivity to the so-called nitro-vasodilators, such as nitroglycerine and nitroprusside, as well as to adenosine is fully functional, although data regarding the preterm neonate are lacking (Åden 2001; Fredholm and Svenningsson 2003).

In terms of the latency of the vascular response in response to sensory stimulation, this is dependent on the cortical area which is involved in processing. As for the mere vascular latency (time from *in situ* neuronal cortical activation and haemodynamic response), studies of the visual cortex from Malonek and Grinvald have shown that it occurs within the first 3 seconds and is highly localized to individual cortical columns. However, there is a later phase of the vascular response that is less localized, spreading over distances of 3 to 5 mm (Malonek and Grinvald 1996).

The vessels that supply the upper layers of the cortex seem to be the first to react to neuronal activity. This has been revealed through measurement of the CBF increases from the upper cortical layers to the lower layers (Nielsen et al. 2000). When considering vascular growth, around the 26th week of gestation human cerebral blood vessels begin to grow rapidly, increasing in both density and diameter and reaching a peak in number and size at around 35 weeks. After week 35 their number and size change relatively little, but the structure and their function change dramatically. Extracellular water already begins to be reabsorbed during the first days post-partum and connective tissue changes occur. Gradually the arterial wall increases its thickness through the synthesis and deposition of collagen and elastin. With respect to the haemodynamic response in the developing brain (i.e. the preterm brain between the 24th

and 32nd week) and how it evolves along brain maturation, very little is known and very few studies have been conducted. These factors should be taken into account when conducting functional studies in premature newborn infants.

The brain processing activity is dependent on neurotransmitters. Glutamate is the main neurotransmitter in the brain causing intracellular [Ca^{++}] oscillation in the astrocytes (via group-I metabotropic receptors, mGluRs); these oscillations would in turn regulate the release of vasoactive agents which induce changes in diameter and tone of the arterioles, and thus the blood supply would be adjusted (Zonta et al. 2003).

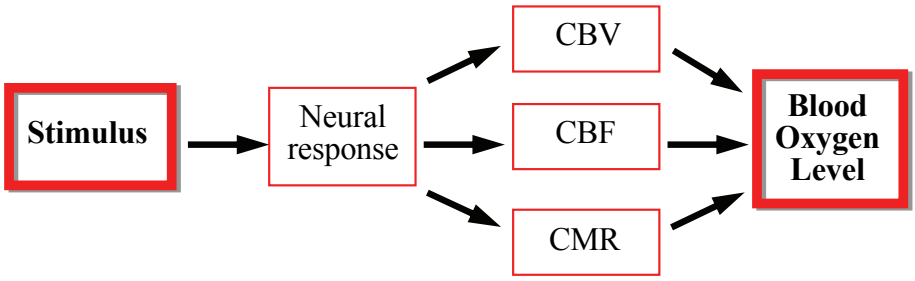


Figure 2.3 *Haemodynamic ramification following a given stimulus that induces a neuronal response. CBV, cerebral blood volume; CBF, cerebral blood flow; CMR, cerebral metabolic rate.*

The combination of neurovascular coupling and metabolic coupling has also to guarantee the appropriate wash-out of the lactate that is produced through glycolysis (Kasischke et al. 2004; Magistretti 2006; Serres et al. 2004). According to the studies by Grinvald and coworkers a short and temporary drop in brain tissue oxygenation may occur during the first 1-3 s of activation (Frostig et al. 1990; Grinvald et al. 1991). The drastic and fairly quick adaptations suggesting a causal relationship between the metabolic demands of local neuronal activity have been recently described with respect to both the density of the capillary network and the placement of the control structures. Such relationships will affect the ultimate spatial resolution obtainable by hemodynamic-based functional brain imaging studies. These relationships will also affect quantitative comparisons of activity levels in different areas of cortex (Harrison et al. 2002). (Figure 2.4).

These findings surely add new insights into the old theory of "capillary recruitment" that was based on the opening of unperfused capillaries which are recruited to maintain adequate glucose transport into neurons (Krogh 1924; Shockley and LaManna 1988; Skov and Pryds 1992).

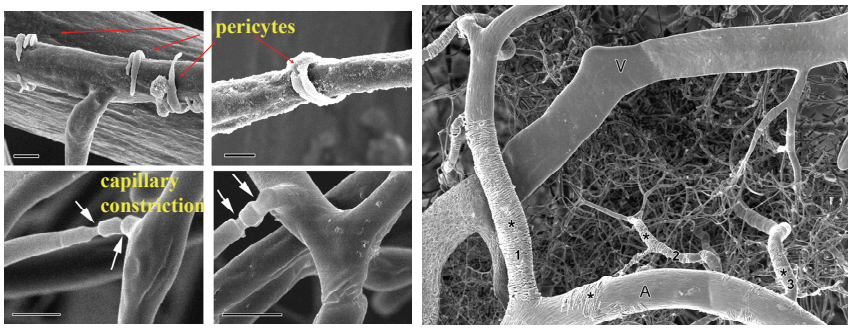


Figure 2.4 *Dynamic and extremely rapid changes in the capillary bed of the cortex following acoustic stimulation in rats. By regulating their diameter mainly through the action of the pericytes and by capillary constriction (arrows) the vascular bed can centralize the blood to the area which is activated. Artery (A) and vein (V) are depictid. Pericytes are present on the artery surface. (From Harrison et al. 2002, with permission).*

When interpreting data of both fMRI (that relies on the BOLD signal) and NIRS, it is important to keep in mind that changes in blood flow may derive from either changes in blood velocity or in blood volume. Villringer and Dirnagl have proposed a quite simple but useful scheme of alternatives that can be associated with increasing neuronal activity and coupled blood flow. If blood flow increases as a result of an increase in blood flow velocity and assuming that the oxygen consumption rate is constant, the vessel content of deoxy-Hb will decrease and that of oxy-Hb will increase. If blood volume increases in the capillary bed due to an increase in blood volume, probably as a result of the action of perycites and vascular diameter re-adjustment (Harrison et al. 2002) and assuming constant oxygen consumption rate, the content in the vessels of deoxy-Hb will remain unchanged while that of oxy-Hb will increase (figure 2.5).

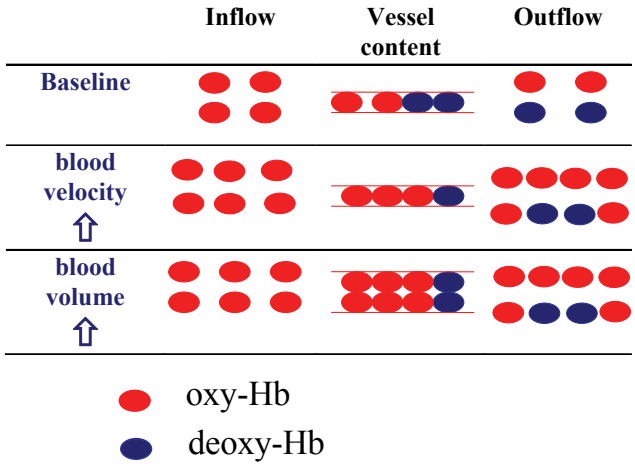


Figure 2.5 *Oxygenation changes in the microcirculation that may accompany neuronal activation in a given cortical area. Red circles represent oxygenated erythrocytes; blue circles represents deoxygenated erythrocytes(Villringer and Dirnagl 1995).*

2.3 NEAR INFRARED SPECTROSCOPY

Near infrared spectroscopy (NIRS) is an optical technique based on the properties of near infrared light to diffuse throughout biological tissue. NIRS physical principles have been previously extensively reviewed in detail. Although light has been used in chemistry and medicine since long time, probably the first to used light for diagnostic purposes were Cutler and colleagues at the beginning of the 20th for the diagnosis of breast cancer by transillumination (Cutler 1929). Butler and Norris in the 1960s were the first to specifically study the near infrared spectrum and its diffusion in plant and human tissue (Butler and Norris 1960).

The principles of near infrared light for assessing tissue oxygenation were introduced by Jöbsis about twenty-five years ago. During the last two decades several studies have addressed the feasibility of this method in monitoring the changes of cerebral oxygenation in newborns at risk of brain damage. Particularly during the last 8 years there has been am enormous increase in applying NIRS and optical imaging in the study of functional development of normal and abnormal neonatal and paediatric brain. Optical techniques such as NIRS have several advantages: they can image both neuronal and haemodynamic activity, and, in some cases, anatomical details. They are safe, can be adapted to a number of experimental and practical conditions, are portable and are easy to combine with other techniques, such as electrophysiological, neuromagnetic, and MR imaging methods. For these reasons NIRS is well suited very well to use with newborn infants and small children.

2.3.1 NIRS principles

This technique depends on the absorption of light in the near infrared spectrum (around 700 e 1000 nm) as it irradiates a certain region of the head. Light in the visible region of the spectrum, (with wavelength between 450 and 700 nm) is strongly attenuated in tissue and therefore fails to penetrate more than approximately 1cm of tissue. However, at NIR wavelength the absorption of the light is significantly lower than at visible wavelengths, and with a very sensitive instrumentation it is possible to detect light which has traversed up to 8 cm of tissue (figure 2.6). The NIR wavelength allows light to penetrate into the tissue and to be absorbed by the natural chromophores: oxyhaemoglobin [Hb O_2], deoxyhaemoglobin [Hb H] and cytochrome oxidase in the redox status [Cyt_{ox}]. A chromophore is a substance that absorbs light at a given wavelength.

NIRS' precursor is oxymetry. Oxymetry relies on the fact that haemoglobin's optical properties change depending on its oxygenation status.

$$saO_2 = HbO_2/(HbH + HbO_2) \times 100\%$$

To understand the basic of spectroscopy we can refer to the so-called "cuvette method". In this simplified example a light source illuminates (I_0) a homogeneous dye (c). A single photon interacts with the substance c, which is not changing with time. The absorption (attenuated energy, A) of light measured at the other side of the cuvette, after the photons have travelled a constant distance d within the substance c, can be calculated using the Beer Lambert law (figure 2.7).

The Beer Lambert law states that the attenuation of an absorbing compound dissolved in a non-absorbent solvent is directly proportional to the product of the concentration of the compound and the optical path length factor.

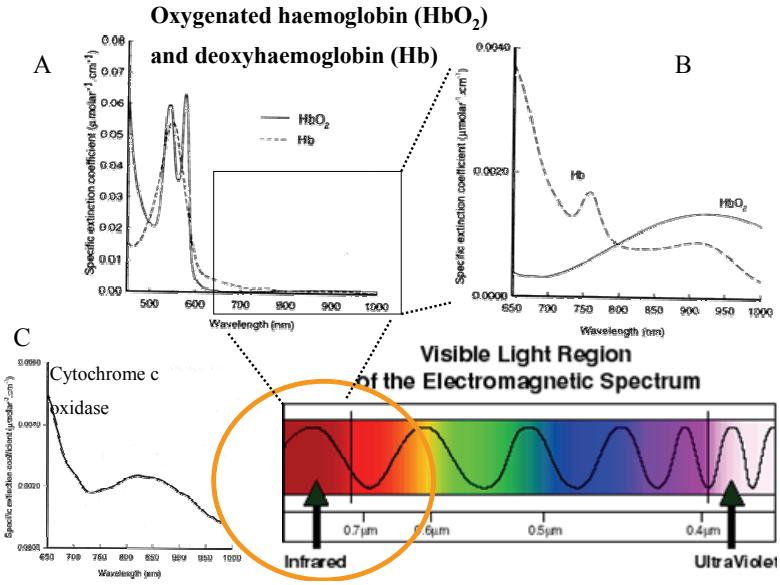
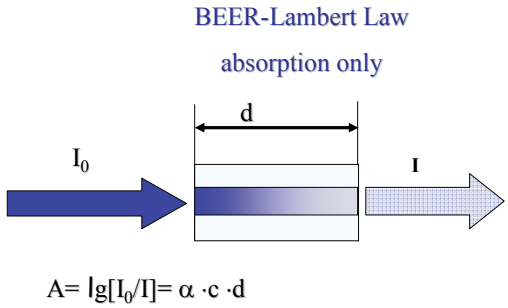


Figure 2.6 *The absorption spectra of oxy- and deoxy-haemoglobin in the near infrared wavelength range. Panel A shows the whole spectrum of visible and invisible light. Panel B shows the NIR light spectrum, between around 650 and 1000 nm of wavelength. In panel C the absorption spectrum of another chromophore – cytochrome C oxidase – is depicted.*



A = Attenuation α = extinction coefficient

d = distance c= concentration of absorbing dye

I_0 = light **in** I = light **out**

Figure 2.7 *Basic arrangement for in vitro spectroscopy. The "cuvette method". In this case only absorption is considered.*

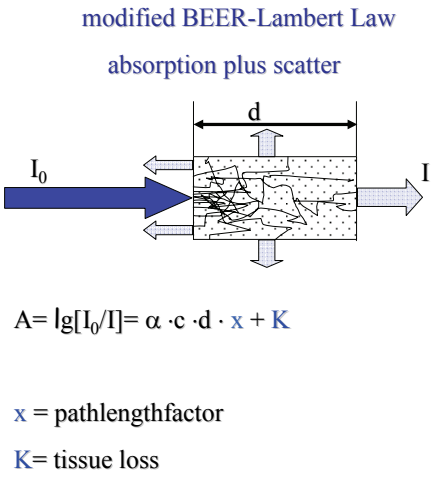


Figure 2.8 *The figure depicts a modified "cuvette method" (see the text for the description)*

Figure 2.8 presents the simplified "cuvette method" in which photons propagate through a more complex substance than a simple, homogeneous medium such as that in figure 2.7. In this model photons do not only travel through the dye from the source straight to the receiver, but are also scattered and lost. This example is an attempt to reproduce the propagation of light in living tissue. Here the model takes into account the actual distance that the photons travel, which is no longer the distance d, but is modified by a path length factor (x) that is a function of an additional factor, i.e. scattering. Undoubtedly the patterns of scatter can be very different, and they are significantly influenced by the particle size, distribution and concentration. Concerning NIR light, photons can travel through the tissue and here undergo a different kind of scattering: 1) some of photons travel almost "unscattered" and therefore follow the shortest path from the light source to the receiver; 2) a fraction of photons is scattered and despite that their course is changed, the direction towards their receiver is maintained. These photons reach the receiver but their path is longer then the mere distance emitter-receiver, altered by a factor that is specific for the examined tissue (path length factor, table 2.1).

Figure 2.9 presents how to calculate changes in the attenuation and therefore in the concentration of chromophores. Assuming that the medium is unchanged (K and x are constant), by subtracting measurements obtained at a certain time-point (t=2) and at a previous time-point (t=1), it is possible to calculate changes in the concentration of the chromophore.

2.3.1.1 Measurement in tissue

Figure 2.8 gets fairly close to the kind of propagation (absorption and scattering, above all) and of how the attenuation is calculated in a living tissue. Nevertheless in living tissue factor d (that in practice is the distance between the emitter and the receiver) has to be corrected according to a differential path length factor (DPF) in order to estimate a differential path length, i.e. the actual distance that photons are travelling in the tissue before finally reaching the recipient optode. While individual photon path lengths cannot be measured, several groups have developed methods to

calculate the mean optical path length in different *in vivo* experimental setups. The "time-of-flight" method was adopted by Delpy and coworkers in 1988 to measure the DPF in cerebral tissue (Delpy et al. 1988). By measuring the mean transit time and using the speed of light in a vacuum, the mean optical path length can be calculated. Other less expensive methods have also been developed. One can either measure the phase delay of intensity modulated light (Lakowicz et al. 1992), or compare absorption changes against absorption for water at a known concentration. These methods made it possible to calculate DPF mean values for different tissues such as neonatal brain, adult brain, and also forearm, and calf muscles (Cope et al. 1988; Cope et al. 1989; Duncan et al. 1996; Duncan et al. 1995; van der Zee et al. 1992).

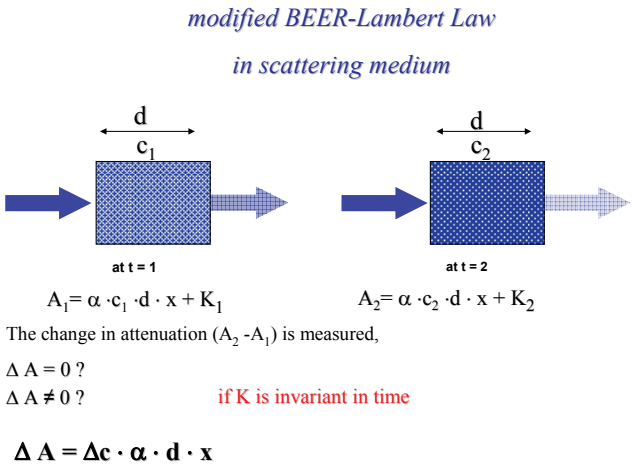


Figure 2.9 *Changes in the attenuation and therefore in the concentration of chromophores.*

The factor K, which mainly depends on the geometry of the sensor and the surface where it lies, as well as on tissue scattering, is a related to the loss of photons (due to scattering). It is significantly different depending on the subject and the part of the brain that is illuminated by the NIR light. As it is extremely complicated to calculate a value for K, during any single measurement one has to assume that this term is relatively constant. To satisfy this condition optodes cannot be moved during the course of the experiment (figure 2.9)(Cope and Delpy 1988).

There is a multitude of studies carried out during the last 20 years, which calculated the different path length factors depending on at which age brain tissue was investigated. It transpires that the newborn brain has a lower DPF then the adult, but there is no significance difference between the term and the preterm neonate. This fact indicates that the trajectory of photons travelling from the emitter to the receiver optode is shorter in the newborn brain than in the adult. It should be mentioned that all these studies take into account one or two wavelengths when calculating the DPF, and this might be a limitation as NIR light is generated at four different path lengths in some of the commercially available devices.

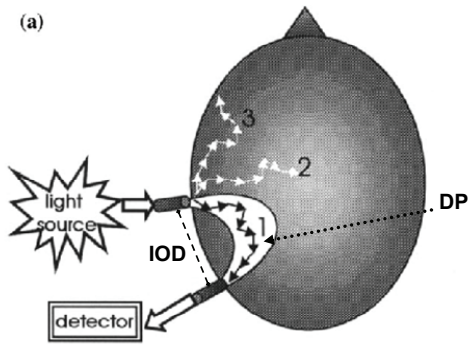


Figure 2.10 *Trajectory of photons during a hypothetical NIRS test. Photon 1 is scattered and reaches the detector. Photon 2 is absorbed after a number of scattering events. Photon 3 leaves the head without being detected. DP indicates the differential path length (in practice the product of inter-optode space – IOD – and DPF).*

2.3.2 Instrument

Many different devices are commercially available. Most of them can count on 2 channels, although there is a growing interest in multichannel devices.

2.3.3 Laser diodes and safety

In our studies we have been using a NIRO 300 apparatus (Hamamatsu Photonics, Japan). The NIRO 300 is equipped with 2 channels. The NIRO-300 Monitor is a quantitative oxygenation monitor for cerebral tissue and muscle tissue; using the spatial resolved spectroscopy method (SRS) it can provide quantitative data for the tissue oxygenation index (TOI) in % and for the tissue haemoglobin index (THI) in a.u., the latter can also be displayed as normalized THI (nTHI) which stands for the percentage change of total haemoglobin. This method will be not discussed further in details in this thesis as it was just marginally used and analysed in our studies. NIRO 300 uses the Beer-Lambert method (see above) and provides at the same time the relative changes of [Hb O_2], [Hb H] and [Hb tot] in micromoles. This simultaneous use of SRS and Beer-Lambert data thus delivers a detailed description of the haemodynamic situation. As it is a two channel system bi-hemispheric data can be obtained as used in studies 2, 3 and 4. Monolateral recording from two different areas in the same hemisphere is also possible (study 3). Due to online checking of the measurement conditions plus the simultaneous use of SRS and the Beer-Lambert method the system allows a redundant check and all data are highly reliable. The NIRO 300 can measure up to a speed of 1/6 sec per data. Each channel is composed of an emitter and a receiver probe. The emitter carries the infrared light that is generated by four pulsed laser diodes, which produce light at wavelengths 775, 810, 850, 910 nm. The pulse frequency of each diode is approximately 2 kHz, each pulse having a duration of 100 nsec. The average output power from the emission probe is about 1 mW, and the irradiation intensity to the patient is included in the Class 1 (IEC 825: 1993). The typical power density at the patient attachment has been considered to be less than 0.05 mW/mm^2.

2.3.4 Optodes

The NIR light is produced by the laser diodes and carried to-and-from a spectrometer via an optical fibre (a bundle of glass fibres). NIRO 300 has two optodes

per couple, an *emission probe* (emitter) (fibre: 1.5 mm(Ø) fibre bundle; head: 8 mm(Ø)x 4 mm (H), cable: 3 mm(Ø)x 2m (L) and a detection probe (receiver) 20 mm(Ø)x 8 mm (H), cable: 3 mm(Ø)x 2m (L). The positioning of the optodes on the skin of the baby is completely painless and does not provoke any tissue alteration. (Figure 2.11).

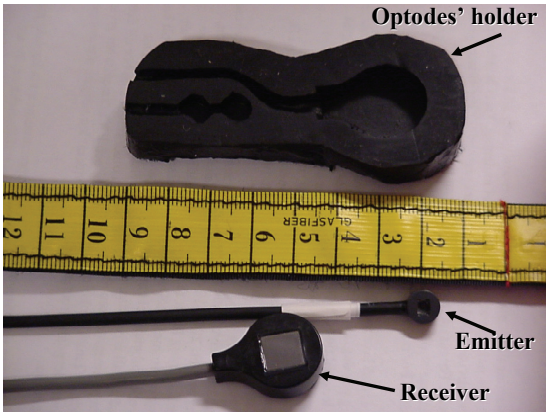


Figure 2.11 *NIRS detail: the emitting and receiving probes are shown. Together they compose one channel. The figure also depicts a type of rubber holder which maintains the distance between the optodes at a constant length of either 5 or 4 cm.*

2.3.5 Sample time

The system is able of sampling data every 0.5, 1, 2, 5, 10, 30, or 60 seconds. Recently a fast sampling mode has become available which allows 6 data collected per second. The device samples every 500 msec and averages the data into the sampling time given above. An event marker allows specific points during the measurement period to be recorded. In our studies we have mainly used a sampling time of 1 or 2 Hz.

2.3.6 Position

The optodes can be placed in a special dark, semi-rigid rubber holder to avoid interference from external light (figure 2.11). The inter-optodes distance of 4 or 5 cm is prefixed and remains stable during the entire recording. Two couples of optodes can be used simultaneously to monitor two different regions of the body. Theoretically when the optodes are positioned at shorter distances between, light penetrates less deeply into the tissue. This should be the aim when conducting functional studies and thus seeking to only explore the cortical response. With increase of in functional studies and in the awareness of the limited spatial resolution of NIRS, the need emerges to standardize with certain precision the position of the optodes. Our research group as well as other groups that conduct research in this area, use system the international 10-20 EEG system as a reference. If not using this system, the location of the optodes should accurately refer to anatomical reference points (figure 2.12).

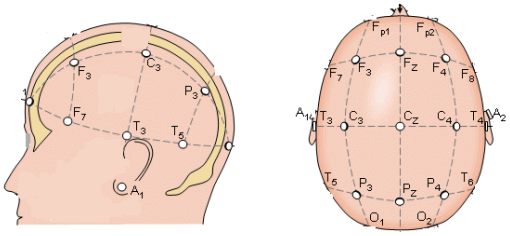


Figure 2.12 *The 10-20 international EEG system can be used as a reference for optode placement in NIRS functional studies.*

2.3.7 Differential Pathlength Factor (DPF) and units

An essential factor for the calculation of the changes in the haemoglobin and other chromophores is the estimation of the real distance which the photons have to cover. Previous experiments have calculated the time-of-flight of the photon through the tissue. In this way it has been possible to indirectly estimate quite accurately the distance inside the tissue travelled by the photons. Based on these studies a differential path length factor (DPF) has been created according to the different properties of the various corporeal districts (head, arm, etc.) or tissues (brain, muscle, etc.). In the newborn head, for example, we established a DPF of 4.2. The changes in the [Hb H], [Hb O_2], and [Cyt_{ox}] are measured in μMol/L (table 2.1).

2.4 APPLICATION OF NIR-BASED METHOD

NIR-based methods in the newborn have been applied in multiple conditions and following different procedures in both clinical and functional settings. Since 1986 about 1100 articles have been published regarding use of near infrared technique to assess tissue oxygenation in humans. About 450 of these reported experiments assessed the brain. Treatments which might have deleterious collateral effects on cerebral circulation include surfactant administration (Fahnenstich et al. 1991; Roll et al. 2000b; Skov et al. 1992), caffeine and theophilline therapy (Dani et al. 2000), mechanical ventilation (van Wijk et al. 2003), blood transfusion in NICU (Victor et al. 2006; Wardle et al. 2002), endotracheal suctioning (Kohlhauser et al. 2000; Mosca et al. 1997a), blood transfusions (Menke et al. 2004; Raj et al. 2004), nitric oxide (Takei et al. 1999), ibuprofen/ndomethacin (Mosca et al. 1999; Mosca et al. 1997b), hypothermia (Kadoi et al. 1999), surgery (Hayashida et al. 2004; Kunihara et al. 2001; Murata et al. 2003; Shin'oka et al. 2000; Wardle et al. 1998; Zaramella et al. 2006), umbilical catheter blood sampling (Roll et al. 2000a; 2006). Pathologies such as head trauma, some types of tumors, asphyxia and hypoxia, hydrocephalus, all of which need strict brain monitoring, have been reported to be well monitored using this technique, (Asgari et al. 2003; Buchvald et al. 1999; Meek et al. 1999; Saliba et al. 1997; Seelbach-Gobel 1997; Soul et al. 2004; Soul et al. 2000; Toet et al. 2006; Wyatt 1994). NIRS has also been used to calculate the relationships between cerebral haemoglobin variations, blood flow, oxygen delivery, oxygen consumption, venous saturation, and fractional oxygen extraction (OEF) in preterm newborn infants (Kissack et al. 2005; Wardle et al. 2000; Yoxall and Weindling 1998).

Subject	age	DPF	Notes	λ nm	Ref.
neonate	preterm 24 wks	4.39 ± 0.28	post-mortem < 12 d	λ 783	(Wyatt et al. 1990b)
neonate	preterm 31 wks	3.85 ± 0.57	post-mortem < 16d	λ 783	(van der Zee et al. 1992)
neonate	35 – 48 wks	4.66 ± 1.01	corrected age	λ 730	(Cooper et al. 1996)
neonate	35 – 48 wks	3.91 ± 0,75	corrected age	λ 830	(Cooper et al. 1996)
neonate	term < 16d	4.99 ± 0.45		λ 807	(Duncan et al. 1995)
infants	1 – 3 y	3.78 ± 0.31		λ 731	(Benaron et al. 1995)
infants	1 – 3 y	3.71 ± 0.30		λ 816	(Benaron et al. 1995)
adult	22 – 54 y	5.93 ± 0.42		λ 761	(van der Zee et al. 1992)
adult	21-59 y	6.26 ± 0.88		λ 807	(Duncan et al. 1995)

Table 2.1 *Different path length factors in different groups of subjects in brain tissue. λ indicates the NIR wavelength that was used for the calculation*

Concerning functional application NIRS has been initially adopted for the detection of sequential brain activation in the prefrontal cortex during mental tasks, and for mapping the human motor cortex. Hirth and co-workers applied non-invasive multi-site NIRS to assess oxygenation changes during performance of a sequential finger opposition task in five healthy human adults and determined a correlation between Hb variation and cortex activation (Hirth et al. 1997; Hirth et al. 1996). Hoshi and co-workers studied brain functional mapping using NIRS. They showed the existence of different fluctuation patterns of cerebral oxygenated [oxy-Hb] and deoxygenated haemoglobin, [deoxy-Hb] during the resting period, related to the cerebral region investigated (Hoshi et al. 1994; Hoshi and Tamura 1997).

Due to its characteristics of non-invasiveness, handiness for cot-side use, NIRS has recently been used to study brain functional activation in newborn infants. The first report in newborn is by Meek and co-workers who assessed the activation of the visual cortex after repeated light stimulation in the newborn infants, demonstrating how NIRS can detect regional blood changes under brain activation (Meek et al. 1998). During the last 5 years the assessment of the development of the newborn brain, especially when the infant is born preterm, has become a new and fascinating application field of NIR-based methods. To monitor functional and haemodynamic changes in the developing brain when exposed to sensory stimulation and to compare these findings to those observed in newborn infants born at term, would represent an enormous step forward in the understanding of the complex mechanisms regulating early learning and sensory processing. NIR-based method has been successfully used to this aim, both by our group and other in the world (Aslin and Mehler 2005; Bartocci et al. 2006b; Bartocci et al. 2001; Bartocci et al. 2000; Bortfeld et al. 2006; Chen et al. 2002; Meek et al. 1998; Sakatani et al. 1999; Taga et al. 2003a; Taga et al. 2003b; Toronov et al. 2000; Wilcox

et al. 2005; Wolf et al. 2002b). Comparisons with functional magnetic resonance imaging (fMRI) (Chen et al. 2003; Kleinschmidt et al. 1996; Punwani et al. 1997; Punwani et al. 1998; Wolf et al. 1999) and positron emission tomography (PET) (Villringer and Chance 1997) and electroencephalogram (EEG) has been performed and there is fairly good concordance in the results.

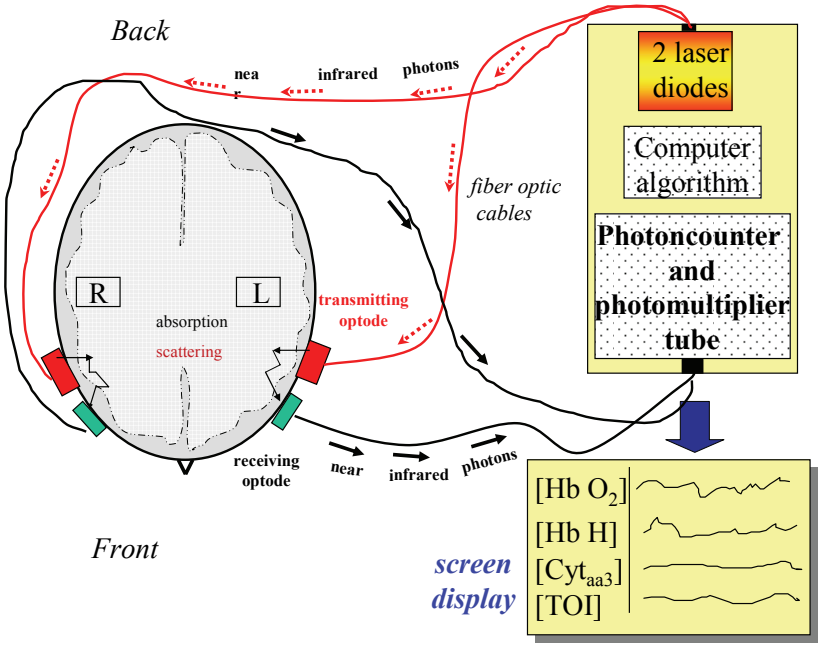


Figure 2.13 *NIRS set-up during measurement with a double channel device. In this example, channel 1 and channel 2 are shown. Each channel consists of an emitter (transmitting optode) and a receiver (receiving optode) probe. The emitter transmits NIR light to the tissue from a laser diode. The receiver transfers the attenuated light to the photon-counter and photomultiplier. Data are analysed and displayed as curves on the screen.*

2.4.1 Limitations of the technique

The major limitation of this technique is its poor spatial resolution. Due to the unknown trajectory (this implies an unknown path length) of the photon travelling within the tissue from the emitter to the receiver optodes, it is only possible to calculate relative changes in the haemoglobin concentration and not absolute values. Concerning functional studies, the anatomical variations of cortical circumvolutions and the different forms and convexity of the cranial bones represent a variable that makes it difficult to state how deep in the brain NIR light can penetrate. Whereas in adults, because of the thicker skull, measurements taken from structures located at more than 5 cm distance from the surface of the head are precluded, in neonates deeper structures of the brain might be feasible.

Environmental aspects are also very important when conducting NIRS functional studies. The surrounding environment must be kept as constant as possible in order to be able to isolate the chosen stimulation and thus to focus on the specific response.

Skin vascularization has been addressed as a potential bias in the calculation of intra-cerebral haemodynamics, despite this should only regard the calculation of absolute values of the natural chromophores. Recent data suggest that the contribution of skin blood flow to NIR measurements might be significant, potentially confounding interpretation of the NIR-derived signal during conditions where both skin and muscle blood flows are elevated concomitantly. This is unlikely during cerebral functional studies, but can occur when NIRS is applied to other regions and for other purposes (e.g. muscle oxygenation during high-intensity and/or prolonged exercise studies) (Davis et al. 2006; Klaessens et al. 2005; Mancini et al. 1994).

3 STUDIES OF SMELL PERCEPTION – STUDIES 1 & 2

Odors and other chemical signals guide and control many behavioural aspects of most animals. Olfaction is essential for neonatal adaptation in mammals, including man (Winberg and Porter 1998). Olfactory signals help the newborn baby localize and attach to the nipple during the first sucking bout during the first postnatal hours (Varendi et al. 1994). Birth and the first successive hours represent a crucial period in the learning mechanism of olfaction. During this period the odors of the mother and that of the newborn meet each other, and changes in the sensory processing of olfactory signals occur on both sides (Kendrick et al. 1992), (Kendrick et al. 1997). The mechanisms leading the newborn baby to recognize certain odors are still unclear, as well as the brain cortical structures involved in processing odors directly after birth. To date, it has been observed that in normal adult subjects olfactory stimuli are processed in the lateral and anterior orbito-frontal gyri of the frontal lobe (Levy et al. 1997) (Sobel et al. 1998; Sobel et al. 1997), even if differences might be related to the type of the odorant and whether it is pleasant or aversive (Zald and Pardo 1997; Zatorre 2002; Zatorre and Jones-Gotman 1991).

3.1 ANATOMICAL ORGANIZATION OF THE OLFACTORY SYSTEM

The olfactory system is specialized to detect volatile chemicals called odorants drawn into the nasal cavity during breathing or spreading into it from the oropharynx during eating. In vision-oriented species, such as humans, this sense seems to be less important than others, but we can still distinguish thousands of different odors, perhaps as many as 10,000, some at remarkably low concentrations (Buck 1996a; 1996b; 2000). There are two olfactory systems: the main olfactory system and the vomeronasal organ (VNO), an accessory olfactory system. The function of the latter in humans is still a matter of debate.

3.1.1 Main Olfactory System

3.1.1.1 Nose development

The fetal nose develops around the 6^{th} - 8^{th} week of gestation. During the third trimester of pregnancy the fetal chemosensory system completes its development. Premature babies above 28wks show behavioural responses to odorants.

3.1.1.2 From the nose to the olfactory bulb

The olfactory region in the nasal cavity consists of cilia projecting out of the olfactory epithelium into a layer of mucus which is about 60 microns thick. The mucus secretion, produced by Bowman's glands included in the olfactory epithelium, is rich in lipids and is the vehicle of volatile odorants to the olfactory receptors. Each olfactory sensory cell (neuron) has 8-20 cilia that are extensions 30-200 microns in length, on which receptors are located. The olfactory epithelium is a unique sensory structure that has the intrinsic ability to renew its receptor neurons naturally throughout the vertebrate lifetime. The olfactory epithelium is also a neurogenetic matrix that generates various cell populations during embryonic development and into adulthood. Some of these cell types migrate to the forebrain and therefore contribute to brain formation. Particular molecules are involved in cell migration and continuous guidance of the axons of the newly formed receptor neurons to their proper target synapses in the glomeruli of the bulb. The olfactory receptor neurons have a turnover of approximately 40 days. The neuronal cells within the epithelium form axons that are bundled in groups of 10-100 and which penetrate the ethmoidal cribriform plate, reaching the olfactory bulb where they converge to post-synaptic cells to form synaptic structures called glomeruli. The

glomeruli are connected in groups that converge into mitral cells. The total convergence is estimated to be 1000:1 (Scott 1991). The olfactory bulb also contains interneurons (many of them called granule cells, present in other regions of the brain as well), and tufted cells, smaller than mitral cells, but supplied with an analogous organiztaion of axon and dendrites.

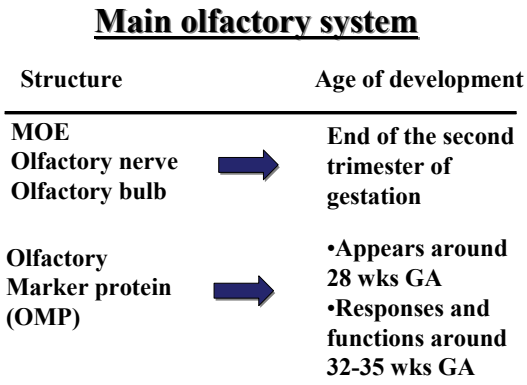


Figure 3.1 *Developmental stages of the olfactory system before birth. MOE, main olfactory epithelium*

3.1.1.3 From the bulb to the cortex

Mitral and tufted cell axons proceed into the olfactory tract, the principal central projection pathway for the olfactory system, giving off collaterals to cells of the anterior olfactory nucleus. Fibres from the anterior olfactory nucleus project back through the olfactory tract to both olfactory bulbs. Crossing fibres in the olfactory nucleus project through the anterior part of the anterior commissure. At the posterior end of the orbital frontal cortex, where the olfactory tract approaches to the base of the brain, some of its fibres end in the anterior perforated substance. Other fibres curve laterally as the olfactory tract.

The lateral olfactory tract travels along the edge of the anterior perforated substance, and as soon as it reaches its lateral border, curves up onto the surface of the temporal lobe near the uncus. Fibres of the olfactory tract terminate into the primary olfactory cortex and a portion of the amygdala. The primary olfactory cortex consists of (i) the piriform cortex (adjacent to the lateral olfactory tract), (ii) a part of the amygdala cortex (periamygdaloid cortex) and (iii) a small zone of the parahippocampal gyrus. In many animals the anterior olfactory nucleus and olfactory tubercle can have a ”cortex-like” structure, classified as a part of the primary cortex.

The primary structures receiving olfactory information project in turn to the hypothalamus and limbic system (hippocampus, other parts of amygdala). Though olfaction is mostly transmitted without any thalamus relay between receptors and cortex, a small part of the thalamus is involved in the connection from the olfactory bulb to the olfactory associative cortex (paleocortex). This area is located posteriorly on the orbital surface of the frontal lobe, extending onto the anterior insula (close to the gustatory cortex). The primary olfactory cortex sends information to this cortical area through a relay in the hypothalamus (the dorsomedial nucleus) as well as via direct projections. The result of this connection net is that olfactory receptor neurons end up in the ipsilateral cerebral hemisphere, unlike the somatic and motor systems in which a given part of the body is mostly represented in the contralateral cerebral hemisphere

3.1.2 Trigeminal component

It must also be recognised that the olfactory epithelium contains another sensory system in the form of trigeminal nerve receptors. Unlike the olfactory receptors which are extremely localized to a small area in humans, the trigeminal variants are widely spread throughout multiple areas of the body. The eyes, nose and mouth have higher sensitivities to chemical trigeminal stimulants than other areas of the body. The trigeminal receptors are responsible for the sensations of hot, cold and tingling. Developmental stages of the trigeminal system are shown in figure 3.2.

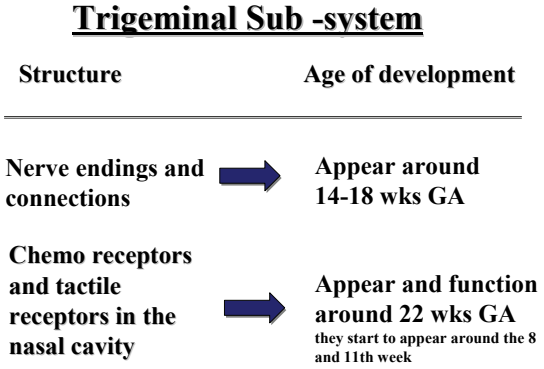


Figure 3.2 *Trigeminal system developmental phases during fetal life*

3.1.3 Vomeronasal organ (VNO)

Although this thesis did not aim to assess the function of the VNO in newborns, it should be mentioned that a further aspect to be considered in odor-mediated behaviour is the perception of pheromones. In animals, these chemical signals provide intra-species information about gender, mating, dominance and reproduction, and regulate social and sexual behaviours as well as influencing hormonal secretion. Pheromones are usually thought to activate a separate chemosensory epithelium. In humans the vomeronasal organ (VNO) is located in the ventromedial face of a blind-ended tubular structure lacated at the base of the nasal septum (Dulac 1997). Recent studies of genes encoding putative pheromone receptors (Dulac and Axel 1995), and signal transduction by VNO neurons (Berghard and Buck 1996; Liman 1996; Liman and Corey 1996) demonstrated that the vomeronasal and the main olfactory system belong to evolutionary distinct chemosensory systems. The VNO neurons project to the accessory olfactory bulb which in turn send fibres to the bed nucleus of the stria terminalis and to the amygdala, within a different area from the zone connected to the main olfactory pathway(Winans and Scalia 1970). The vomeronasal nucleus in the amygdala projects its neurons to the hypothalamus, which regulates both behavioural and reproductive functions as well as neuroendocrine stimulation.

How odorant-related innate behavioural responses are mediated is still in most cases unclear, as is their function at birth and during the first months during lactation. However, several studies suggest that a balanced contribution from both main olfactory epithelium and the vomeronasal organ is fundamental for normal behaviour.

3.1.4 Developmental phases

The main steps of the olfactory development are schematically indicated in figure 3.1, 3.2 and 3.3. The olfactory system is well structured from the 32nd - 34th weeks of

gestation and distinct behavioural responses to olfactory stimuli have been observed from the 32nd week of gestation(Sarnat 1978). Other components of the olfactory system, such as trigeminal and accessory subsystems, seem to already be responsive at 30 weeks of gestation or even earlier. Cortical processing in preterm infants in response to olfactory stimuli, particularly when these are unfamiliar and likely unpleasant, is still unclear.

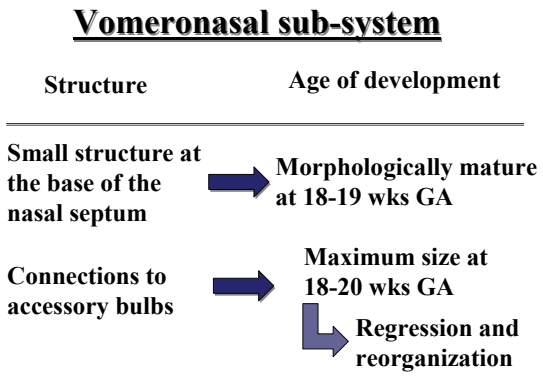


Figure 3.3 Developmental stages of the vomeronasal system

3.1.5 Conductive and sensorineural disruption of olfactory perception

Despite its involution throughout life in humans, the sense of smell can sometimes be of diagnostic interest. Two kinds of processes can disrupt smell perception: processes that prevent odorant from reaching the olfactory epithelium (conductive olfactory deficit), and processes damaging olfactory receptor neurons or part of the olfactory cortex (sensorineural olfactory deficit). Conductive olfactory deficits can be caused by nasal polyps, septal deviations and inflammation. Sensorineural olfactory deficits are mostly consequences of neurodegenerative conditions (Parkinson's or Alzheimer's diseases), or head injuries affecting the olfactory fila through the cribriform plate and/or the olfactory areas. As for newborns, and specifically the intensive care newborn, intubation and mechanical ventilation could damage the olfactory mucosa and consequently lead to disruption of smell perception. Moreover, an active neonatal resuscitation may be associated with anoxic-hypoxic-hyperoxic events, which could lead to a damage of the sensorineural olfactory structures, and to disorders of odor processing (Binienda et al. 1996; Zilstorff-Pedersen 1955). On the other hand it has been shown that the introduction of a pleasant odor in the incubator is of therapeutic value in the treatment of apneas unresponsive to caffeine and doxapram in preterm newborn (Marlier et al. 2005).

3.2 CLINICALLY ORIENTED STUDIES

The olfactory discrimination capacity and its importance in the early newborn-mother relationship have recently been investigated and reviewed by Varendi (Varendi 2001). Her findings and those from other groups reveal the key role of amniotic fluid and maternal breast odors in guiding and attracting the baby during the first hours of life, and in influencing behavioural states. Experiment of Schaal and co-workers, concerning amniotic fluid odor, indicated that newborns can recognize the AF odor, and that they remain attracted towards it for at least 2 days after birth (Schaal et al.

1998). A further analysis of the pathways which an odorous stimulus follows up to its recognition, memorization as well as of its behavioural effects, is of interest.

A recent article reported that there is a dissociation between brain regions activated by olfactory exploration (sniffing) and regions activated by olfactory content (smell) in human olfactory perception (Sobel et al. 1998). In adults, it has been observed through fMRI studies that a smell stimulus mainly activates the lateral and anterior orbito-frontal gyri of the frontal lobe (Sobel et al. 1998). Studies by Zald and co-workers, demonstrated that there is a significantly greater increase of the cerebral blood flow in the orbito-frontal cortex (Brodmann's area 47) when the olfactory stimulus is aversive than when it is pleasant leading to speculation of differences in lateralization of the signal processing (Zald and Pardo 1997; 2000).

To our knowledge there are few studies dedicated to the evaluation of cortical activity during smell perception in newborn infants, and of how this can change during the first hours of life. This fact is manly due to the lack of feasible and reliable methods to assess brain cortical activity non-invasively in the newborn.

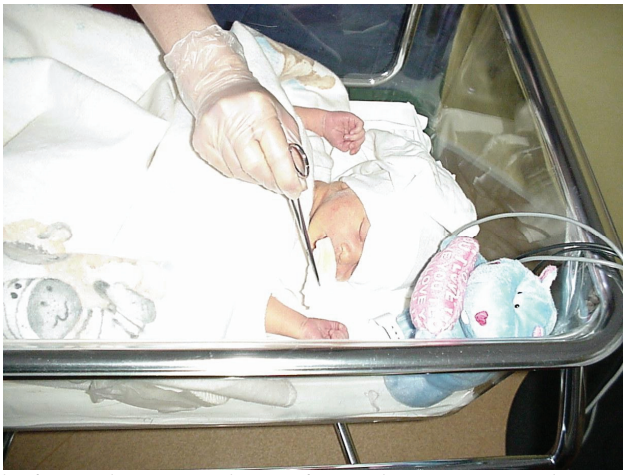


Figure 3.4 *The figure shows a newborn infant undergoing a test where a pad soaked in colostrum is presented in front of the nose. NIRS optodes are placed onto the head and are gently held by a soft elastic bandage*

The methodological approaches for studying olfactory processing have been modified during the last three decades, increasingly sophisticated techniques being adopted. A large body of studies has recently been based on measuring circulatory changes in the cerebral cortex in response to an olfactory stimulus in adult subjects. Although the dilemma of the correlation (coupling) between cortical activity, blood flow and metabolism is still open, all these methods are based on the assumption that increases in cerebral cortical blood flow are coupled with increases in neuronal function, underling a cortical processing. The techniques mostly utilised for this type of functional approach are fMRI (Cerf-Ducastel and Murphy 2001; de Araujo et al. 2003; Kettenmann et al. 1997; Levy et al. 1999; Rolls et al. 2003; Sobel et al. 1998; Sobel et al. 1997) and PET(Dade et al. 1998; Gulyas et al. 2004; Kareken et al. 2004; Royet et al. 2001; Savic 2002; 2001; Savic and Gulyas 2000; Savic et al. 2002; Small et al. 1997; Zald and Pardo 2000). Despite the spatial resolution of fMRI and PET being more accurate than NIRS, these methods cannot be easily used in neonates due to the need of sedation, transportation of the baby and their relative invasiveness. Due to its

characteristics that have been described in the previous chapters, NIRS is suitable to study brain functional activation during olfactory stimulation in newborn infants.

3.3 AIM

The primary aim of these studies was to use NIRS to monitor the activity of the olfactory cortex as mirrored by the haemodynamic response when newborns were exposed to a biologically meaningful stimulus such as the smell of colostrum and to other odors, using vanilla as a positive control and distilled water as a neutral control. Study 2 was especially aimed to assess the haemodynamic response in a particular group of newborns, i.e. those admitted to the NICU who are routinely exposed to strong odors such as disinfectant and remover substances.

3.4 PANEL EVALUATION OF THE SMELL

Despite there being several studies that have addressed intensity and hedonic significance of odors in newborn infants by assessing behavioural reactions and face expression, it is still very difficult to rate intensity and hedonic value in this category of patients. It is also interesting to have a comparison between adult and newborn judgment. For these reasons we have included in our studies a panel evaluation by adult subjects (6 males and 6 females) for rating of intensity and hedonic values of two substances commonly used in the NICU, Neomidil® and Remove®. The panel was also asked to rate three other control fluids: colostrum, vanilla and sterile water. For rating of intensity we used a 20 cm long visual analogue scale. The panel judged the intensity of the disinfectant significantly stronger than the smell of water and colostrum ($p< 0.001$ for both), but similar to that of vanilla. As regards to hedonic value, water and colostrum were rated as neutral ($p< 0.01$ vs. Neomidil® and Remove®), Neomidil® and Remove® as unpleasant and vanilla as pleasant ($p<0.001$) (figure 3.5).

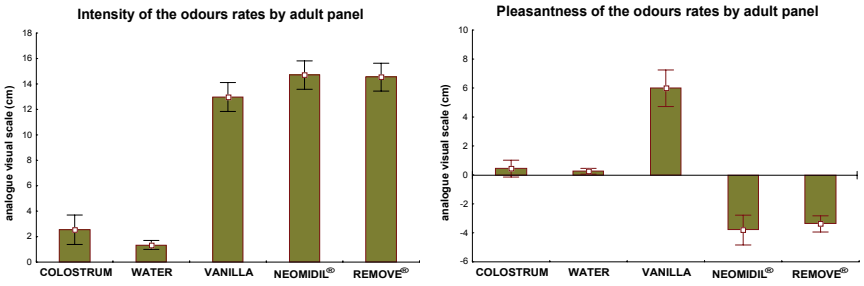


Figure 3.5 *Adult panel judgment. Left panel: intensity score. Vanilla, Neomidil, and Remove were scored significantly more intense then colostrum and water ($p <0.001$ for all comparisons). Right panel: pleasantness value. Neomidil and Remove were scored as unpleasant by the panelists as compared to vanilla ($p<0.001$), colostrum ($p<0.01$), and water ($p<0.01$). Columns represent mean values and whiskers standard errors. Statistical analysis has been performed by using ANOVA and Newman-Keuls post-hoc comparison (Statistica®, Tulsa, USA, 1998).*

3.5 METHODS

3.5.1 Study group

A total of 43 neonates were monitored. Twenty-three healthy, full-term newborn infants were included in study 1. They were all delivered vaginally by healthy, non-smoking mothers after an uneventful pregnancy (mean duration 39.4 weeks, min. 37, max 41, SD 1.3). In study 2 twenty preterm newborn infants with a mean gestational age (GA) of 33.7 (max 37, min 30) at testing, and a mean post-conceptional (PC) age of 35.5 weeks (max 37.3, min 32), and a mean postnatal age (PN) of 12.5 days (max 35, min 0.75), were studied. Of these, fifteen were admitted to the Neonatal Intensive Care Unit, Department of Pediatrics, Gaslini Institute, University of Genoa, (mean GA 33.1, max 36, min 30 weeks), and five to the Neonatal Intensive Care Unit, Astrid Lindgren's Children's Hospital, Karolinska Institute, Stockholm (mean GA 35.4, max 37, min 33 weeks). The local Ethics Committee approved the study and parental informed consent was obtained.

3.5.2 Environmental settings

In study 1 the neonates were studied at a postnatal age varying between 6 hours and 192 hours (median 48.0 h, SEM 10.1 h), when lying in the bed in the supine position and being in a quiet, awake state (Prechtl 1974). All babies were exclusively breast-fed and at least 30 min had elapsed since the last feed. In the original study group of 30 babies, 7 fell asleep during the test and were excluded, leaving 23 babies with a male/female ratio of 12/11 in the study group. Babies whose mothers had used scented soaps, perfumes, or deodorants during the three days before the experiment were not eligible. The room temperature was kept around 22-24˚C, the light was dim and the noise level was reduced as much as possible.
In study 2 The babies were studied when they were lying in their incubators in the supine position and were in a quiet, awake state (Prechtl 1974). The incubator temperature was kept constant around 29-30°C, the light was dim and the noise level was reduced as much as possible. The elapsed time between the last handling and the experiment was at least 1/2 hour. At the time of the experiment all babies were in a stable condition breathing spontaneously, without any need of oxygen supplementation or infusion. All were fed with mother's milk, given by bottle, and at least 2 hours had passed since the last meal (mean 2.9, max 4, min 2, SD 0.47). Cranial ultrasound scans were normal in all babies. Heart rate, respiration and peripheral oxygen saturation were continuously monitored during the examination (HP monitor, Germany). In both studies parents were allowed to be present during the experiment session, but they were asked not to touch or talk to their baby during the test.

3.5.3 Odor preparation

In study 1 we used as odorant sources (i) the own mother’s colostrum, manually expressed into a plastic cup just before the test; (ii) vanilla essence (4-hydroxy-3-methoxybenzaldehyde) dissolved in an oily solution, commercially available (Body Shop International PLC, West Sussex BM 17 7LR England); (iii) distilled water as a negative control. The substance to be tested was soaked into a cotton bud sized about 1.5 x 0.5 cm attached to the end of a 20 cm long stick. The cotton buds were soaked in a room separated from the testing room. The vanilla smell was judged as being very strong and the colostrum smell as very weak by the adult nose. As in many other studies of olfaction, vanilla was chosen as a positive control because of its properties to

activate mainly the primary olfactory system, with very little effect on the trigeminal system (Kobal and Hummel 1998; Kobal et al. 1989).

In study 2 we combined two different sessions of experiments. In the NICU at Genoa Children's Hospital we tested 15 babies with the smell of a disinfectant solution (Neomidil®, Pharmec) routinely used to sterilize the skin before blood sampling. This solution is composed of benzalconio chlorate: g 0.25%, ethylic alcohol: g 66.29%, excipients: essentially lemon oil, acetone, iso-propilic alcohol, camphor, purified water. In the NICU at Karolinska Hospital, Stockholm we monitored 5 neonates when exposed to the smell of a detergent frequently used to remove tape from the skin (Remove®, Smith & Nephew). This substance mainly contains dipropylene glycol methyl ether (45-55%), water and mineral essences. The whole study population originally consisted of 30 babies, but in 10 babies the recordings could not be evaluated due to movement artefacts on the NIRS trace. The compounds to be tested were soaked into a 2x2 cm cotton pad and presented to the babies at a distance of about 2 cm from the nostrils for 10 seconds. Care was taken not to touch the skin of the baby.

The responses were compared to those registered after exposure to colostrum as a biologically meaningful odor, vanilla (*vanilla essence,* 4-hydroxy-3-methoxy-benzaldehyde, dissolved in oily solution, commercially available, Body Shop International PLC, West Sussex BM 17 7LR England) a probably pleasant odor, and water as a negative control.

3.5.4 Odor stimulation

In study 1, after placing NIRS optodes on the head, we monitored a 30 s period baseline definition, after a 90s starting period. Each infant received each stimulus in the following order: 1 Control, 2 colostrum, and 3 vanilla (figure 3.6). The smell stimuli were administered by moving the cotton bud slowly from one nostril to the other at a distance of around 1-2 cm. Particular attention was paid to prevent the tip from touching the nose. Each exposure lasted 30 seconds. There was a two-minute interval between each exposure. Before administering a new stimulus, NIRS baseline was re-defined (figure 3.6).

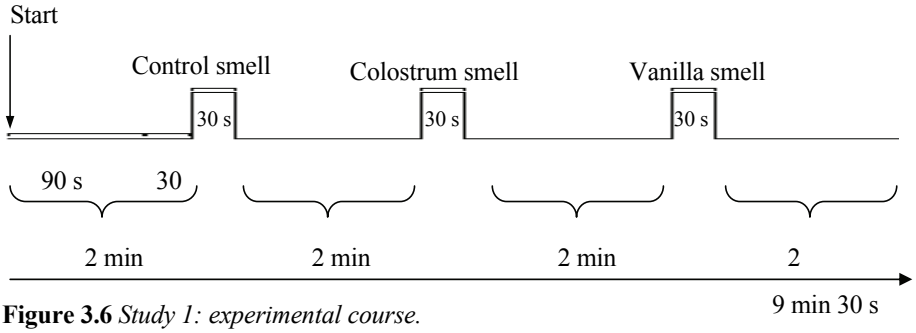


Figure 3.6 *Study 1: experimental course.*

In study 2 exposure to the stimuli was shorter because of ethical reasons and also in the attempt to reproduce the exposure that the infant experiences when these substances are used in daily practice. After NIRS optodes were positioned on the head, a start period of about 2 minutes elapsed, during which the signal was adjusted and stabilised. A 30 s period of NIRS baseline was then defined. The little pad soaked in the substance to be tested was then moved slowly from one nostril to the other, at a distance of around 2 cm, for 10 seconds (figure 3.7).

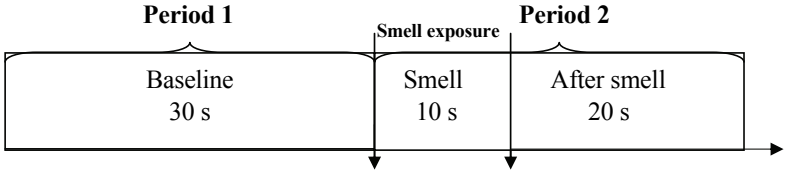


Figure 3.7 *Study 2: experimental course.*

3.5.5 Data Storing

A trigger signal was marked on the NIRS device at the beginning and end of the stimulation period in each experiment. Through an RS232 A/D board NIRS data were transferred online to a PC.

3.5.6 NIRS settings

In study 1 we had only one channel available for recording. That is the reason why we analysed just one side of the brain. The optodes were placed in a special dark semi-rigid rubber holder, which kept a constant inter-optode distance of 4 cm during recording. They were positioned over the left orbito-frontal gyrus of the frontal lobe. The emitting optode was placed 2 cm above the mid-point of the line connecting the external angle of the left eye to the homolateral tragus (slightly anterior to the T3 position according to the international EEG 10-20 system) (figure 2.12). We established a differential path-length factor (DPF) of 4.2 according to previous studies (see above). The sampling rate was performed every 0.5 seconds. In study 2 we used the same position as in study 1, but as we had the possibility to use two channels we could position the optodes bilaterally. NIRS technical features have been discussed previously.

3.5.7 Data analysis and Statistics

In both studies statistical analysis was performed using the program Statistica® (version 1998, StatSoft, Inc. USA), and a p-value <0.05 was considered as being statistically significant.

In study 1 we have basically compared three 60-value epochs, one for each odor exposure, for statistical analysis. Two different approaches to the values were used. First, the mean values of each subject's recordings during the three different odor conditions (control-colostrum-vanilla) were compared. Secondly, an averaged value for each single measurement of the 60 in total was calculated from all 23 babies, for all three stimuli. In this way it was possible to represent the average changes in [Hb O_2] for each olfactory stimulation. To compare these curves, and to calculate the latency interval from the onset of the stimulus to recorded changes of [Hb O_2] during which the values were likely to be similar, a time series stepwise analysis was conducted. For each sampling point (every 0.5 sec) following the onset of odor exposure, the mean values obtained from the 23 babies were compared in order to extract the cut-off point at which the three averaged curves became significantly different.

Analysis of variance (two way ANOVA for repeated measurements) and subsequently, Student –Newman-Keuls post- testing, were used to compare the changes of [Hb O_2] in response to the three different stimuli. Computationally the Newman-Keuls test used for post-hoc comparisons sorts the means into ascending order. For each pair of means the program then assesses the probability according to the null hypothesis (no differences between means in the population) of obtaining differences between means of this (or greater) magnitude, given the respective number of samples.

Thus it actually tests the significance of ranges, given the respective number of samples. An analysis of variance for repeated measurement was also performed for each second's mean values of the averaged curve.

To better represent the averaged curves an "aggregated index" was used. This specified the number of consecutive observations from which the mean was calculated. The Spearman Rank correlation was used to examine whether postnatal age or gestational age were related to the magnitude of changes of [Hb O_2]. The Mann – Whitney U test was applied to evaluate whether there were any sex differences in the magnitude of the changes of [Hb O_2].

In study 2 two periods were compared for each baby (figure 3.7). Period 1 (P1) consisted of a 30 s baseline period before exposure to the odor. Period 2 (P2) included the 10 s period of exposure and the following 20 s period. As sampling was performed every second, each of the periods thus contained 30 numerical values corresponding to [Hb O_2] and 30 values for [Hb H]. [Hb tot] was calculated off-line as [Hb O_2] + [Hb H] (Wyatt et al. 1986). Also, $[Hb\ O_2]_{diff}$, $[Hb\ H]_{diff}$ and $[Hb\ tot]_{diff}$ were calculated by subtracting the mean values of period 1 from the mean values of period 2 for each subject. An average value for each single measurement of the 30 in total was calculated from all 20 babies, both in the basal condition and after stimulation. In this way it was possible to derive average curves describing the haemodynamic changes in the left and right hemisphere during the basal condition (period 1) and during/after exposure (period 2). An average curve with mean values of [Hb] diff for each baby was Also calculated. To compare changes in the same hemisphere related to the exposure, the P1 average curves of each side were compared to the P2 average curves of the same side, for [Hb O_2], [Hb H] and [Hb tot]. This analysis was performed using the Student t-test for dependent samples. Secondly, to compare the differences between the two hemispheres, P1 and P2 average curves were compared with the contralateral ones. For this analysis the Student t-test for dependent samples was used.

To calculate the latency interval from exposure to the stimulus to significant changes in $[Hb\ tot]_{diff}$, a time series stepwise analysis was carried out. For each sample point (every 1 s) following the onset of odor exposure, the mean values obtained from the 20 babies were compared in order to extract the cut-off point at which the average curves became significantly different from the baseline. This was done for values for both sides. A comparison of $[Hb]_{diff}$ on the left side with $[Hb]_{diff}$ on the right side was also used to evaluate inter-hemispheric differences of the changes in haemoglobin concentration. This was performed using the Student t-test for independent variables. To measure the relationship between the variables gestational age, postnatal age, post-conceptional age, against $[Hb]_{diff}$, correlation matrices were used.

3.6 RESULTS

3.6.1 Study 1

In study 1, during exposure to vanilla each infant had a marked increase in [HbO_2] over the left orbito-frontal region, slowly tapering after the end of the exposure. During exposure to distilled water (as the negative control) only small fluctuations around the baseline were recorded. The response to the smell of colostrum was variable; in some babies there was an increase in [Hb O_2] similar to that elicited by vanilla, in others only minor or no changes were recorded. Clearly positive responses to colostrum and vanilla are presented in figure 3.8. During exposure to colostrum and to vanilla, but not to water, we could observe sniffing movements of the alae nasi.

For each type of stimulus we averaged all [HbO_2] values collected every 0.5-sec, i.e. 60 registrations during each exposure, figure 3.9. Comparison of the changes during

exposure to water, colostrum and vanilla revealed a significant difference (ANOVA for repeated measurement $p < 0.00001$). The post-hoc comparison also yielded a difference between groups (control vs. colostrum $p = 0.0004$; control vs. vanilla $p = 0.0001$; colostrum vs. vanilla $p = 0.0001$).

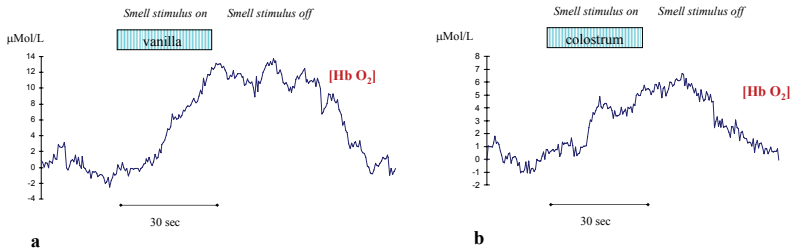


Figure 3.8 *Changes in [Hb O_2] during exposure to vanilla (a) and colostrum (b) in one baby 7 hours post-partum.*

The average curve for [Hb O_2] changes during colostrum exposure revealed an increase from the baseline starting around 6-8 seconds after onset ($p = 0.002$). The difference was significant after 6 and 8 sec, but not after 7 sec. After vanilla exposure a significant difference from baseline was evident after 5 seconds ($p = 0.03$).

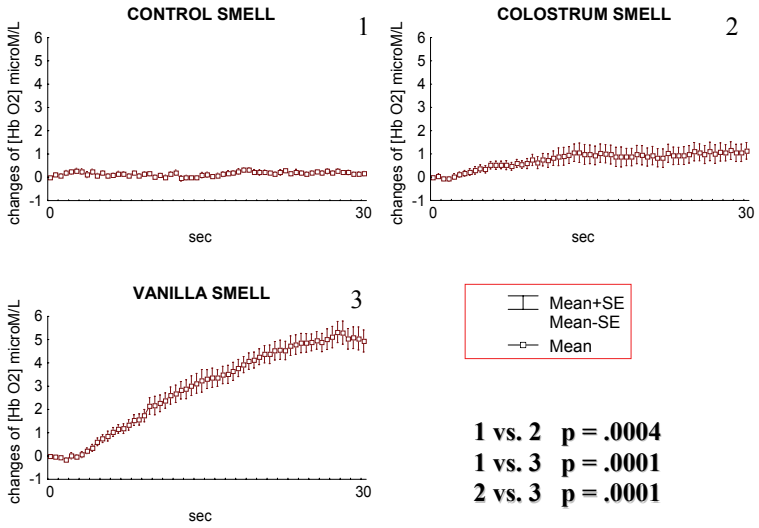


Figure 3.9 *Average curves (means ± SEM) of [Hb O_2] changes during smell exposures. The means were derived from 23 values at each 0.5 sec recording. ANOVA for repeated measurements showed a significant difference between the three responses ($p < 0.0001$). Newman - Keuls post-hoc comparison evidenced statistically significant differences (see legend in the figure).*

A preliminary look at the individual [Hb O_2] curves obtained during exposure to colostrum smell gave the impression that an increase in [Hb O_2] was mainly apparent in the youngest individuals. This was confirmed by demonstration of a statistically

significant negative correlation between changes in [Hb O_2] and postnatal age (r=-0.64 p=0.001 with 95% confidence interval) (figure 3.10). Those babies exhibiting the greatest increase in [Hb O_2] were between 6 and 24 hours old at testing. During exposure to vanilla the [Hb O_2] changes did not correlate with postnatal age (r= 0.40, p = 0.9).

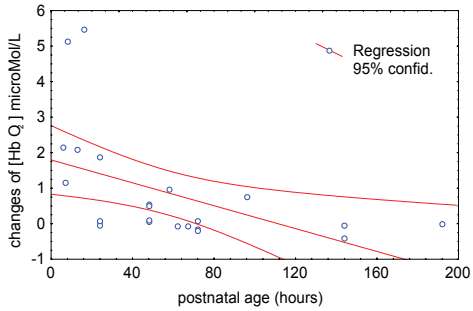


Figure 3.10 *Changes of [Hb O_2] vs. postnatal age during colostrum exposure. Changes of [Hb O_2] were inversely correlated to postnatal age (r=-0.64 p=0.001 with 95% confidence interval). As a matter of fact an increase in [Hb O_2] was only evident in infants being 24h old or less at exposure. For vanilla there were no similar age-related differences.*

In nine infants who were 24 hours or younger at testing the [Hb O_2] response to colostrum, vanilla and water were compared as earlier for all 23 babies. There was a significant difference between all three test conditions (ANOVA for repeated measurements p= 0.0001). According to the post-hoc comparison with Newman - Keuls test the changes between each smell test were statistically different (control vs. colostrum p= 0.009; control vs. vanilla p= 0.0002; colostrum vs. vanilla p= 0.01). In the 14 babies older than 24 hours there were no significant differences between the changes of [Hb O_2] during control and colostrum exposure (Newman - Keuls post hoc comparison, p= 0.8).
For all cases and exposures examined, changes in [Hb tot] were essentially the same as for [Hb O_2]. No sex differences in the changes of [Hb O_2] were observed during the test (Mann - Whitney U test p= 0.6).

3.6.2 Study 2

In study 2 it was possible to follow both heart rate and respiratory patterns as the newborns were admitted to the NICU.

3.6.2.1 Circulatory/respiratory responses to olfactory exposure

For neither of the two smell stimuli tested, heart rate, respiration and peripheral oxygen changed significantly during periods 1 and 2.

3.6.2.2 NIRS data in response to olfactory exposure

[Hb O_2] and [Hb tot] changed almost in parallel during NIRS registrations due to the small changes in [Hb H]. Thus we usually limit our reports to [Hb O_2] changes.

Neomidil® odor (15 cases) Before exposure to Neomidil® odor, (period 1), only minor fluctuations around the baseline of [Hb O_2], [Hb H], and [Hb tot] were observed, similar on both left and right sides. After exposure 13/15 babies responded with a significant decrease in [Hb O_2], and [Hb tot] on both sides ($p<0.0001$). The changes of [Hb H] were different: on the right side there was an insignificant increase compared to the baseline, while on the left side there was a significant decrease ($p<0.05$). A representative case is depicted in figure 3.11.

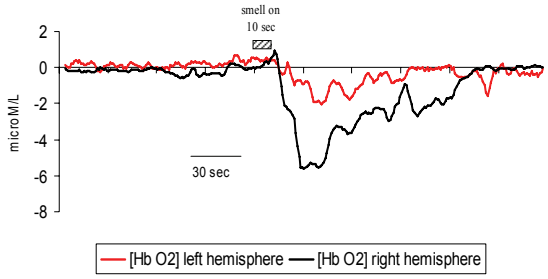


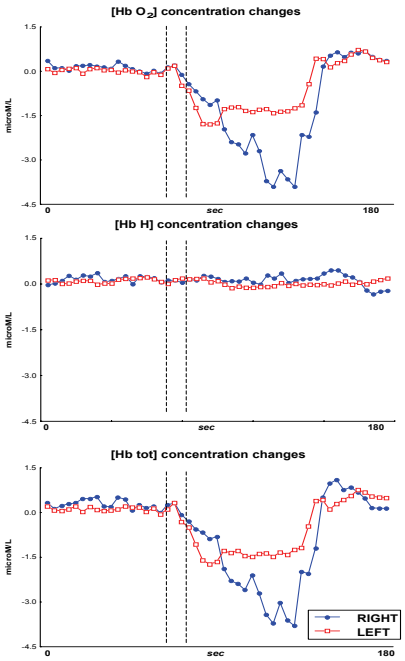
Figure 3.11 *Representative case of a newborn exposed to Remove. Although in some cases it was possible to follow the NIRS trace until the signal re-settled itself around the baseline, in most cases this was impossible because of movement artefacts.*

The difference between left and right response was highly significant ($[Hb\ H]_{diff}$ left vs. right $p < 0.0001$). A comparison between left and right sides for $[Hb\ O_2]_{diff}$ and $[Hb\ tot]_{diff}$ showed a significantly larger decrease for both on the right side ($p<0.01$ for $[Hb\ O_2]_{diff}$, and $p<0.05$ for $[Hb\ tot]_{diff}$ respectively) (figures 3.12 and 3.13). With regards to $[Hb\ tot]_{diff}$ on the right side, there was a clear tendency to a diminished response in the older babies (r-value = 0.44, with 95% confidence interval). This trend was only apparent for the right side; on the left side in fact, the r-value was -0.09.

The decrease from the baseline of $[Hb\ O_2]_{diff}$ started around 4 seconds after onset of exposure ($p<0.05$) on the right side and around 6 sec on the left side ($p<0.05$) (figure 3.13).

Remove® odor (5 cases) Analysis of the baseline did not revealed any major changes in any of the NIRS variables, and left- and right-side recordings were not significantly different. After exposure to Remove 4/5 babies responded by showing a decrease in the $[Hb\ O_2]$, and [Hb tot] especially in the right hemisphere ($p < 0.01$ Student t-test). As far [Hb H], a minor decrease was observed on the left side after the exposure to Remove®, while a consistent increase was recorded on the right side.

When comparing the [Hb H] differences there was a significantly higher value of [Hb H] on the right side ($p<0.0001$). Gestational age, postnatal age, and post-conceptional age were unrelated to the magnitude of the $[HbO_2]$, [Hb H], and [Hb tot] changes. Similarly to the Neomidil® findings, $[Hb\ O_2]_{diff}$ started to decrease around 4 seconds after onset of exposure to Remove® on both right and left sides ($p<0.05$ for both).



[Hb O_2]

[Hb H]

[Hb tot]

Figure 3.12 *Changes of [Hb O_2] (a), [Hb H] (b) and [Hb tot] (c) in one case after a 10 s exposure (dotted lines) to Neomidil®. The decrease was more pronounced on the right side (solid circles) than on the left side (open squares). An analogous trace was seen after exposure to Remove®. [Hb tot] changes paralleled those of [Hb O_2].*

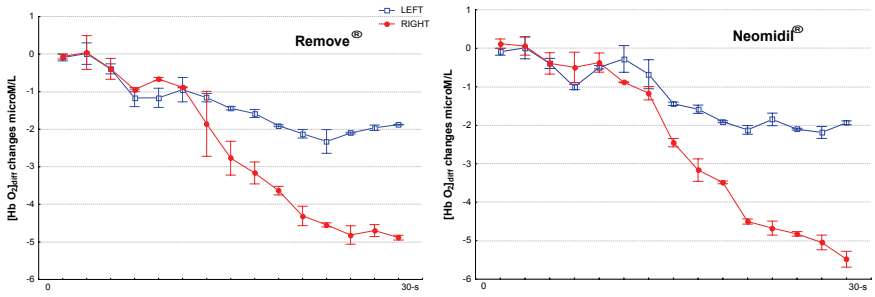


Figure 3.13 *Changes of [Hb O_2]$_{diff}$ after exposure to the two tested substances Remove® (left panel) and Neomidil® (right panel). A 30s period (corresponding to the differences period 2 - period 1) has been analyzed. The curves represent averaged line plots for [Hb O_2]$_{diff}$ changes both on the right (full squares) and the left (open circles) sides.*

3.7 DISCUSSION

The main finding of these studies was that the NIRS technique could be used in the neonatal period to record changes in blood circulation coupled to neuronal activity in an area that likely includes the orbito-frontal cortex, during exposure to a biologically meaningful odor such as colostrum as well as to artificial odors such as vanilla, remove® and neomidil®.

The first biologically interesting finding was that the olfactory response to colostrum, but not to vanilla and water, was inversely correlated to postnatal age. This was unexpected since the smell of milk, as well as that of the mother's breast, elicits an approach behavior in the newborn well beyond the first postpartum day (Macfarlane 1975). A possible explanation for this finding might be that the older the baby the more experience it has of taste and smell of colostrum, and the response might have been successively modified in strength or localization by coupling to recognized food (habituation). Another possibility is that a putative odorous compound is more concentrated in the first few drops of colostrum than a few days later, when milk secretion has increased, as occurs for other compounds such as antibodies (Carlsson et al. 1976). A third possibility is that the very early colostrum smells differently from the late colostrum. In rats the circulating oxytocin triggered the release of some unidentified substance that was attractive for the pups (Singh and Hofer 1976). If a corresponding mechanism exists in humans, the surge in circulating oxytocin during delivery might have influenced the smell of early colostrum.

We have taken into account the important role that catecholamines play in olfactory learning (Kawai et al. 1999; Keverne et al. 1993), as it has been suggested that adrenergic pathways might influence signalling by the olfactory epithelium. This could be possible *via* double action of the olfactory receptor neuron: an increase in the threshold of the spike train of action potentials and an augmented firing rate in response to the stimuli. Thus a high concentration of adrenaline, which modulates signal encoding of the olfactory epithelial adrenergic neurons, might enhance the high olfactory response of the newborn just around birth. The hypothesis that such a mechanism influenced the different responses among the youngest and the somewhat older infants does not fit adequately with our studied population. Firstly, the surge in catecholamines following vaginal delivery decreases markedly within about an hour after birth, stabilizing at a blood concentration which was probably in the same range in all the babies we studied. Secondly, such a mechanism should have influenced both colostrum and vanilla responses. Conversely, the influence of catecholamines on smell perception should be taken into account in all stress conditions that the newborn infants undergo when they are in the intensive care units.

In study 2 we demonstrated that the strong and unpleasant (to adults) odors of solutions commonly used in NICUs might elicit a decrease in blood oxygenation in an area which likely includes the orbito-frontal olfactory area. When interpreting these results we have to be aware that both the studied population and the tested odors were extraordinary; the population consisted of premature newborns, although all in stable conditions. Concerning the odor, substances containing different odorous compounds may have multiple targets in the brain cortex, some represented in the olfactory areas, some in areas related to them, or others not directly related. The two very strong substances that we have tested contain compounds such as acetone, camphor and alcohol both able to activate the trigeminal system that is fully receptive already at 22 weeks of gestation. Trigeminal system contribution to the modulation of cerebral haemodynamics can be both indirect and direct. Parasympathetic projections arising from the sphenopalatine ganglion, which in turn is innervated by trigeminal fibres (Suzuki et al. 1989), distribute through the ethmoid nerve to the cerebral vessels

(Suzuki et al. 1988; Walters et al. 1986). A considerable number of sensory trigeminal projections directly innervates the cerebral blood vessels as collaterals from the same fibres that innervate the nasal cavity (Finger and Böttger 1993; Fusco et al. 1994; Major and Silver 1999). These fibres project to second order neurons in the trigeminal nucleus caudalis and its caudal extension into C1 and C2 cervical spinal cord segments (Kaube et al. 1993). According to our observations, trigemino-vascular system activation may play a major role by causing a redistribution of the cerebral blood flow towards certain areas of the brain, such as regions of the brainstem and upper cervical spinal cord, or the superior sagittal sinus, thus determining a [Hb O_2] and [Hb tot] decrease in the region that we have illuminated. We have therefore interpreted the haemodynamic changes as the result of a dynamic, physiological regulation of regional CBF based upon the olfactory- and trigeminus-related areas of the brain.

Another aspect to be considered when interpreting the present data is that unpleasant odorants can lead to cortical deactivation (Brauchli et al. 1995). Fransson and co-workers have recently investigated the correlation between deactivation and cerebral haemodynamics by MRI, and they concluded that a state of cortical deactivation is associated with a decreased MR-signal, indicating a decrease in blood supply (Fransson et al. 1999). As for the NIRS technique, a decrease of [Hb tot] has been defined as deactivation (Benaron et al. 2000; Obrig et al. 1997; Obrig and Villringer 1997; Obrig et al. 2000). If our data are considered in this perspective, the recorded decrease in [Hb O_2] and [Hb tot] might be related to a cortical deactivation due to a likely unpleasant smell stimulus. This finding would be supported by the [Hb H] increase on the right side, but not on the left in which a [Hb H] decrease was observed. On neither side did the [Hb H] changes affect [Hb tot] concentration significantly.

The drastic functional response following exposure underlines the sensibility and competence of the olfactory system in premature babies. The biological significance of exposure to unpleasant odors is unknown, but with regards to the closed connections between the olfactory cortex and centres regulating emotional state, it might be worth considering. This raises the question as to whether or not and to what degree certain odor substances should be used in NICUs. Further investigations are needed to understand whether exposure to artificial, unpleasant odors can affect the development of the brain and its capacity to process smells.

4 STUDIES OF PAIN PERCEPTION – STUDY 3

Although it is unanimously recognized that newborn infants can feel pain, according to the latest survey about 33 to 45% of painful/discomfort procedures performed in NICU are not adequately treated (Rohrmeister et al. 2003; Simons et al. 2003).

There have been a tremendous increase in knowledge of neonatal pain during the last decade (Anand and Scalzo 2000; Fitzgerald 2005; Fitzgerald and Beggs 2001; Fitzgerald et al. 1988; Walker and Howard 2002). What basically brought me and my colleagues to conduct a study with NIRS and pain processing in neonates has been the awareness that despite new data confirming that neurological structures involved in pain perception are already sufficiently developed at 24 weeks of gestation (and maybe earlier) to process nociceptive stimuli, there is almost no evidence of supraspinal pain processing in newborn infants (Klimach and Cooke 1988).

4.1 PAIN IN NICU AND DEVELOPMENTAL ASPECTS

Recurrent neonatal pain and stress occur routinely during neonatal intensive care, particularly among the extremely low birth weight preterm neonates (Johnston et al. 1997; Simons et al. 2003; Stevens et al. 2003). Analgesic or anaesthetic drugs, whose long-term effects are not yet fully understood (Anand and Soriano 2004), may attenuate prolonged pain but may not be effective for the acute pain caused by invasive procedures (Carbajal et al. 2005; Liu et al. 2004). Moreover, a painful procedure such as venipuncture for blood sampling is not routinely preceded by any analgesic measures (Simons et al. 2003) and its repercussions on the development of the cerebral cortex or sub-cortical structures is not yet understood. Recent studies of adult subjects report that painful stimuli are associated with circulatory and metabolic changes in specific cortical and subcortical areas (Ohara et al. 2004; Ringler et al. 2003). Objective evidence for the supraspinal processing of pain in human neonates, particularly those born preterm, is currently lacking. Furthermore, investigation of the mechanisms regulating cerebral haemodynamics in the cortical areas processing pain stimuli might be of great help in understanding the influence of pain on neonatal brain plasticity and cognitive or behavioural outcomes (Anand et al. 1999; Anand 2000; Winberg 1998).

According to its definition, “Pain is a subjective sensory and emotional experience that requires the presence of consciousness to permit recognition of a stimulus as unpleasant”. Consequently, approaching the question of neonatal pain one cannot avoid facing a wide and problematic issue: “When does consciousness emerge?” As we have recently reported (Bartocci et al. 2006a), the “Hard Problem” (Searle 1997) in this case may be the hump from cerebral neurovascular events to the subjective feeling of pain. However, the traditional dogma that preterm responses to noxious stimuli are only reflex must be rejected (Lloyd-Thomas and Fitzgerald 1996). The pain system is certainly functional in preterm and term neonates (Fitzgerald 2005), and their responses to pain are complex and nuanced forms of ‘‘self-expression’’(Beacham 2004). Furthermore, effective mechanisms of hyperalgesia, allodynia, and referred pain also exist in neonates (Andrews and Fitzgerald 1999; 2002; Fitzgerald et al. 1989; Taddio et al. 2002). According to these discoveries, ‘pain’ and ‘nociception’ might be reformulated into more dynamic concepts, maturing in parallel with thalamocortical connections which gradually emerge soon after 20 weeks of gestation (Anand 2006).

Previous studies have demonstrated that the number of nociceptive nerve fibers in the skin of the neonate is similar to and possibly even greater than the number

present in the adult (Fitzgerald et al. 1988). Despite incomplete myelination of pain fibers, pain transmission is preserved. The short distances in the immature brain compensate any slowing of velocity that may be caused by the lack of myelinisation. It is also been shown that there is an abundance of pain neurotransmitters in the newborn and fetal brains, a part of which implicated in painful stimuli processing, as well as there being receptive fields of neurons in the somatosensory cortex (Fitzgerald 2005; Fitzgerald and Beggs 2001). From all these reports it appears that pain transmission and neurotransmitters are extremely well developed at birth and in the preterm neonate and that modulatory and inhibitory circuits are still immature and partially lacking. This is mainly due (i) to a delay in the maturation of descending inhibitory pathways from supraspinal areas, (ii) to a delayed maturation of interneurons in the "substantia gelatinosa, and (iii) to a possible deficiency of inhibitory neurotransmitters (Fitzgerald 2005; Fitzgerald and Beggs 2001).

•Sensory receptors in the moth already at week 7 of gestation
•Receptors on the skin from week 20
•Synapses develop aroud week 20
•Endorfins present at week 20
•Nociceptive connections develop between week 22 and 24
•Myelinisering till brainstem, thalamus at week 30 NICU

Table 4.1 *Important developmental steps of the nociceptive/pain system*

4.2 AIM

To study the patterns of supraspinal pain processing in neonates we hypothesized that acute pain activates the somatosensory cortex in preterm neonates, associated with significant metabolic changes in the cortical areas associated with pain processing which can be measured using the NIRS technique. To compare the cortical processing of tactile and painful stimulation we recorded the responses during gentle cleaning of the skin (tactile stimulation) and during venipuncture (acute pain). The NIRS device used in this study recorded from two channels, placed bilaterally over both somatosensory areas. To test for the specificity of these responses we compared the somatosensory cortex with the occipital cortex, which is not primarily involved in sensory processing for painful or tactile stimuli.

4.3 METHODS

4.3.1 Study group

This prospective study included forty vaginally delivered newborn infants who fulfilled the inclusion criteria and required blood sampling in the NICUs at Karolinska Hospital (Stockholm, Sweden) or Gaslini Hospital (Genoa, Italy). Cranial and cardiac ultrasonography were performed on all subjects. The inclusion criteria selected

neonates born after 26 weeks of gestation, postnatal age (PNA) more than 24 hours (h), absence of congenital malformations affecting the brain circulation or the cardiovascular system, ongoing intubation and mechanical ventilation, who had not received analgesic, anesthetic, or sedative drugs duirng the preceding 24 h (e.g. morphine, fentanyl, phenobarbital, midazolam).

The local Ethics Committee approved the research protocol. Informed consent was obtained from the parents of all enrolled subjects and around 90% of the parents approached for this study gave their consent.

All subjects	**Number of patients**	**Mean**	**Median**	**Minimum**	**Maximum**	**SD**
Gestational age (weeks)	40	32.0	32.0	28.0	36.0	3.0
Postnatal age (hours)	40	30.7	30.0	25.0	42.0	4.8
Weight (grams)	40	1899.5	1772.5	1200.0	3100.0	590.3
Venepuncture duration (seconds)	40	48.3	49.0	35.0	60.0	6.7
Females						
Gestational age	20	32.7	33	28.0	36.0	3.0
Postnatal age	20	29.5	28.5	25.0	40.0	4.3
Weight	20	1958.0	2005	1200.0	3010.0	567.0
Venepuncture duration	20	49.8	50	35.0	60.0	6.9
Males						
Gestational age	20	31.4	31	28.0	36.0	2.9
Postnatal age	20	32.0	30	25.0	42.0	5.2
Weight	20	1841.0	1595	1200.0	3100.0	621.9
Venepuncture duration	20	46.7	45.5	39.0	59.0	6.2

Table 4.2 Characteristics of the neonates included in study 3.

4.3.2 Study Procedure

We analyzed the recordings from 40 subjects (20 females and 20 males), as described in table 4.1 and 4.2 and figure 4.1. For 29 neonates (15 females), the 2 pairs of optodes were positioned over the somatosensory cortex symmetrically on each side of the head. The emitter optode was placed about 2 cm below and slightly posterior to the C3/C4 position (according to the international EEG 10-20 system (Kuboyama et al. 2005; Mehagnoul-Schipper et al. 2002; Schroeter et al. 2002; Suzuki et al. 2005). The receiver optode was consequently positioned 4 cm above the emitter optode in order to illuminate the primary somatosensory cortex and underlying structures (figure 4.2, 4.3).

Eleven newborns were monitored with the 2 pairs of optodes placed on the same side of the head: one pair overlying the somatosensory cortex (as described above) and the other pair overlying the occipital cortex. In these 11 newborns, the tactile and painful stimuli were applied contralaterally to the side where the optodes were placed.

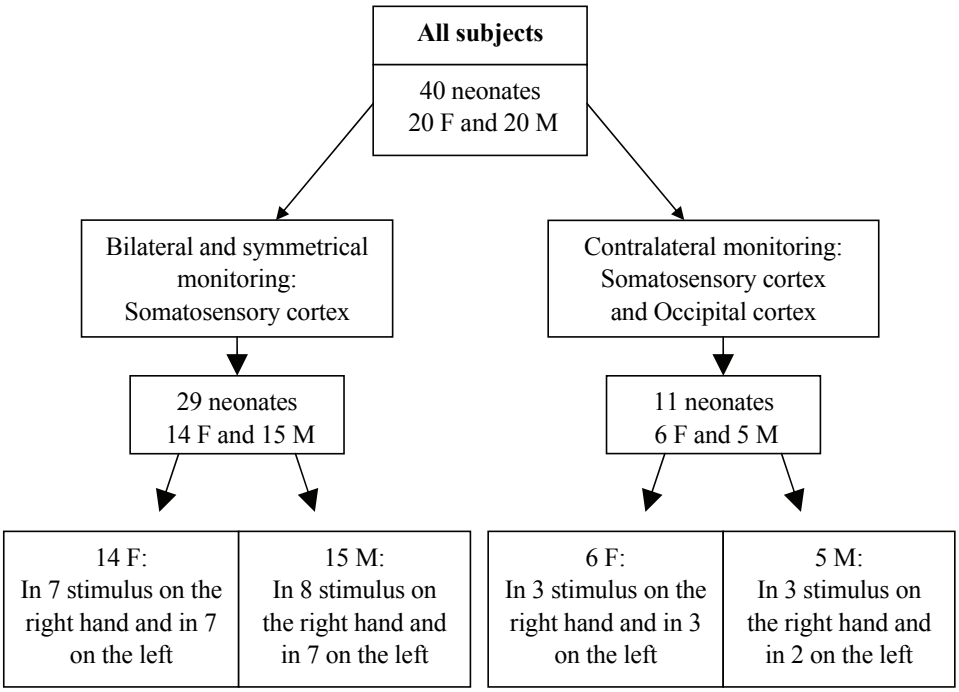


Figure 4.1 Flow diagram describing the study population. (F: females, M: males)

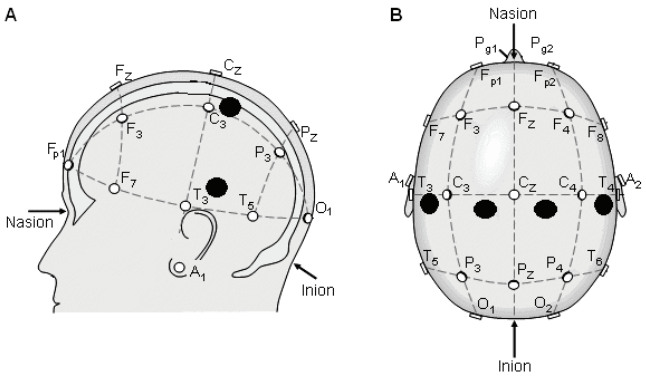


Figure 4.2 *Optode position in study 3 in 29 subjects who were monitored with two couples of optodes (emitter/receiver each couple), positioned symmetrically, bilaterally in order to illuminate the somatosensory cortex. Position of NIRS optodes is indicated by dark circles placed in reference to the international EEG system (open circles). A) Viewed from the left side. B) Viewed from the top.*

The study interval was divided into 3 main periods, period 0 (P_0 = baseline), period 1 (P_1 = tactile stimulus) for cleaning and period 2 (P_2 = painful stimulus) for the venipuncture. Neonates were gently handled in their cot, the NIRS probes were placed on the head and the venipuncture site was identified visually. When the infant was in a quiet, waken and stable condition (behavioral state 3 according to Prechtl) (Prechtl 1974) the various haemodynamic signals including NIRS were recorded for a baseline period of at least 60 seconds (P_0). During period 1 the dorsum of the right or left hand was stimulated for 30 seconds by disinfecting the skin with an alcohol-soaked, cotton pad at room temperature. The onset of tactile stimulation was marked on the NIRS recording and the following 60 seconds were stored (P_1). During this period the neonate was quiet. Venipuncture was performed by an expert neonatal nurse using a standard needle size (24 gauges, 0.75 inch) for all newborns and a 60 second-period following the needle insertion was recorded for analysis (P_2). The environment around the infant during the experiment course was as quiet as possible, any kind of visual, auditory and olfactory external stimulation being reduced. Those newborns that showed prolonged episodes of crying and movements affecting the NIRS signal were not considered for further analysis, thus excluding 6 of the 46 subjects monitored.

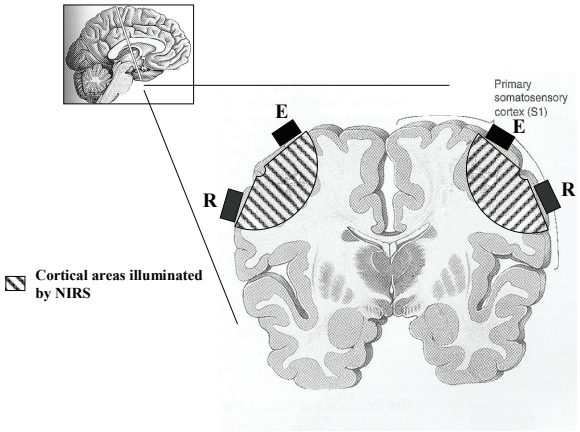


Figure 4.3 *The position of NIRS optodes (E – emitter, R – receiver) viewed with reference to a coronal section of the brain. The NIRS technique does not allow a good spatial resolution of the areas that are illuminated by these optodes. NIRS signal changes depend on the position of the optodes, the type of tissue and the age of the subject. This figure illustrates the cortical regions (dashed areas) illuminated by the near infrared light between the emitter ("E") and the receiver ("R") optodes bilaterally. In the preterm newborn brain, because of a thinner cortical mantle and lower tissue density (scalp, skull, dura and brain), the infrared light may penetrate much deeper, with signals from the primary somatosensory cortex and parts of the secondary somatosensory cortex, insula, cingulate cortex, thalamus, and the amygdala.*

4.3.3 Recording and data analyses

4.3.3.1 Heart rate and arterial oxygen saturations

Heart rate (HR) and arterial oxygen saturation (SaO_2) data were recorded using an HP monitoring system (Hewlett-Packard, Boeblingen, Germany) simultaneously with the NIRS data. Respiration was observed during the procedure. HR and SaO_2 average values were averaged at baseline and at 10, 20, 30, 40, 50 and 60 seconds following the tactile and painful stimuli, respectively. Repeated measures ANOVA and Newman-Keuls post-hoc tests were used to compare these values throughout the

experiment. Differences between males and female were analyzed using the Student's t-test for independent samples.

4.3.3.2 Near Infrared Spectroscopy

NIRS data were sampled every second during the baseline (P_0), tactile (P_1), and pain stimulation (P_2) periods and exported to a computer via an RS232 digital output. As the sampling occurred every second, each of these periods contained 60 numerical values corresponding to the [HbO_2] and [HbH] concentrations. [Hbtot] was calculated off-line by adding the [HbO_2] and [HbH] values (Wyatt et al. 1990a). Values during the period P_0 were subtracted from the P_1 and P_2 values for each neonate to calculate the $[HbO_2]_{diff}$, $[HbH]_{diff}$ and $[Hbtot]_{diff}$ values for tactile vs. baseline and painful vs. baseline periods, respectively. An average for each measurement (from the 60 measured values per period) was calculated for each neonate to describe the haemodynamic changes in the left and right hemispheres during each study period (P_0, P_1, P_2).

Using repeated measures ANOVA and Newman-Keuls post-hoc comparisons, the P_0 curves for each side were compared to the P_1 and P_2 curves of the same side for [HbO_2], [HbH] and [Hbtot]. Student's t-tests for independent variables were used to compare $[HbO_2]_{diff}$ values between the parietal and occipital cortices (in neonates where both sets of optodes were positioned contralateral to the stimuli), to compare $[HbO_2]_{diff}$ values between the ipsilateral and contralateral side, to evaluate inter-hemispheric differences depending on whether the left or the right hand was stimulated, and to compare the differences between male and female infants. Correlations between GA, BW, PNA, duration of the venipuncture and magnitude of the NIRS response were performed by linear regression analyses. Data were analyzed using the program Statistica® (6.0, StatSoft 2001, Inc., Tulsa, USA), and p-values of less than 0.05 were considered as being significant.

4.4 RESULTS

4.4.1 Study group

The neonates were born at 28-36 weeks of gestation (mean 32.0) and were studied at 25-42 hours (mean 30.7) after birth (table 4.2). The painful stimulation lasted for 35-60 seconds. No differences occurred between male and female infants in the GA (males 31.4 vs. females 32.7 wks), PNA (males 32.0 vs. females 29.5 h), BW (males 1841 vs. females 1958 grams), or duration of venipuncture (males 46.7 vs. females 49.8 s). The GA, PNA, BW and duration of the venipuncture were also similar among neonates receiving left or right hand stimulation.

4.4.2 Heart rate and arterial oxygen saturation

During tactile stimulation no significant changes occurred in the HR ($p>0.05$) and SaO_2 ($p>0.05$) values. The HR increased significantly with acute pain (repeated measures ANOVA $p<0.001$) with differences from baseline level noted at 10 and 20 seconds following venipuncture, but not at 30 seconds (baseline 138.5 bpm (±11.5); after venipuncture, at P10: 171.8 bpm (±11.2) and P20: 174.9 (±15.5); post-hoc Newman-Keuls tests, $p<0.001$). No differences occurred in the HR responses between female and male infants or between neonates receiving painful stimulation on the right versus the left hand ($p> 0.05$). The SaO_2 values decreased significantly (repeated measures ANOVA $p<0.0001$), with differences noted from 10 to 40 seconds following venipuncture (baseline 96.1% (±2.3); after venipuncture, at P10 92.3% (±1.9), at P20 89.2% (±1.0), at P30 91.6% (±2.3) and at P40 91.4 (±1.9); *post hoc* Newman-Keuls tests, $p<0.001$). No differences occurred in SaO_2 values between male and female

neonates or between the two sides of the venipuncture ($p > 0.05$). None of the subjects showed apnoeic episodes during the experiment. No correlation between the magnitude of HR increase and GA or PNA was determined.

4.4.3 NIRS data

Neuronal activation within cortical areas is coupled with regional changes in cerebral blood volume (r-CBV), thus reflected in [HbO_2] changes measured by NIRS. We observed increases in the [HbO_2] concentrations in both hemispheres following tactile stimulation, with further increases following painful stimulation (figure 4.4, 4.5).

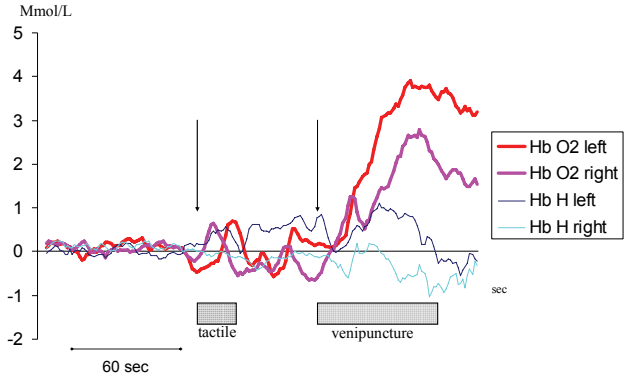


Figure 4.4 *Example of time course of the NIRS data in response to tactile and painful stimuli applied to the left hand in one male subject born at 28 weeks of gestation. He had suffered from a mild respiratory distress at birth and at the time of the experiment (34 hours post-partum) he was in stable condition without need of oxygen. [Hb O_2] concentration changes on the left and right sides are shown, respectively, as red and pink thick lines. [Hb H] concentration changes on the left and right sides are shown, respectively, as in dark and light blue thin lines. The arrows indicate the beginning of the tactile and painful (venipuncture) stimulation, which are indicated by the grey boxes.*

The response to pain was greater in the male neonates than in female preterm neonates ($p<0.05$ for the left hemisphere and $p<0.001$ for the right hemisphere; figure 4.6). Following tactile stimulation no differences were recorded between the [HbO_2] changes on the right and left sides ($p>0.5$ after stimulation of the right hand; $p>0.5$ after stimulation of the left hand). In contrast, venipuncture of the right hand stimulated greater [HbO_2] increases in the left hemisphere as compared to the right hemisphere ($p<0.01$), whereas venipuncture in the left hand resulted in no differences between the right and left hemispheres ($p>0.1$; figure 4.7). The latency between the skin puncture and a significant increase in [HbO_2] compared to the baseline was 2 seconds both in females and in males (repeated measures ANOVA, $p<0.0001$ and post-hoc Newman-Keuls test, $p<0.01$).

The [HbO_2] increases after pain were directly correlated with postnatal age ($r = 0.75$ for the left hemisphere, $p<0.0001$; $r = 0.67$ for the right hemisphere, $p<0.0001$). The magnitude of pain-induced [HbO_2] increases were negatively correlated with the GA ($r = -0.53$ for the left hemisphere, $p<0.01$; $r = -0.42$ for the right hemisphere, $p<0.05$).

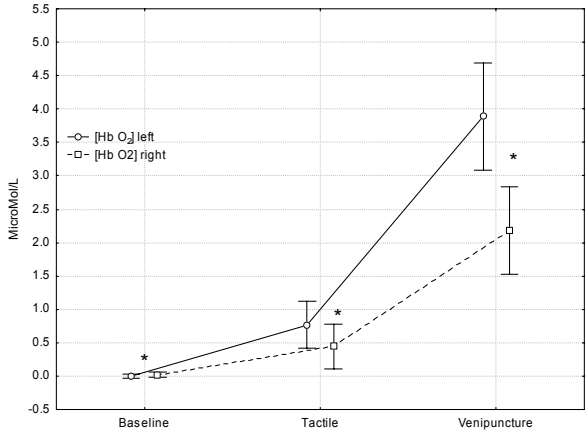


Figure 4.5 *Changes in [HbO_2] values occurring on the left side (circles and continuous line) and on the right side (squares and dotted line) in all subjects, regardless of which hand was being stimulated. The figure depicts significant [HbO_2] increases from baseline in both hemispheres following tactile and painful stimulation (does not denote differences between the two hemispheres) (Wilk's lambda=0.1945, $F_{(4, 36)}$=37.28, p<0.0001; Newman Keuls post-hoc tests: baseline vs. tactile p<0.001; baseline vs. venipuncture p<0.001; tactile vs. venipuncture p<0.001). Vertical bars denote the 95% confidence intervals and the middle points denote the mean values.*

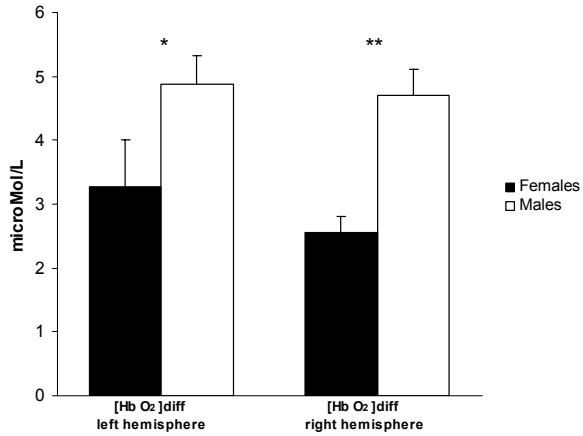


Figure 4.6 *Comparison of the cortical [HbO_2] increases between the female (black columns) and male (white columns) neonates following venipuncture. Males had a more pronounced increase in [HbO_2] compared to females (*p<0.05 on the left and **p<0.001 on the right; Student's t-tests). Columns denote mean values and bars standard deviations.*

No correlation was evident between the [HbO_2] increases and the duration of venipuncture (r = -0.17 for the left hemisphere, p>0.1; r = -0.12 for the right hemisphere, p>0.5), or between [HbO_2] increases and BW (r = -0.34 for the left hemisphere, p>0.05; r = -0.18 for the right hemisphere, p>0.1).

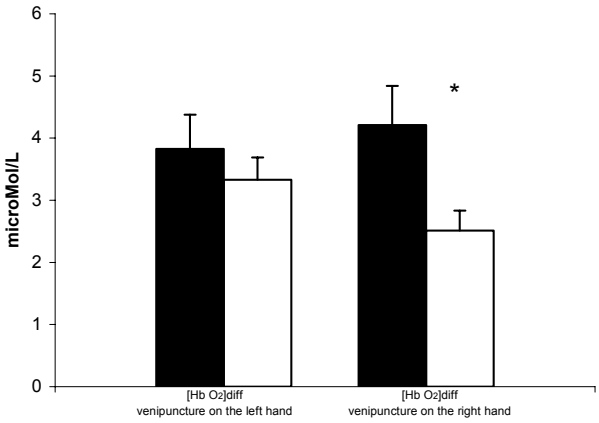


Figure 4.7 *Differences in cortical [HbO_2] changes following venipuncture of the left or the right hands. Black bars denote [HbO_2] increases on the left hemisphere and white bars on the right hemisphere. When the venipuncture occurred in the right hand there was a more pronounced increase in [HbO_2] over the left hemisphere (*p<0.01, Student's t-test), but no significant differences occurred following venipuncture of the left hand (p>0.05). Columns denote mean values and bars standard deviations.*

Neonates monitored with NIRS on the contralateral side (n=11) from venipuncture had significant [HbO_2] increases over the somatosensory cortex (ANOVA p<0.01, baseline vs. tactile p<0.05; baseline vs. pain p<0.01; tactile vs. pain p<0.01, Newman-Keuls post-hoc tests), but no significant changes occurred over the occipital cortex (ANOVA p>0.05; baseline vs. tactile p>0.1; baseline vs. pain p>0.1; tactile vs. pain p>0.5; Newman-Keuls post-hoc tests) (figure 4.8).

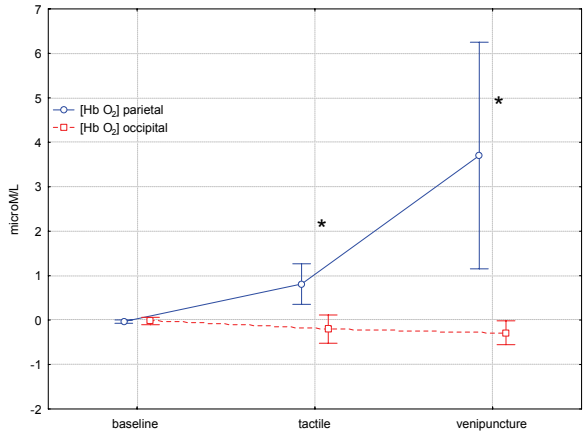


Figure 4.8 *Differences between cortical [HbO_2] values during simultaneous recordings from pairs of optodes located over the somatosensory cortex (continuous line) and the occipital cortex (dotted line). Significant changes occurred over the parietal area (Repeated measures ANOVA, *p<0.01; baseline vs. tactile p<0.05; baseline vs. pain p<0.01; tactile vs. pain p= 0.01, Newman-Keuls post-hoc tests), but not over the occipital area. Data from 11 neonates presented as mean values and 95% confidence intervals (vertical bars).*

4.5 DISCUSSION

In preterm newborn infants unilateral tactile or painful stimuli elicited bilateral increases in cortical [HbO_2] concentrations, implying increases in regional cerebral blood flow that resulted from changes in metabolic activity within the somatosensory cortical areas. Responses to pain were greater in preterm boys than in girls and were greater over the left hemisphere when venipuncture occurred on the right side. The latency between venipuncture and cortical activity was comparable to that of adults (Spiegel et al. 1996) and was similar in male and female neonates. A negative correlation was apparent between pain-induced cortical activity and gestational age, consistent with the ontogeny of pain thresholds in preterm neonates (Fitzgerald et al. 1988), but a positive correlation occurred with postnatal age, consistent with a postnatal decay of fetal inhibition (Greenough et al. 1990).

The main novelty of these findings is that graded cortical responses to tactile and noxious stimuli occur, implying that preterm infants experience pain, and not only nociception. The essential physiological substrate for our study was defined by the early studies of Hrbeck and Karlberg who they showed that somatosensory evoked potentials with distinct, constant components are fully developed by 29 weeks of gestation (Hrbek et al. 1973; Klimach and Cooke 1988). Multiple observations, such as the cortical lateralization, the latency and duration of these responses and their localization to the somatosensory (but not occipital) cortex, support the concept that preterm infants consciously process acute pain.

Bucher and coworkers reported that r-CBV, as reflected by [Hbtot] variations, may increase, decrease or remain unchanged after heel puncture in preterm babies, with wide differences among subjects (Bucher et al. 1995). In contrast, another reported that [Hbtot] and [HbH] increased during crying in newborn infants (Brazy 1988). Unilateral somatosensory activation using NIRS in full-term infants has been reported in abstract form (Meek 2000), whereas functional cortical activity following heelsticks in seven neonates at 29-42 weeks GA was recently presented (Slater et al. 2004). In contrast, our studies included a larger sample size and data were only obtained from preterm neonates, with tightly controlled gestational and postnatal ages.

In our studies, NIRS optodes were placed over the post-central gyrus (figure 4.2, 4.3) bilaterally to illuminate the somatosensory cortex and contiguous cortical areas that are postulated to be involved in pain processing from adult studies (Narsinghani and Anand 2000; Price 2000). We also studied 11 newborns by placing pairs of optodes over the contralateral parietal and occipital cortices to exclude the possibility that haemodynamic changes in the somatosensory cortex may reflect global increases in cerebral blood flow resulting from an increased cardiac output following pain (Morison et al. 2001). Auditory (Sakatani et al. 1999), visual (Meek et al. 1995; Meek et al. 1998) and olfactory (Bartocci et al. 2000) stimuli, which can confound pain-induced NIRS responses were reduced as much as possible during these studies and recordings with movement artefact were discarded.

The changes in HR and SaO_2 following pain are consistent with previous studies (Morison et al. 2001; Van Cleve et al. 1995), but do not explain the [Hb O_2] changes noted over the somatosensory cortex. The [Hb O_2] changes persisted over the entire 60-second period which was analyzed, whereas changes in HR and SaO_2 were transient, lasting only 20-30 seconds. Moreover, SaO_2 were decreased following venipuncture whereas cortical [Hb O_2] values increased significantly. In many neonates, though not reported in the results, the [Hb tot] values remained significantly above the baseline for 2-3 min, well after the changes in HR and SaO_2 had subsided.

Gender differences in pain-related cortical activation also occur in adult subjects. Noxious thermal stimulation, as recorded by PET in the contralateral prefrontal cortex,

insula and thalamus, was perceived with greater intensity and produced greater cortical activity in adult women than in men (Paulson et al. 1998), whereas visceral pain caused greater activation of supraspinal areas in men than in women (Berman et al. 2000). Gender-based differences in pain occur during the neonatal period (Guinsburg et al. 2000), but do not persist in pre-pubertal children (Hogeweg et al. 1996). Female preterm neonates showed more robust facial expressions of pain than did males following acute pain, but no differences occurred with multidimensional assessments of pain in the same study (Guinsburg et al. 2000). The greater cortical activation observed in males, both contralaterally and ipsilaterally to the venipuncture, implies increased neuronal activation in the underlying brain regions. This activation can either lead to surround-activation or surround-inhibition of adjacent neurons (Derdikman et al. 2003), or may be coupled to other neuronal circuits involved in pain modulation or pain affect (Coghill et al. 2003; Hofbauer et al. 2001; Singer et al. 2004). Hormonal differences may also explain these gender-related neurovascular differences in neonates.

Another factor that has to be kept in mind as being a possible explanation of gender-related neurovascular difference is linked to hormonal differences. Reproductive hormones may potentially affect pain perception, potentially including estrogen and oxytocin among others. In adult subjects estrogens play a role in migraine headaches by affecting dural vasculature directly and perhaps indirectly by altering the levels of ionized magnesium (Lichten et al. 1996). Multiple studies report that estrogens can have either excitatory or inhibitory effects within the CNS, mediated via different estrogen receptors activating different types of neurons; for example, with inhibition of EAA-induced activity in the solitary tract nuclei by 17-betaestradiol (Kelly and Levin 2001; Woolley 1999a; 1999b; Xue and Hay 2003). Oxytocin and hypocretin may also modulate sensory input, particularly in regions of the brain and spinal cord related to pain perception and autonomic tone (Bodnar et al. 1984; van den Pol 1999). Further investigations using fMRI or other imaging techniques are required to identify specific structures involved in the gender-based differences of supraspinal pain processing in preterm neonates.

Crying begins to influence cerebral haemodynamics after a time span of about 5 minutes, perhaps related to changes in venous return or intrathoracic pressure, rather than to a functional response. Our observations revealed a functional activation of the somatosensory cortex during the initial, "silent" phase, between the pain stimulus and a behavioural response. The latency and duration of these responses are also consistent with the parallel-serial model of affective pain (Price 2000) and with recent mapping of cortical activity following acute pain in adult subjects (Ibinson et al. 2004; Ohara et al. 2004).

The number of painful procedures in the NICU may determine the patterns of subsequent pain processing in preterm neonates (Johnston and Stevens 1996). We therefore studied all neonates between 25 and 42 h after birth, so that they were not exposed to widely disparate numbers of painful stimuli. A gradual loss of fetal inhibition or an increased excitability resulting from early pain exposure may explain why the postnatally older newborns responded with more pronounced increases in [HbO_2] (Liu et al. 2004; Taddio et al. 2002). Multiple lines of clinical evidence, anatomical and neurophysiological data indicate lower pain thresholds in early development (Anand 1998; Fitzgerald et al. 1988), consistent with greater cortical [HbO_2] responses in more immature preterm neonates following venipuncture.

Bilateral activation of cortical areas occurred in all infants, regardless of the side of venipuncture. We postulate that the NIRS beam was not exclusively confined to the S1 area, but also sampled other regions such as the S2 cortex/anterior insula, the ventral premotor area and the anterior cingulate cortex, areas that are implicated in bilateral pain processing (Coghill et al. 1999; Ibinson et al. 2004; Ohara et al. 2004). Changes in

the NIRS signal are coupled to neuronal firing activity in synaptic terminals, regardless of whether excitatory or inhibitory neurotransmitters are released, therefore any correlation with pain intensity or other perceptual qualities of acute pain is speculative and cannot be based on these data.

There is widespread uncertainty about the cognitive ability or sensory awareness of newborn infants (Zelazo 2004). Clinicians or scientists may characterize premature infants essentially as unconscious automata, only capable of reflexive responses (Anand et al. 1999; Zelazo 2004). The lateralization of pain processing, the latency and duration of these responses, their gradations across gestational age and postnatal age, and the neuroanatomical location of these responses (parietal versus occipital), suggest that preterm infants may be consciously processing acute pain from venipuncture.

From a constructionist perspective, according to Edelman, of the conscious processing of pain (in its widest meaning) the supraspinal processing of noxious stimuli implying activation of cortical neuronal circuits is the first step towards the formation of the primary consciousness (Edelman 2004; Seth and Baars 2005; Seth et al. 2005). We assert that beginning from neonatal life and probably before the third trimester of gestation, brain cortical structures start to build re-entrant loops connecting value-category memory to current perceptual categorization. The re-entrant linkage (to frontal, temporal and parietal areas and in turn to amygdala and hippocampus) is the crucial evolutionary development that results in primary consciousness (Edelman 2004). We would like to emphasize that the awareness of pain in preterm infants has been neglected for decades since the introduction of modern neonatal care and must be taken into consideration given the enormous repercussions that early pain can have in later life (Anand 2000; Anand and Scalzo 2000).

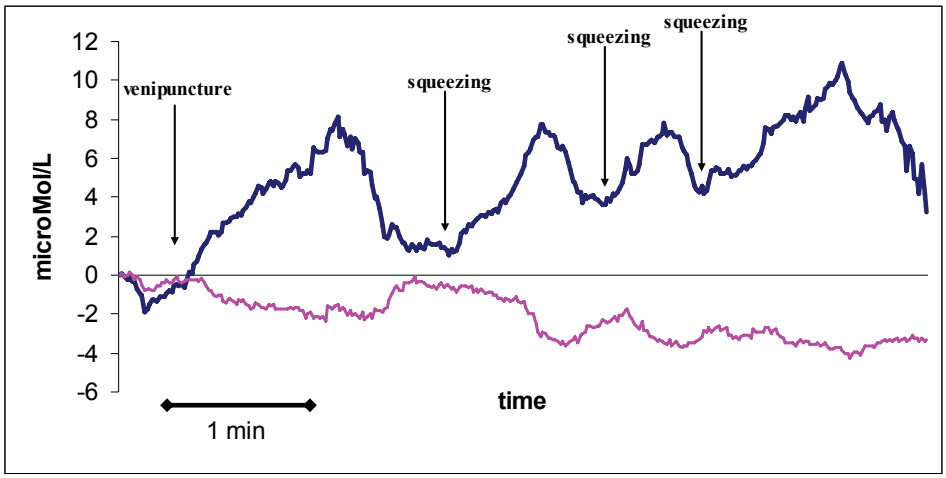


Figure 4.9 *This figure shows the [HbO_2] (blue line) and [Hb H] (pink line) during a heel lancing. The NIRS optode was placed onto the left somatosensory cortex. The heel lancing was performed on the right foot. Any squeezing of the heel (arrows) corresponds to an increase in the [Hb O2] and a slight decrease in [Hb H] suggesting cortical pain processing. (Data not published).*

In conclusion, using a non-invasive technique to monitor preterm neonates, we determined that tactile and painful stimuli specifically activate somatosensory cortical areas. Male neonates show greater responses to pain than the female neonates, whereas all preterm newborns at lower gestations and older postnatal ages showed progressively

increasing cortical responses to venipuncture. We propose that collateral pain circuits are activated simultaneously in the preterm neonate. Future correlations with behavioural responses and also with other methods of studying functional cortical activity will elucidate the clinical importance of these findings.

5 STUDY ON AUDITORY PERCEPTION AND ANAESTHESIA – STUDY 4

5.1 FUNCTIONAL STUDIES ON AUDITORY PERCEPTION IN INFANTS

While perceiving smell or pain is essential for the infant, enjoying music is regarded as an epiphenomenon not essential for survival. Nevertheless music has en extraordinary ability to evoke powerful emotions and psychological and physiological effects on humans. When a sound, speech or a melody is perceived by normal subjects, it elicits a cortical response in different areas of the cerebral cortex. It is evident that the activation of cortical neurons is somehow related to haemodynamic changes recorded in that area. The mechanisms that couple haemodynamic changes and neuronal function during auditory stimulation either basic sounds or more complex melodies are still not yet fully understood.

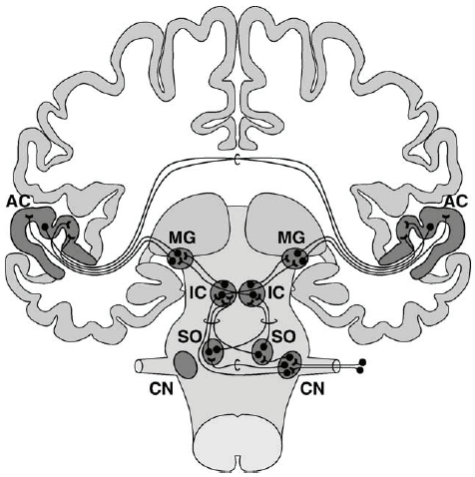


Figure 5.1 *The major processing centres of the central auditory system. From (Langers et al. 2005) See text for comment.*

Neural impulses generated in the inner ear are brought to the lower brainstem via the cochlear nerve through the ipsilateral cochlear nucleus (CN). From the CN, impulses travel along at least two auditory pathways (one analyzing the content and one the location of the sound). From the dorsal subdivision of the dorsal nucleus the *monaural* pathway originates. Here spectral features of the incoming sound are enhanced and fed directly to the inferior colliculus on the contralateral side. In parallel, nerve fibers of the binaural pathway project from the anterior ventral subdivision of the CN towards the ipsilateral as well as the contralateral superior olivary complexes (SO). Its medial and lateral subdivisions are involved in the detection of temporal differences (time delay) and intensity of the sound stimulus between the left and the right ears. The outgoing signals are transmitted upwards to the inferior colliculi (IC). Both excitatory input from the contralateral ear and inhibitory input from the ipsilateral ear convey to the IC, which is the brainstem target of almost all auditory pathways, and where important integrative information is processed (sound frequencies, integration of cues for sound localization). From the inferior colliculus via the medial geniculate (MG) complex of the thalamus in the

rostral midbrain, the auditory pathway projects to the primary auditory cortex (AC) in the upper surfaces of both temporal lobes. Through the corpus callosum it is possible that information is interchanged between the left and right hemispheres (Langers et al. 2005; Moore 1991; Nieuwenhuys 1984). The human primary auditory cortex is located deep within the lateral fissure on a small patch on the transverse gyrus of Heshl, having distinctive cyto- and myelo-architectonic features (Galaburda and Sanides 1980; Howard et al. 2000; Roland 1997; Seldon 1981a; 1981b). It is known that the auditory cortex is tonotopically organized (Pantev et al. 1995; Pantev et al. 1988). Recent data have demonstrated the existence of multiple interconnected auditory areas in the superior temporal gyrus similar to those areas previously described in other primates (Howard et al. 2000; Merzenich et al. 1975), although the location, boundaries and organization of these areas vary considerably between subjects (Merzenich et al. 1975).

Recent investigation of the development of auditory cortices in fetuses and newborns show that from the 16th week of gestation to the 4th postnatal month the cortex progresses from a marginal layer and an undifferentiated cortical plate to incipient lamination (Moore and Guan 2001). Probably starting from the 22nd fetal week a two-tiered band of neurofilament-immunoreactive axons develops in layer I. During the subsequent period to the 4th postnatal month the number of immunopositive axons in this layer is greatly reduced. Between the middle of the first year of life and age 3 years the laminar pattern of cytoarchitecture becomes fully mature and a network of immunostained axons develops in layers VI, V, IV, and IIIc. This axonal plexus in the deep cortical layers continues to increase in density until age 5. By 11-12 years of age, overall axonal density is equivalent to that in young adulthood. The emergence of auditory cortical function is thus a long process that already starts *in utero* around the 16th week of gestation. From the 29th week of gestation a decrease of the latency in the response to auditory stimulation has been observed (Holst et al. 2005; Lengle et al. 2001), suggesting a more prompt responsiveness to the stimuli (Fellman and Huotilainen 2006).

5.2 NIRS APPLIED TO FUNCTIONAL STUDY OF AUDITORY PERCEPTION

Recent studies have demonstrated the reliability of NIRS in monitoring haemodynamic changes in the fronto/temporal region during auditory stimulation in both adults (Minagawa-Kawai et al. 2002; Ohnishi et al. 1997; Sakatani et al. 1998), infants (Bortfeld et al. 2006) and neonates (Chen et al. 2002; Saito et al. 2006; Sakatani et al. 1999; Zaramella et al. 2001). In their study, Sakatani and co-workers used as auditory stimulation the sound of a popular piano (60 db) in a population comprising 28 preterm and term newborns. The sound was presented by earphones to the baby lying in their bed, and no particular discomfort for the babies who underwent the study was reported (Sakatani et al. 1999). Zaramella and co-workers have also assessed auditory cortical haemodynamic response in newborns and determined a similar NIRS pattern (Zaramella et al. 2001). In their study they applied an Audiometric sound stimulation was a tonal sweep with a frequency increasing from 2 to 4 kHz, intensity 90 dB SPL applied 5 cm away from the external auditory meatus. Despite that the stimulation was relatively short, the intensity was rather high, when compared to normal sound levels in the NICU. In this study the response recorded by NIRS was a marked increase in [Hb O_2] and [Hb tot].

Our preliminary results obtained by exposure of newborn infants in NICU to mother's voice, also confirmed that NIRS is a suitable technique to assess activation-related haemodynamic changes is response to auditory stimulation (figure 5.3).

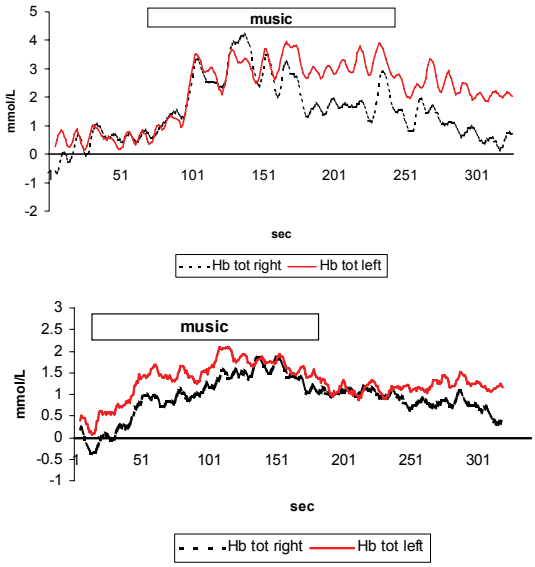


Figure 5.2 *Changes in [Hb tot] on the left and the right hemisphere in response to auditory stimulation as assessed by 2 pairs of NIRS optodes located bilaterally and symmetrically onto the parietal region in two adult subjects (personal data, not published)*

The idea to conduct a study in infants during anaesthesia was primed by recent reports that patients undergoing general anaesthesia for elective surgery are able to perceive and remember sounds to which they were exposed during the operation (Liu and Tan 2000). Moreover, the latest studies have also shown the beneficial effect of intra-operative exposure to music (Kain et al. 2001; Kiviniemi 1994; Lepage et al. 2001; Nilsson et al. 2001).

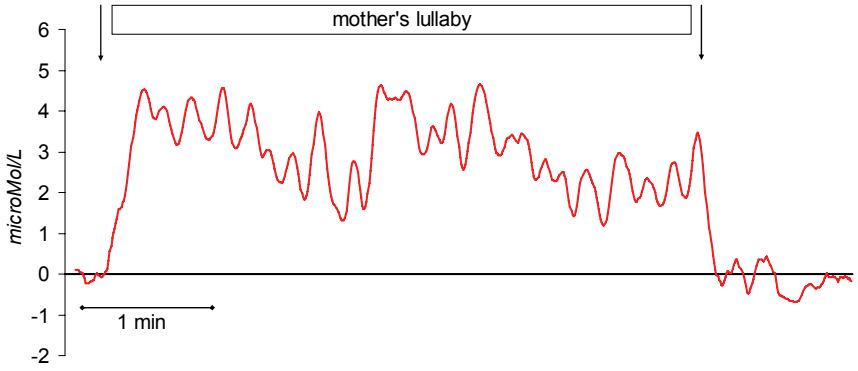


Figure 5.3 *Changes in [Hb tot] in response to auditory stimulation as assessed by 1 pair of NIRS optodes located onto the left parietal region in a preterm newborn born at 30 weeks of gestation. The mother was asked to sing a lullaby close to the incubator. NIRS recorded optical density changes. Hb tot changes are shown during a period of about 5 min (personal data, not published)*

5.3 CEREBRAL ACTIVATION, CONSCIOUSNESS AND SENSORY STIMULATION

As anaesthesia is by definition the loss of sensation and conscious awareness, we considered it extremely interesting to assess auditory sensory responses in young infants undergoing anaesthesia for elective surgery. When interventions such as anaesthesia, induce a decrease in level of consciousness, an assessment of awareness and mental status is not possible, and instrumental monitoring of brain oxygenation becomes essential. The most recent studies of awareness measured by post-operative recall were conducted in the United States, and showed an occurrence of 0.13% (Sebel et al. 2004). This data was comparable to those reported in other countries (Sandin et al. 2000). In newborns and infants it is extremely difficult to assess the level of awareness during anaesthesia, much more than in older subjects who are able to report to the clinician during the postoperative period sounds or noises or words that they might eventually remember or have perceived (i.e. recall test).

Despite that several studies have focused on the molecular and cellular actions of anesthetic drugs, very little is known how the brain responds to them and to what extent they may be affected by basic processes to integrate sensory information such as learning. It is also been demonstrated that during general anaesthesia the human brain may be primed or at least temporally activated by auditory information. Priming during anaesthesia seems to be a significant phenomenon and is part of the evidence that unconscious processing may occur in the brain (Andrade 1995; Andrade et al. 1994) (Ghoneim & Block, 92, 97).

Different groups have recently used imaging techniques to assess brain metabolic and functional responses to anaesthesia. Alkire and coworkers have demonstrated that thalamus metabolism drastically decreases during anaesthesia, suggesting unconsciousness (Alkire et al. 2000; Alkire et al. 2005). The right prefrontal and parietal cortices seem to be involved in anaesthesia-related amnesia (Veselis et al. 2002). Kerssens and collegues reported that 1% sevoflurane can induce suppression of auditory induced activation (Kerssnens et al., 2005).

How anaesthesia interacts with brain basic haemodynamic auto-regulation is still unclear. It is also unclear how anaesthetic drugs can affect sensory processing ability. Induction of anaesthesia represents a particularly interesting circumstance that suits perfectly to this kind of study. In fact, in a relatively short time span a young infant passes from the conscious to what we think is an unconscious status.

Very recently NIRS has been used to assess cortical activity in infants ages 6 to 9 months during exposure to linguistic stimulation paired with visual stimuli. One finding of the study was that [HbO_2] increased as registered by the optodes positioned over the left temporal region during the auditory stimulation (Bortfeld et al. 2006).

5.4 AIM

Taken together all these previous findings support the main hypothesis of this research: that infants might also be sensitive to auditory stimuli during anaesthesia. To test this hypothesis we presented an auditory stimulation before and after the induction of anaesthesia to young infants and we simultaneously recorded activation-coupled haemodynamic changes using NIRS. The aim of the study was to assess differences in activation pattern in response to auditory stimuli before and after the induction of anaesthesia with sevoflurane.

5.5 METHODS

5.5.1 Inclusion criteria

All infants below 2 years of age undergoing elective abdominal or thoracic surgery at Astrid Lindgren's Hospital have were eligible for inclusion in the study according to the following criteria: born full-term, absence of major anatomical malformation, absence of PV-IVH, no history of perinatal asphyxia, no need of mechanical ventilation prior the operation, no history of auditory problems and no familiarity for congenital deafness.

5.5.2 Procedure

5.5.2.1 Study group

Participants were 7 infants between 8 and 24 months, 4 male and 3 female, mean age 14.3 months (SD 7.02), (mean male age: 14.5, mean female age: 13.3) (table 5.1). Two additional infants were tested, but eliminated from the sample because of large artefact in the NIRS trace mainly due to movements. Parents were approached by telephone and then two investigators have a meeting where they explained the experimental course and aim of the study. We did not have any refusal from the parents that we have contacted.

Case	Sex	Age (months)	Type of operation
1	F	23	Soave
2	M	9	Plexus brachialis
3	F	8	Plexus brachialis
4	F	9	Plexus brachialis
5	M	16	Vescicostomi
6	M	9	Homerus fracture
7	M	24	Hernia inguinalis

Table 5.1 *Study population*

5.5.2.2 Approach to the infant and optode position

After pre-medication had been given, the patient was transported to the operation room. The parents were allowed to be present although not talking to their child, nor giving any sort of sensory stimulation (e.g. touching). The NIRS optodes were positioned being the International 10-20 EEG system. The emitter was located slightly anterior to the T3 and T4 on the left and right sides of the head, respectively. The receiver was 4 cm apart slightly posterior to T3 and T4, respectively, onto the left and right temporo/parietal regions of the skull (figure 5.4). Earphones were comfortably placed and connected to a CD player (figure 5.5).

5.5.2.3 Auditory stimulation

The noise level will not exceed 50 dB, rather below the limit of 60 dB suggested by British and American Neonatal Society (Nzama et al. 1995; Oehler 1993). For this study we chose to use as auditory stimulus a classical melody from G. F. Händel: *Water music Suite No. 1 in F major*, HWV 348: no 6 (Academy of St. Martin-in-the-fields; Sir Neville Marriner-Conductor; Grammy Foundation, 2000). All parents agreed in choice of this melody for their children.

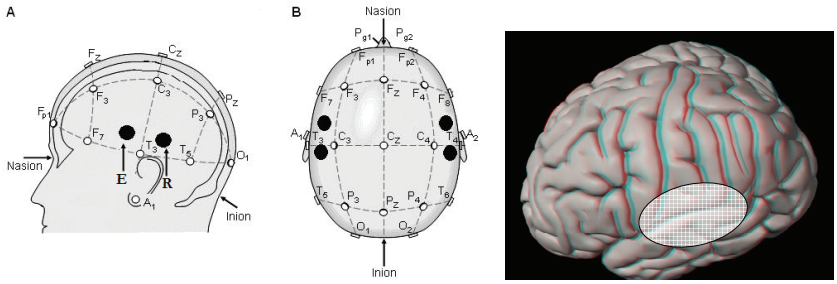


Figure 5.4 *Optode position during the NIRS and anaesthesia study. E, emitter; R, receiver. A: view from the left side; B: view from the right side. The panel on the left shows the brain cortical area that is likely illuminated with the adopted optode position.*

5.5.2.4 Induction of anesthesia

Under controlled condition an air oxygen mixture via a circle system (face mask connected to a semiclodsed anaesthetic circuit) with an increasing dose (1% → 2% → 3%) of sevoflurane was administered to the child. The stepwise increases of the gas concentration are marked on the NIRS recording. A facemask was applied and held manually by the anaesthetist. Haemodynamic responses to the different gas concentration were followed by NIRS.

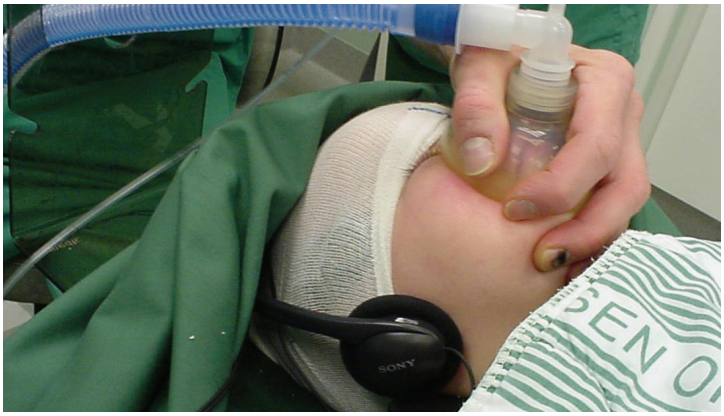


Figure 5.5 *The infant was comfortably lying on the operation table. The auditory stimulus was delivered through earphones. The anaesthetist was sitting just behind the infant's head. The parents were allowed to sit by the side of the operation table.*

5.5.2.5 Haemodynamic parameters and analysis

By use of a HP monitoring system (Hewlett Packard, Germany) heart rate (HR), end tidal volume CO_2 (etCO2) and end tidal gas concentration (etSEVO) were monitored during the experiment simultaneously with the NIRS data. Respiration was observed during the procedure and was calculated visually. Four data for each test period – P0, P1 and P2 – were calculated for HR, saO2, EtCO2 and used for statistical analysis. The Wilcoxon matched pairs nonparametric test was used to compare values throughout the experiment.

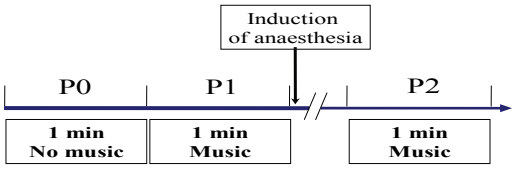


Figure 5.6 *Experimental course. See text for details.*

5.5.2.6 NIRS recording and analysis

At least 1 minute NIRS trace was collected while no music was played. This period was used as baseline. During period P1 the child was calm, sleeping, not dreaming (no REM) and was exposed to the music. During P2 the child was under anaesthesia and exposed to the same piece of music as in P1. During all experiments extra sensory stimulation was avoided. Movements of the child were marked on the NIRS trace.

NIRS data were sampled every second during the baseline (P0), music (P1), and music under anaesthesia (P2) periods and exported to a computer via an RS232 digital output. As the sampling occurred every second each of these periods contained 60 numerical values corresponding to the $[HbO_2]$ and [HbH] concentrations. [Hbtot] was calculated off-line by adding the $[HbO_2]$ and [HbH] values (Wyatt et al. 1990a). Analysis of variance was used to calculate differences between the three periods P0, P1 and P2. The Newman-Keuls post-hoc comparison was then used to analyse differences between each single period P0 vs. P1; P1 vs. P2 and P2 vs. P0. Values during the period P0 were subtracted from the P1 and P2 values for each neonate, to calculate the $[HbO_2]_{diff}$, $[HbH]_{diff}$ and $[Hbtot]_{diff}$ values for [P1-P0] and [P2-P0] and [P2-P1] periods, respectively. To assess possible lateralization the Student's t-tests for independent variables was used to compare $[HbO_2]_{diff}$ values of the left and right hemispheres. We used the program Statistica® (6.0, StatSoft 2001, Inc., Tulsa, USA), and p-values of less than 0.05 were considered significant.

5.6 RESULTS

5.6.1 Haemodynamic parameters

SaO_2 and RR did not change significantly during the test. HR did not change significantly except between P1 when we observed a slightly but significant increase ($p=0.04$, Wilcoxon matched pairs Test). $EtCO_2$ remained fairly stable during the test, although a minor decrease was recorded between P0 and P1 ($p=0.03$, Wilcoxon matched Pairs Test).

5.6.2 NIRS data

We have mainly considered $[HbO_2]$ changes for analysis, as it has been previously shown that these are a reliable indicator of cortical activation (Bortfeld et al. 2006). During the three periods of the test ANOVA gave significant differences (p

<0.001) on both left and right sides (figure 5.7). During P0 (no music) with the infant in a resting condition (sleeping, no REM), we did not observe significant changes of [HbO_2], in either the left or right hemispheres (mean [Hb O_2] on the right 0.05 mmol/l ± 0.17; mean [Hb O_2] on the left 0.08 mmol/l ± 0.34). During P1 [Hb O_2] increased significantly: mean [Hb O_2] on the right 1.54 mmol/l ± 0.87 SD; mean [Hb O_2] on the left 1.70 mmol/l ± 1.05 SD. Post-hoc comparison (Newman Keuls) revealed a significant difference between [Hb O_2] changes during P1 and P0 on both the right and left side ($p<0.001$). No significant changes of [Hb H] were observed during all 3 periods. [Hb tot] changes followed [Hb O2] changes. During P2 [HbO_2] increased significantly on the left side (mean 1.46 mmol/l ± 0.32 (SD), although the increase was less pronounced then in P1. On the contrary, we noted a decrease in [Hb O_2] on the right side during P2 (mean -0.35 mmol/l ± 0.32). Post-hoc comparison (Newman Keuls) revealed significant differences between [Hb O_2] changes during P2 and P0 on both the right and left sides ($p<0.001$) as well as during P2 and P1 on the right side, but not on the left side. This finding was confirmed when comparing $[HbO_2]_{diff}$ on the left and right side. There was no significant difference in the magnitude of the increase during P1 between left and right hemispheres. During P2 there was a significant difference between the magnitudes of the [HbO_2] increase on the left side compared with the right side.

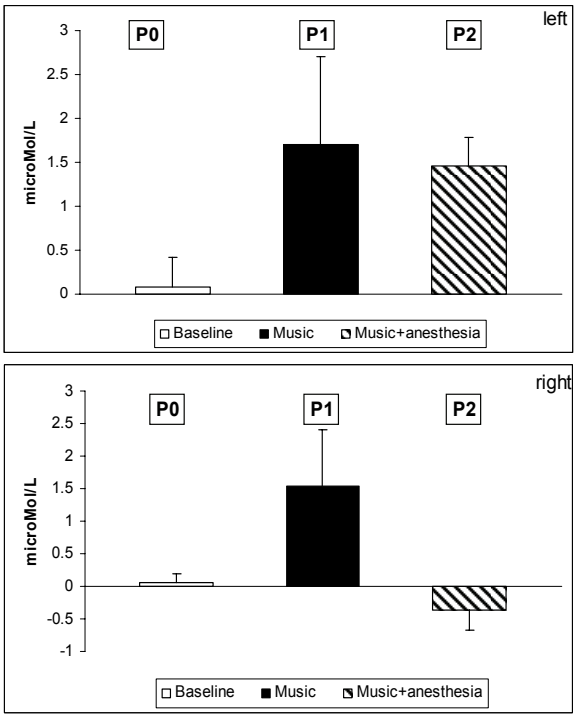


Figure 5.7 *[Hb O_2] changes during the different phases of the test. Mean and SD are shown. Top panel shows [Hb O_2] changes occurring on the left auditory areas; bottom panel shows [Hb O_2] changes occurring on the right auditory areas.*

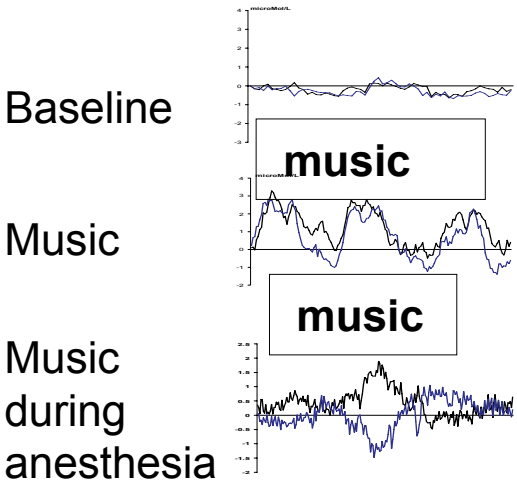


Figure 5.8 *Explicative case. Top panel depict NIRS small fluctuations in [Hb O_2] in absence of auditory stimulation. Middle panel describes [HbO_2] increase during musical stimulation without sevoflurane. Bottom panel shows [Hb O_2] changes during musical stimulation under the effects of sevoflurane. Changes in [HbO_2] are expressed in microMol/L (Y axis). Changes in [HbO_2] on the left are indicated in black; changes in [HbO_2] on the right are indicated in blue.*

5.7 DISCUSSION

The main finding of the study was that music stimulation elicited a bilateral HbO_2 increase during sleeping similar to that previously reported in concious subjects (Sakatani et al. 1999; Zaramella et al. 2001) suggesting that the infant perceives the auditory stimulus and likely processes it. Additionally, when the infant is anaesthetised exposure to the same auditory stimulation could still be perceived as suggested by the increase of [HbO_2] in one hemisphere.

It has been said that the human brain has an intrinsic interest in music (Walker 1979). This concept has developed throughout the last 2 decades, i.e. since functional studies have impressively demonstrated the profound cortical responses that music elicits in brain structures. Despite the biological significance of music and its properties during anaesthesia, there are few functional studies that have addressed music processing in children (Overy et al. 2004).

We preferred to assess cortical responses to a likely pleasant melody, such as the Water music by Handel, instead of assessing single and simpler sounds or pitches, firstly because we consider this kind of music may sooth the infant during the first phase of the experiment. We are aware that the response to such a complex stimulus is much more difficult to interpret from both a qualitative and quantitative point of view. The optode position that we have chosen likely allowed illumination of the area of the superios temporal gyrus (figure 5.4), which in previous studies with fMRI and PET was found to be significantly activated during melody processing (Overy et al. 2004)Zatorre 94. NIR technique reliability has been also demonstrated by comparison with event-related potential (ERPs) to studying language function (Horovitz and Gore 2004).

5.7.1 Auditory processing during pre-anaesthesia

When the infants were exposed to music during P1 they were sleeping. No REMs could be noted. Yet we clearly recorded a bilateral increase in [HbO_2] in both hemispheres, suggesting cortical processing. This finding that we observed in all subjects, was in accordance with previous data where NIRS was used to assess brain activation in neonates (Sakatani et al. 1999). The question of what this increase means still remains unanswered. The perception of sound is a complex process that involves many cortical areas and pathways (Belin et al. 2000). Conversely, music has been known for a long time to be closely related to our subconscious. Walker raised the question of whether music may "have an unconscious content" (Walker 1979). Nevertheless the ability of the brain to perceive sound and speech during an unconscious state has been demonstrated (Koelsch et al. 2006b). In this perspective our findings corroborate the belief that when the child falls asleep they have the capacity to perceive auditory stimuli, suggesting that the gap between conscious and full unconscious states may comprise of several steps during which cortical processing ability is still preserved.

According to fMRI studies children differ from adults in music processing (Koelsch et al. 2005). The mean age in our study was 14.3 months which represents a transitional dynamic developmental phase of the auditory areas. In fact from about the age of 5 months, mature connections grow in the lower infragranular layers of the auditory cortex, reaching the adult conformation at the age of 5 years (Moore and Guan 2001). Concerning these developmental phases there is solid evidence from AEPs studies indicating the maturational process depends on cortical synaptic activity and is influenced by specific auditory experiences (Eggermont 1992; Eggermont and Ponton 2003; Trainor et al. 2003). Adults and older children seem to process melodies and rhythms differently than do younger infants, being more "sensitive" in the right hemisphere to melodies and in the left to rhythm. In our study there were no differences in the magnitude of the activation measured as increase in [HbO2], between the left and the right cortices, thus suggesting any sign of lateralization. This may support the hypothesis that hemispheric specialization of music processing may develop later in life (Overy et al. 2004; Trainor and Trehub 1992).

One aspect that should also be considered is the emotional component of music processing. As we tested only one type of melody we do not know whether the infant perceived it as being pleasant or unpleasant, and we can only speculate that emotion processing might play a role in the activation pattern that we observed. Typically, consonant music such as the Water music should be perceived as being pleasant. Several cortical areas seem to be particularly active during pleasant music processing (Koelsch et al. 2006a), among which the Rolandic operculum, the Heschl´s gyrus and insula are areas that were illuminated in our setting. We do not know whether NIR light has penetrated deep enough to also illuminate the parahyppocampal cortex and amygdale that have been seen to be highly involved in hedonic processing of music (Gosselin et al. 2006).

5.7.2 Auditory processing during anaesthesia

It has been recently demonstrated that sevoflurane can markedly attenuate brain responses to repeated auditory word stimulation in in adults, as indicated by BOLD fMRI (Kerssens et al. 2005). This response suppression was seen in a dose dependent manner. What also emerged from Kerssens' study was that recognition memory performance following exposure to auditory stimulation under different sevoflurane concentrations did not show a dose-dependent pattern. In our experiment we observed an increase in [HbO_2] during musical stimulation in the left hemisphere, and a

decrease in the right hemisphere. Our data support the hypothesis that even during deep anaesthesia cortical structures, likely including STG, insula, middle temporal gyrus, may be involved in music processing. While it is difficult to explain with fMRI data why subjects undergoing deep anaesthesia are able to remember sounds, our data might help in understanding of the underlying mechanisms of this phenomenon. According to the latest studies there is evidence that perceptual priming during anaesthesia can occur without conscious awareness (Deeprose and Andrade 2006). According to our data left hemispheric structures located in the temporo-parietal region may be responsible for perceptual auditory implicit memory.

We conclude that sevoflurane attenuates cortical responses to music stimulation. Nevertheless, during deep anaesthesia infants demonstrated an increase in the left hemisphere suggesting music processing, which may be associated with perceptual memory and learning. The decrease in the right hemisphere might suggest deactivation, but this is just a speculation and needs further studies.

6 CONCLUSION AND FUTURE DIRECTIONS

The NIRS method can detect blood flow fluctuations in the cerebral micro-vasculature, that are coupled with cortical neuronal activation in newborn and adult subjects (Bartocci et al. 2000; Benaron et al. 2000; Kleinschmidt et al. 1996; Meek et al. 1998; Obrig and Villringer 1997; Sakatani et al. 1999).

These studies and other studies that have been carried out in parallel by other groups, demonstrate that NIRS is a suitable technique to assess cortical activation in response to varying forms of sensory stimulation in human infants. The technique is likely to play an important role in providing new insights into the ontogeny of cortical function, as well as possibly providing a sensitive means for the early detection of perinatal cortical impairment.

NIRS as well as other functional techniques, cannot identify the emergence of emotions, but can recognize cerebral haemodynamic correlates of emotion-processing over time. In this perspective haemodynamic techniques can be applied to the study of the emergence of consciousness. I our first studies we have provided information that sensory stimulation such as smell, pain and acoustic are processed at a cortical level. We do not know whether the newborn infant, particularly the preterm is subjectively aware or conscious about smell or pain, but I have demonstrated in this thesis that these modalities are processed in the cortex where we believe the consciousness is handled. This can have profound developmental implications in both healthy and sick neonates treated in NICU. In our fourth study we have shown that despite deep anaesthesia and loss of apparent consciousness, the infant is able to process, sensory (music) stimulation. These data enhance the idea that cognitive functions may exist also during anaesthesia.

Future studies should essentially be aimed in three directions: i) technical implementation of the device. To develop easy-to-use multi-channel NIRS devices would enormously help the understanding of haemodynamic-related neuronal activation in neonatal population, as these subjects are not easy to assess by other functional techniques. ii) Methodological improvement by studying simultaneously with two techniques which explore both the haemodynamic-related events (NIRS) and electrical events (EEG, aEEG) in order to better understand coupling mechanisms and improve spatial resolution. iii) Repeated tests on the same subjects exposed to the same kind of sensory stimulation, both in short and long run, in order to understand how much can be retained in their brain and influence future developmental junctures.

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