

Linköping University Medical Dissertations  
No. 943

# Stress in infants and parents

Studies of salivary cortisol, behaviour and  
psychometric measures

**Evalotte Mörelius**



**Linköpings universitet**  
**FACULTY OF HEALTH SCIENCES**

Department of Molecular and Clinical Medicine  
Division of Pediatrics, Linköping University  
581 85 Linköping, Sweden  
2006

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Linköping University Medical Dissertations: No. 943

ISBN: 91-85497-78-9

ISSN: 0345-0082

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Printed in Sweden by LiU-tryck, Linköping, Sweden, 2006

# Preface

Imagine that we could say that all preterm infants born in week 25 behave in a certain manner and need a well-defined amount of nursing and medical care to achieve a special outcome. How easy it would be to work with infants if they all behaved in the same way and needed the same kind of care. However, the charm and the beauty of caring for preterm and newborn infants is the individuality of each infant.

Researchers today are challenged by identifying ways to assess preterm infants in terms of current strengths, vulnerabilities, prognosis, and recommendations for treatment, support, and care. In this thesis I focus on the stress response. The stress response is studied in infants and parents during routine neonatal care. However, "...good experimental work with complex human behavior - involving, as it does, incomplete control of the subject, his life history, and his environment - is difficult, tantalizing, and frustrating" (Nowlis & Nowlis 1956, p. 345). This is a beginning and from here on it is important to continue and further investigate the concept of stress in newborn infants.

Seymore Levine has written: "The neonate plays by different rules than the adult" (Levine 2000, p. 156). However, I would like to add: the preterm infant plays by different rules than the full-term infant.

Linköping March 2006  
Evalotte Pålsson Mörelius



I would like to dedicate this book to all the infants of the world.

To myself,

One step closer to knowing  
One step closer to knowing  
One step closer to knowing  
To knowing, to knowing, to knowing

Bono



# Abstract

The life of a preterm infant admitted to a neonatal intensive care unit may be stressful from the moment of birth. Ever since Hans Selye's initial characterisation of the biological stress response, cortisol has been frequently measured as an indicator of stress responsivity. However, research of the stress response and cortisol in infants, especially those who are preterm and/or ill, has been scarce basically because of methodological issues.

The first aim with this thesis was to investigate the acute stress response, as measured by salivary cortisol and behaviour, for preterm infants, healthy infants, and infants at high psychosocial risk in response to certain defined handling procedures. The second aim was to investigate the stress response, as measured by salivary cortisol and psychometric measures, for parents present during the handling procedure of their infants. The intention was to perform all investigations in an as naturally occurring situation as possible, which means that the studied procedures would have been performed irrespectively of the research.

The present thesis includes six original articles. The results of the first study demonstrate that it is feasible to collect sufficient amounts of saliva and to analyse salivary cortisol in neonates using the presented method of collection and analysis. The second study shows that preterm infants, usually cared for in incubators, show no signs of discomfort and have variable cortisol responses during skin-to-skin care with their mothers. The mothers, however, experience stress and low control before their first skin-to-skin care with their preterm infant and do not relax completely until after the session. In the third study we found that preterm infants have higher baseline salivary cortisol as compared to healthy full-term infants. Moreover, preterm infants have higher and sustained pain response during a nappy change as compared to healthy full-term infants. The results of the fourth study shows that infants younger than three months, living in psychosocial high-risk families, have increased cortisol responses during a nappy change, performed by the mother. However, support with the aim of improving mother-infant interaction, dampens the stress response. The results of the fifth study show that oral sweet-tasting solution in combination with a pacifier dampen the levels of the stress hormone cortisol in three months old infants during routine immunisation. Moreover, parents experience more self-rated emotional stress before immunisation if it is their first child who is being immunised. The sixth paper shows that the material used for saliva collection (cotton buds with wooden or plastic sticks) is of importance when saliva is collected but for practical reasons not centrifuged within 24 hours prior to cortisol analyse.

The present thesis shows that it is practically feasible to collect saliva and to analyse the stress hormone cortisol in infants. The interpretation of infants' and parents' salivary cortisol responses to different handling procedures are discussed in relation to short- and long-term consequences, neonatal intensive care, preterm birth, attachment, mood, and pain.



## List of papers

This thesis is based on the following research papers, referred to in the text by their Roman numerals:

- I. Mörelius E, Nelson N, Theodorsson E. Salivary cortisol and administration of concentrated oral glucose in newborn infants: improved detection limit and smaller sample volumes without glucose interference. *Scandinavian Journal of Clinical & Laboratory Investigation* 2004;64:113-118
- II. Mörelius E, Theodorsson E, Nelson N. Salivary cortisol and mood and pain profiles during skin-to-skin care for an unselected group of mothers and infants in neonatal intensive care. *Pediatrics* 2005;116:1105-1113
- III. Mörelius E, Hellström-Westas L, Carlén C, Norman E, Nelson N. Is a nappy change stressful to neonates? *Early Human Development* 2006;26: in press
- IV. Mörelius E, Nelson N, Gustafsson PA. Salivary cortisol response in mother-infant dyads at psychosocial high-risk. *Child: Care, Health & Development* 2006;32:in press
- V. Mörelius E, Theodorsson E, Nelson N. Stress at three-month immunization: A randomized, placebo-controlled trial of parents' and infants' salivary cortisol response in relation to the use of pacifier and oral glucose. In manuscript
- VI. Mörelius E, Nelson N, Theodorsson E. Saliva collection using cotton buds with wooden sticks: a note of caution. *Scandinavian Journal of Clinical & Laboratory Investigation* 2006;66:15-18



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## Abbreviations

ACTH	Adrenocorticotrophic hormone
ADHD	Attention deficit hyperactivity disorder
BSA	Bovine serum albumin
CPAP	Continuous positive airway pressure
CPM	Counts per minute
CRIB	Clinical risk index for babies
CRH	Corticotrophin-releasing hormone
DPNB	Dorsal penile nerve block
EEG	Electroencephalography
EMG	Electromyography
EMLA®	Eutectic mixture of local anaesthetics
Fr	Friedman statistical test
G	G-force
GA	Gestational age
GAS	General adaptation syndrome
HPA	Hypothalamic-pituitary-adrenal
HR	Heart rate
IVH	Intraventricular haemorrhage
KC	Kangaroo care
KMC	Kangaroo mother care
NBSA	Neonatal behavioural assessment scale
NICU	Neonatal intensive care unit
NIDCAP®	Newborn individualised developmental care and assessment program
NIPS	Neonatal infant pain scale
NNS	Non-nutritive sucking
NSB	Non-specific binding
PIPP	Premature infant pain profile
PN	Partus normalis
PTSD	Post traumatic stress syndrome
Q1	First quartile
Q3	Third quartile
RCT	Randomised controlled trial
RIA	Radioimmunoassay
SAM	Sympatico-adrenomedullary system
SaO <sub>2</sub>	Oxygen saturation
SHRP	Stress hypo-responsive period
SSC	Skin-to-skin care
TcpO <sub>2</sub>	Transcutaneous oxygen pressure measurement
VAS	Visual analogue scale
Wi	Wilcoxon signed ranks test
VLBW	Very low birthweight

# Background

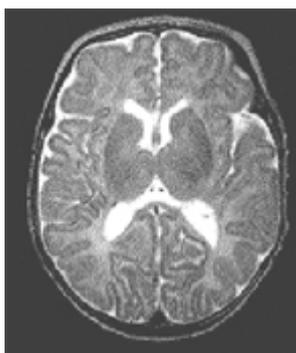
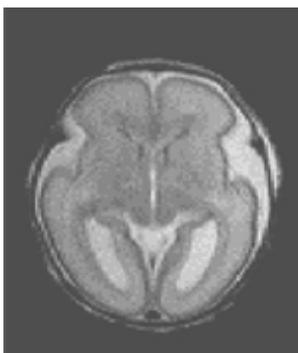
## The infant and neonatal intensive care

### *The preterm infant*

During the last two decades, the decrease in infant mortality and morbidity, even for the very smallest preterm newborns, has been considerable due to remarkable improvements in neonatal intensive care (Rennie 1996; Bylund et al. 1998; Serenius et al. 2004b; 2004a; Wilson-Costello et al. 2005; Vohr et al. 2005). Simultaneously, with these improvements has come a surge of interest in the functional capabilities of preterm infants (Als 1986; Glover and Fisk 1999; Whitfield and Grunau 2000).

All organ systems are functional but more or less immature in the preterm infant. The immature brain for instance, differs from the mature brain not only because it lacks some of the components that are prominent in the adult brain, such as myelin, but also because it possesses certain temporary structures which normally regress postnatally (Johnston 1995; Blows 2003) (Picture 1).

It is difficult for a preterm infant to maintain neurobehavioral organisation and to self-regulate (Als 1986; Als et al. 1986). The immature infant is commonly having bradycardias, periodic breathing, desaturations, body temperature instability, and changes in skin colour as well as purposeless and energy-depleting movements. A preterm infant has difficulties to gain deep sleep and spends most of the time in an active sleep state and cannot attain, maintain, and withdraw from attentiveness at will. A mature infant can achieve and maintain balance by sucking or hand-to-mouth manoeuvres while a preterm infant needs help and support to accomplish the same (Als 1986; Als et al. 1986). Even though the neurobehavioural organisation stabilise with increasing maturation there is still a huge difference in organisation and capacity between healthy preterm infants with gestational age (GA) of 34 weeks and full-term infants (Mouradian et al. 2000).



**Picture1** *Picture to the left illustrates the brain of a preterm infant born and imaged at 25 weeks GA, picture to the right illustrates the brain of a full-term infant born and imaged at 40 weeks GA. Images are reprinted with permission from the Neonatal Research Group, Hammersmith Hospital, London.*

### ***Stress in infants***

The life of the infant is inherently stressful from the moment of birth. When a baby is born stress is advantageous for the infant's respiratory adaptation (Walters and Olver 1978; Wallace et al. 1995) and low levels of the stress hormone cortisol can cause low blood pressure (Helbock et al. 1993) and pulmonary morbidity in preterm infants (Watterberg and Scott 1995; Korte et al. 1996). On the other hand high levels of cortisol may predict intraventricular haemorrhage (IVH) (Korte et al. 1996).

A sick infant is not only sick and subject to necessary painful and invasive procedures but also bombarded with stimuli from the environment and daily handling procedures (for instance; nappy change, feeding, repositioning, weighing, and personal hygiene care). In one study it was reported that during a 24-hour observation period very low birthweight (VLBW) infants were handled on average more than 200 times (Murdock 1984). When preterm infants are exposed to a cascade of procedures they may become hypersensitive to physical stimuli whereas procedures that are not normally viewed as noxious are perceived as painful (Fitzgerald et al. 1989; Whitfield and Grunau 2000). Several studies indicate that preterm infants in relation to common handling situations have symptoms of stress as increased stress hormone levels or heart rate, decreased oxygen saturation, and decreased levels of growth hormones (Grunau and Craig 1987; Field 1989; Pokela 1994; Wang 2004). Stress is, as well as pain, a multifaceted phenomenon and in the newborn period it is probably impossible to distinguish one from another.

### ***Pain in infants***

The definition of pain given by the International Association for the Study of Pain (IASP®) is: "An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (<http://www.iasp-pain.org/index.html>). In 1987 and forward, Anand and co-workers published the first scientific papers showing that preterm infants possess the functional nociceptive system required to feel pain, that they feel pain, and benefits from medical pain relief (Anand and Hickey 1987; Anand et al. 1988; Anand and Carr 1989; Anand and Hickey 1992). Earlier, neonates were thought to be incapable of feeling pain, interpret noxious stimuli as painful, and remember pain. Today it is well founded that just due to their undeveloped nervous system and their inability to inhibit noxious stimuli neonates are more sensitive and feel even more pain than older infants (Anand and Carr 1989; Anand 2001). There is also evidence showing that newborn infants acquire memories of pain (Taddio et al. 1995; Taddio et al. 1997).

Preterm infants (< 32 weeks) spending their first weeks in intensive care are less mature in their pain response when reaching 32 weeks post conception as compared to premature infants born at 32 weeks post conception, the more invasive procedures they have been exposed to the more immature pain behaviour (Johnston and Stevens 1996). There are also distinct differences between preterm and full-term infants' pain expression, preterm infants responding less robustly. Moreover, there are differences between newborns and older infants' pain expression. Newborn infants show more upper face actions as compared to two and four months old infants

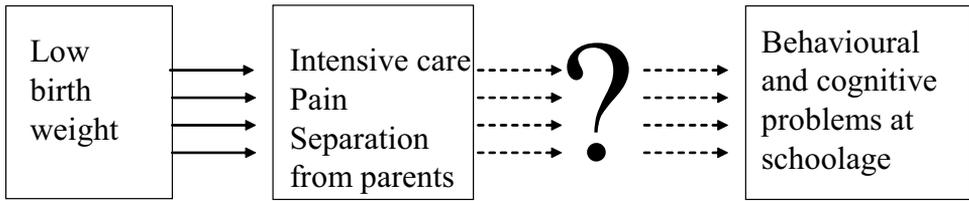


**Picture 2** A preterm infant cared for in an incubator at the neonatal intensive care unit in Linköping.

(Johnston et al. 1993; Johnston et al. 1995). In animal models neonatal pain has been associated with accentuated stress responses, learning deficiencies, and behavioural changes later in life (Anand et al. 1999; Liu et al. 2000). In a study of eight-month-old preterm infants a positive correlation was found between exposure to pain in the neonatal period and higher baseline levels of the stress hormone cortisol at eight months corrected age (Grunau et al. 2004).

### **Aspects on neonatal intensive care**

The environment in a neonatal intensive care unit (NICU) is usually busy (Picture 2). Several infants are cared for in the same room; alarms from cardio respiratory monitors, incubators, and mechanical ventilators sound; several staff members are required in order to support and care for the infants; parents are expected to be by their infant's side; and different medical procedures are performed. The infants in neonatal intensive care are inheritally, developmentally, and/or medically unstable and many of them may suffer from severe medical conditions. They may also suffer from pain from localised infections, inflammations, surgery, skin burns or abrasions caused by transcutaneous probes, monitoring leads, or topical agents. Other sources of pain may be inflammation and hyperalgesia around previous tissue damage (Anand 2001). As a part of their medical care the infants are subject to several different invasive procedures. The most common daily invasive procedures are endotracheal

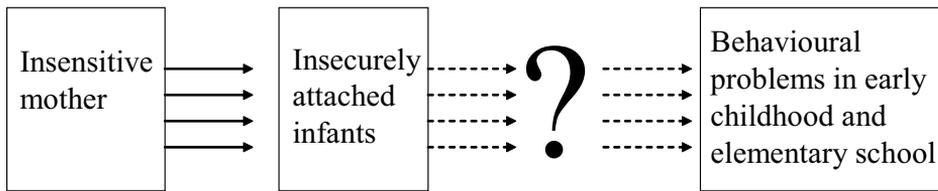


**Figure 1** *Low birthweight leads to intensive care treatment involving pain and separation from parents. It has previously been shown that infants with low birthweight have increased risk of developing behavioural and cognitive problems later in life. However, the links between low birthweight and later problems are not yet completely clarified.*

suctioning, blood sampling, and intravenous cannula insertion (Barker and Rutter 1995; Simons et al. 2003a). The mean number of invasive procedures per infant per day during the first weeks has shown to be more than thirteen, the most immature and sick infants undergo the largest numbers of procedures but do not always receive more continuous opioid infusions or more bolus analgesia (Barker and Rutter 1995; Simons et al. 2003a; Stevens et al. 2003). In a more recent study, however, cumulative morphine exposure since birth was significantly correlated with lower GA and birthweight, higher severity of illness, number of days on mechanical ventilation, and the number of skin breaking procedures (Grunau et al. 2005). Pain can cause harm. Multiple invasive procedures in preterm infants cause significant fluctuations in intracranial blood pressure increasing the risk for IVH (Anand 1998).

### ***Preterm and thereafter...***

When a group of extremely low birthweight infants were studied in southern Sweden the preterm infants were found to have more difficulties to self-regulate at term as compared to full-term controls (Stjernqvist and Svenningsen 1990). They were smaller and had a delay in locomotion and eye-hand coordination at the age of one year and four years (Stjernqvist and Svenningsen 1995). At ten years of age 32 % extremely preterm infants had general behavioural problems and 20 % had attention deficit hyperactivity disorders (ADHD), as compared with 10 and 8 %, respectively, in controls (Stjernqvist and Svenningsen 1999). In a Swedish cohort of the southeast region, VLBW infants (< 1501g) were found to be smaller and have more hospital visits at the age of four as compared to controls. The VLBW infants were still smaller at the age of 9 and 12, had more hyperactivity, and produced poorer in school as compared to controls. However, when controlling for intelligence the differences in school performance and hyperactivity disappeared illustrating a relatively good prognosis for the infants with birthweights < 1501g. Risk factors for VLBW infants were found to be IVH and mechanical ventilation (Bylund et al. 1998; Finnstrom et al. 2003; Leijon et al. 2003) (Fig. 1). An early and long-lasting intervention programme has successfully decreased behavioural problems and the occurrence of low IQ scores (< 75) among three years old VLBW infants in USA (Blair 2002).



**Figure 2** *Previously, it has been shown that infants of insensitive mothers may be insecurely attached and that insecurely attached infants may develop behavioural problems later in life. However, the possible links between insecurely attached infants and behavioural problems need further investigation.*

## Parent-infant relationship

### **Bonding**

Extended mother-infant research by Marshall H. Klaus, John H. Kennell and co-workers focus on the postpartum period as being important for the development of a functional mother-infant relationship (Klaus and Kennell 1970). They suggest that the period immediately after birth is uniquely important and involves a special attachment period for the woman (Klaus et al. 1972). Klaus and Kennell introduced the term bonding defined as rapidly appearing mother-to-infant attachment behaviours. These behaviours include touching, skin-to-skin contact, eye-to-eye contact, and soothing the baby (Klaus et al. 1970). Patterns of initiating, maintaining, and terminating social interaction is established during the first three months of life, why interaction during this early time period may be especially critical (Blehar et al. 1977).

### **Attachment**

An infant will usually become attached around the middle of the first year of life (Ainsworth et al. 1974). Attachment behaviour is defined as any form of behaviour that results in a person attempting to retain proximity to someone differentiated and preferred individual, who is usually conceived stronger and wiser (Bowlby 1953). John Bowlby, influenced by Freud's view that attachment in infancy constitutes a genuine love relationship, developed the attachment theory. He found that an infant needs a warm, intimate, and continuous relationship with his/her mother, or a permanent mother substitute, to grow up in good mental health. The relationship should include satisfaction and enjoyment for both the mother and the infant (Bretherton 1992). According to Ainsworth the attachment figure is a secure base from which an infant can explore the world and return to for reassurance (Bretherton 1992).

Mary Ainsworth, working with Bowlby, concentrated her research on the development of the mother-infant relationship. She found that secure attachment is significantly correlated with maternal sensitivity. Sensitive mothers tend to have securely attached infants while less sensitive mothers are more likely to have infants classified as insecurely attached (Ainsworth et al. 1974). A sensitive mother is characterised by the way she accurately, appropriately, and promptly interprets the baby's communications, signals, wishes, and moods. She shows empathy and

understanding for her baby and responds correctly (Ainsworth et al. 1974). On the other hand, a highly insensitive mother's interventions and initiations of interaction are prompted or shaped largely by signals within herself; she seems geared almost exclusively to her own wishes, moods, and activities (Ainsworth et al. 1974). A mother who is sensitive in her response to her infant's signals in one context tends to be responsive to the infant's signals even in other contexts (Ainsworth 1979) and across ages (Spangler et al. 1994). Insecurely attached children are more likely to have increased levels of the stress hormone cortisol in response to novel situations as compared to securely attached children (Nachmias et al. 1996). Insecure attachment has previously been associated with behavioural problems in early childhood and elementary school (Erickson et al. 1985; Renken et al. 1989; Shaw and Vondra 1995; Munson et al. 2001) (Fig. 2).

### ***Attachment in animal models***

Psychologist Harry Harlow raised infant rhesus monkeys without mothers in order to answer the question "why do infants become attached to their mothers?" Instead of a mother he gave the monkey infants a choice of two types of artificial surrogate mothers made of wood and wires: one surrogate mother gave nutrition from a bottle of milk attached to the torso while the other surrogate mother's torso was wrapped in terry cloth but gave no nutrition. The baby monkeys chose to cling on to the soft terry cloth mother. Harlow concluded that man cannot live by milk alone. Love is an emotion that does not need to be bottle- or spoon-fed (Harlow and Zimmermann 1959).

In rodents, maternal separation during the first weeks of life alters the dam-pup interaction and the pup's sensitisation to stressors. In one early study by Levine neonatal pups that were not disturbed at all showed higher hyper-emotionality as compared to disturbed pups (Levine et al. 1956). Later studies show that pups separated from the dam for 15 minutes a day become more resistant to stressors while pups separated longer (3 hours per day) become more sensitised to stressors as compared to pups not separated at all (Ladd et al. 2000; Levine 2005). Rodent pups separated from their dams for long periods have shown increased anxiety- and fear like behaviour and elevated levels of the stress hormone corticosterone (rodents' version of cortisol) in response to stressors as compared to the pups handled for 15 minutes or not at all (Caldji et al. 1998; Plotsky et al. 2000; Levine 2005). Repeated maternal separations three to six months postpartum have also caused increased levels of the stress hormone cortisol in rhesus monkey offsprings (Sanchez et al. 2001; Sanchez et al. 2005).

Short manipulation of the pup seems to increase dam-pup interaction; short handling causes dams to show a shorter latency to nurse and lick/groom their pup on reunion as compared to dams separated from their pups for longer periods (Caldji et al. 1998; Plotsky et al. 2000). Maternal separation has also shown altered maternal behaviours in goats (Hersher and Richmond 1958). Moreover, uncontrollable varying environmental demands, making macaque mothers psychologically unavailable

for their infants, cause infant behaviours similar to anxious attachment (Rosenblum and Paully 1984).

### ***Parent-infant relationship in neonatal intensive care***

The relationship between a mother and her infant in neonatal intensive care begins with a mother who is usually unprepared psychologically and practically for giving birth to a preterm and/or sick infant and an infant who is physiologically immature. In a descriptive study of mothers' first visits to her baby in the NICU, most mothers were found to demonstrate both verbal and nonverbal (inspection, facial expression, touch) attachment behaviours. The most common attachment behaviour was touching the baby (Tilokskulchai et al. 2002). Mothers of very low birthweight infants experience more psychological distress in the neonatal period as compared to mothers of term infants (Singer et al. 1999). It has been suggested, in an early study, that preterm infants are less active, less responsive, and vocalize and smile less frequently throughout the first year as compared to matched full-term infants (Crnic et al. 1983). Severity of illness and preterm birth have also been associated with insecure attachment (Jeffcoate et al. 1979; Huxley and Warner 1993). However, in more recent Nordic studies, neonatal intensive care treatment did not negatively affect mother-infant interaction (Schermann-Eizirik et al. 1997) and parents did not experience more parental stress at the age of two years as compared to a control group (Tommiska et al. 2002). Moreover, in a prospective study by Goldberg et al. 75 % of low birthweight infants were securely attached at the age of one year (Goldberg et al. 1986). Several other authors state that preterm birth and associated problems have no adverse effects on the development of secure relationships (Field et al. 1978; Frodi and Thompson 1985; Pederson and Moran 1996). However, preterm infants with neurological impairment seem to be at higher risk to develop an insecure quality of attachment (Brisch et al. 2003). One explanation for preterm infants being secure attached at the age of one year despite long-term hospital care and serious illness may be mothers' ability to adapt to and compensate for their infants' limitations (Goldberg et al. 1986). In a comparative study mothers of preterm infants were shown to have more care-taking and affectionate behaviour as compared to mothers of full-term infants (Crawford 1982). It has also been suggested that interaction is dependent on mutual behaviours from the infant and the mother, and that interaction changes as the infant grows (Green et al. 1980). The preterm infant behaves more and more as a full-term infant and contributes more to the mother-infant relationship as he/she grows (Crawford 1982).

## **What can we do in neonatal care to reduce stress in infants?**

### ***Kangaroo care***

In the years around 1970 there were large problems with neonatal mortality at the hospital of Bogotá, Colombia. There were problems with newborn infections, they did not have enough incubators, and surviving preterm infants were often abandoned. To solve the problem with incubators two physicians, Martinez and Rey, asked



**Picture 3** Sanna and her mother during skin-to-skin care.

*Photo by A. Löfgren*

the mothers to stay at the hospital, keep their newborns skin-to-skin, chest-to-chest, under the clothes acting as incubators. The results were amazing: infection rates decreased, neonatal mortality was reduced, and fewer infants were abandoned. Mothers kept carrying their preterm infants in an up-right position, skin-to-skin around the clock; the kangaroo mother care (KMC) was invented (Whitelaw and Sleath 1985). Today there is a proven good comparison between maternal and paternal kangaroo care (Ludington-Hoe et al. 1992; Bauer et al. 1996), thus the concept of KMC is a bit old fashioned; the term kangaroo care (KC) is more commonly used today. Several modifications of the KC are used around the world. For instance, when the term skin-to-skin contact (SSC) is used, it often refers to intermittent periods of KC; the parent holds the preterm infant skin-to-skin for some hours a day, while the infant is cared for in the incubator in between. According to the studies by Ainsworth it is important how the parent holds the baby rather than how long the baby is held, for the development of attachment (Ainsworth 1979) (Picture 3).

In neonatal care it is particularly important for the staff members to support parent-infant bonding. SSC is one way to improve the parent-infant relationship. In a study by Feldman and co-workers parents practicing SSC were found to be more sensitive towards their baby's signals at three months of age as compared to parents of infants treated traditionally (Feldman et al. 2002). It is found that parents' perceptions

of skin-to-skin care is highly individual and changes as time goes by, thus several parents express the wish that they had held their baby sooner (Affonso et al. 1993; Gale et al. 1993; Ludington-Hoe et al. 1994; Neu 1999).

In studies comparing incubator care versus SSC for preterm infants, it is found that SSC has no short or long-term adverse effects on the infant's physiological parameters i.e. oxygen saturation, heart and respiratory rates, and body temperature (Ludington-Hoe et al. 1991; Ludington-Hoe et al. 1994; Bosque et al. 1995; Bauer et al. 1996; Bauer et al. 1997; Christensson et al. 1998; Fohe et al. 2000). The same results are found when comparing SSC with traditional holding (the baby is wrapped in a blanket and held in the parent's arms) (Legault and Goulet 1995; Roberts et al. 2000). Behavioural and developmental outcomes of the infant are improved by SSC (Ohgi et al. 2002; Feldman and Eidelman 2003; Ferber and Makhoul 2004). Skin-to-skin care has also proved to shorten the hospital stay, decrease pain and decrease stress hormone levels in neonates (Charpak et al. 1997; Cattaneo et al. 1998; Gray et al. 2000; Gitau et al. 2002; Johnston et al. 2003; Ludington-Hoe et al. 2005). Table 1 displays short-term physiological effects of SSC for preterm infants.

### ***Developmental care***

Developmental care is an approach that was designed to modify the NICU environment to minimise the stress for the preterm infant (Als 1986; Als et al. 1986). In the early 1970's T. Berry Brazelton developed a behavioural paradigm including tests of motor activity and strength, response to visual, auditory, tactile, and painful stimuli, and measures of irritability and tension. It also attempted to evaluate the interaction between the newborn infant and the caretaker (Als et al. 1977; Brazelton 1984). The newborn individualised developmental care and assessment program (NIDCAP<sup>®</sup>) is a family centred framework partly based on Brazelton's early behavioural observations (Als 1986; Als et al. 1986; Als 1998; Als et al. 2004). NIDCAP<sup>®</sup> encompasses all care and procedures as well as social and physical aspects in the newborn intensive care unit. The goal with NIDCAP<sup>®</sup> is to support the family and each individual infant to be as stable, competent, and well organised as possible. In order to design and provide the best support for each infant the infant's current strengths, vulnerability, and thresholds to disorganisation need to be assessed. External stimuli as light and noise need to be controlled. Procedures need to be well prepared and perhaps synchronised (Als et al. 1986; Als 1998; Als et al. 2004). Previously, NIDCAP<sup>®</sup> support to the preterm infant during routine care handling has proved to reduce pain expressions (Sizun et al. 2002; Catelin et al. 2005). The NICU environment should be warm and friendly. The infant's nest in the bed or incubator needs to be supportive and cosy. Environmental enrichment has earlier proved to reverse the negative effects of maternal separation in both squirrel monkey infants and rodent pups (Harlow and Zimmermann 1959; Coe et al. 1989; Francis et al. 2002).

### ***Positive touch***

It is important to make sure that a sick infant not only is subject to painful and stressful stimuli but also get the chance to experience friendly stimuli, i.e. positive touch. Positive touch is one way to facilitate positive signals to the brain, avoiding

**Table 1** Studies of short-term physiological effects in relation to skin-to-skin care (SSC) in preterm infants.

<b>Authors, year</b>	<b>GA</b>	<b>n</b>	<b>Design</b>	<b>Main results</b>
Acolet et al. 1989	25 - 31	14	10 min SSC vs. 10 min supine horizontal position	Heart rate and oxygen saturation increased during SSC
Ludington 1990	34 - 36	8	One group design	Longer durations of quiet sleep during SSC
Ludington-Hoe et al. 1991	34 - 36	12	One group design: 3h crib - 3h SSC - 3h crib	Heart rate and temperature increased during SSC
De Leeuw et al. 1991	27 - 29	8	One group design: 1h incubator - 1h SSC - 1h incubator	Heart rate, oxygen and temperature remained the same
Ludington-Hoe et al. 1992	34 - 37	11	One group design: 2h of paternal SSC within the first 17h of birth.	Heart rate, respiratory rate and temperature increased
Legault & Goulet	24 - 35	71	One group design: SSC vs. traditional care	SSC produced less variation in oxygen than traditional care
Bauer et al. 1996	28 - 32	11	Pretest-Posttest: before - SSC - after	Temperature increased during maternal as well as paternal SSC

Bauer et al. 1997	25 - 31	22	One group design, first SSC: Incubator - 1h SSC - incubator	Rectal and peripheral skin temperature increased No risk for cold stress
Christensson et al. 1998	33 - 35	80	SSC vs. incubator care in hypothermic infants	SSC infants reached normal temperature faster
Ludington-Hoe et al. 1999	34 - 36	6	SSC the first 6h after birth	Heart rate, respiratory rate and oxygen saturation remained within normal limits, temperature increased
Tornhage et al. 1999	24 - 30	17	One group design: before and during 1h of SSC	Changes in heart rate, tcpO <sub>2</sub> , and temperature were minimal, preterm infants (n=8) tolerated nasogastric tube feeding in SSC
Fohe et al. 2000	25 - 35	53	Pretest - Posttest: incubator - SSC - incubator	Heart rate, oxygen saturation, tcpO <sub>2</sub> , and temperature increased during SSC, respiratory rate decreased
Ludington-Hoe et al. 2000	26 - 35	29	Pretest - Posttest: SSC vs. control	Temperature remained stable 3 hours of SSC
Gitau et al. 2002	26 - 37	14	Pretest - Posttest: SSC vs. no intervention	Salivary cortisol decreased in response to SSC
Ludington-Hoe et al. 2004	33 - 35	24	SSC vs. traditional care	Heart rate, oxygen saturation, and temperature remained within clinically acceptable ranges, no apnea or bradycardia

**Table 2** Randomised controlled studies on oral sweet-tasting solution as a pain reliever in relation to immunisation for infants after the newborn period.

Authors, year	Age, n	Sweet-tasting solution	Additional interventions	Outcome measure	Main results	Comments
Barr et al. 1995	2 and 4 months n = 123	2 groups: 50 % sucrose Water	Parents were not allowed to talk to their infant	Crying-time	Sucrose reduced post injection crying-time	In parents lap Different nurses administered the immunisations
Allen et al. 1996	0.5 - 18 months n = 285	3 groups: 12 % sucrose Water No intervention		Crying-time	Sucrose and water reduced crying-time in infants aged 2 weeks	Placed on an examining table Different nurses administered the immunisations
Lewindon et al. 1998	7 - 38 weeks n = 107	2 groups: 75 % sucrose Water	10 infants used a pacifier, 8 infants received paracetamol, distraction was used	Crying-time Parents and nurse rated infants' pain with VAS	Sucrose reduced crying-time and nurse's pain scoring on VAS	Placed on an examining table Oral polio vaccine before sucrose/water 2 immunisations

Ramenghi et al. 2002	<p>2 - 4 months n = 180</p> <p>4 groups: Glucose 25 % sucrose 50 % sucrose Water</p>	<p>4 infants used a pacifier</p>	<p>Crying-time</p>	<p>50 % sucrose reduced crying-time in infants receiving their 3<sup>rd</sup> immunisation</p>	<p>In parents lap</p>
Reis et al. 2003	<p>2 months n = 116</p> <p>2 groups: Sucrose + tactile stimulation + in parents lap  Standard practice</p>	<p>Tactile stimulation with a pacifier or bottle</p>	<p>Crying-time, heart rate</p>	<p>Interventions reduced crying-time</p>	
Lindh et al. 2003	<p>3 months n = 90</p> <p>2 groups: Glucose + EMLA® Placebo cream</p>	<p>EMLA®</p>	<p>Crying-time, heart pain scale, heart rate variability, parents and nurse rated infants' pain with VAS</p>	<p>EMLA® in combination with glucose reduced crying-time, VAS, and pain scores</p>	<p>In parents lap</p>

the exaggerated development of the neurons sensing negative stimuli in the brain to expand. Studies have shown that massage and comforting behaviour have decreased the number of startle-responses, decreased stress hormone levels, increased weight gain, and shortened hospital stay. Moreover, massage has increased the maturation of the infant's behaviour in terms of habituation, orientation, and motor activity (Jay 1982; Field et al. 1986; Acolet et al. 1993; Scafidi et al. 1993; Diego et al. 2005). It is important, however, to assess the developmental state of the infant before initiating any kind of structured positive touch and to observe the infant during the session in order to make sure that the individual infant consistently benefits from the sensory stimulation of touch (Scafidi et al. 1993).

### ***Pain relief***

Some infants in neonatal intensive care may need continuous pain relieving medication. However, a correct assessment of the infants' individual need is essential in order to provide the right type of medicine and the right dose (Simons et al. 2003b; Anand et al. 2004). In addition, it is important to minimise procedural pain. Ever since Blass and co-workers showed that sweet-tasting solutions were effective to prevent procedural pain in pups (Blass et al. 1987) and newborn infants (Blass and Hoffmeyer 1991) a number of studies have been made with oral sucrose and glucose. One mL of oral glucose (300 mg/mL) before blood sampling is today standard procedure of neonatal care in Sweden (Skogsdahl 1996; Eriksson et al. 1999; Gradin et al. 2002; Gradin et al. 2004). The physiological mechanism behind the effect of oral sweet solution is not yet clarified (Shide and Blass 1989; Gradin and Schollin 2005). Neither is the cut-off age for when the oral sweet solution is no longer effective. Table 2 displays studies made on oral sweet-tasting solutions in infants after the newborn period and up to the age of 12 months. Several studies have also shown the effectiveness of non-nutritive sucking, i.e. the use of a pacifier, to reduce behavioural and physiological pain responses in preterm and term infants (Field and Goldson 1984; Gunnar et al. 1984; Campos 1994; Carbajal et al. 1999; Carbajal et al. 2002; South et al. 2005). Blass and Watt showed that sucking a pacifier reduced pain in relation to heel stick only when the infant sucked at a rate exceeding 32 sucks per minute (Blass and Watt 1999). Other studies have shown that the use of a pacifier in combination with oral sweet-tasting solution is an advantageous combination (Greenberg 2002). The physiological mechanism behind the analgesia of non-nutritive sucking suggests oro-tactile stimulation of mechanoreceptors (Blass and Watt 1999). Non-nutritive sucking may also be a way for the infant to self-regulate (Campos 1994). Skin-to-skin care (Gray et al. 2000; Johnston et al. 2003; Ludington-Hoe et al. 2005), breastfeeding (Gray et al. 2002), EMLA® (Stevens et al. 1999), and facilitated tucking (Ward-Larson et al. 2004) have also been found to reduce procedural pain in neonates.

### ***Support of the parent-infant dyad in a NICU***

Parents of an infant treated in intensive care are in an unfamiliar situation in a strange environment. In a study by Miles and co-workers infant's appearance and behaviour along with alterations in parental role caused by the infant's illness were

found to generate stress in parents of neonatal intensive care treated infants (Miles et al. 1991). In the same study it was shown that parents felt particularly stressed when they sensed that they were not told enough about the infant's care and treatment (Miles et al. 1991). Moreover, parent-infant separation after birth often involve emotional strain, stress, and anxiety for parents (Miles et al. 1991; Seideman et al. 1997; Nystrom and Axelsson 2002).

Parents of intensive care treated infants need information, support and guidance (Shields-Poe and Pinelli 1997; Franck and Spencer 2003). Support to parents has to be individualised and not based exclusively on the severity of the infant's problem. Earlier studies show that parental stress is not associated with the severity of illness (Davis et al. 1998; Morelius et al. 2002) but rather with how parents perceive the severity of their infant's illness (Shields-Poe and Pinelli 1997). As soon as the parents feel ready they should be involved in the care of their baby. It is important that the parents know that they are irreplaceable and that their participation and involvement in the infant's care is necessary for the child (Miles et al. 1991). To avoid separation it is important to invite parents to stay at the hospital with their infant, to stay as close to the infant as possible, and to encourage SSC (Feldman et al. 2002). As soon as it is possible depending on the infant's medical and developmental condition, the parent-infant dyad should be cared for in their home with support from nurses and physicians specialised in neonatal care (NOBAB: <http://www.nobab.se>).

### ***Support of high-risk families***

A psychosocial high-risk family may be defined as a family including one or more of the following criteria: parent with previous alcohol and/or drug abuse problem, mother with psychiatric problems, mother with specific social circumstances of relevance for motherhood, or infant behavioural problems (Svedin et al. 1996).

Infants in psychosocial high-risk families are at risk of being insecurely attached, to develop behaviour problems, and poor mental health (Worobey 1985; Hans and Bernatein 1990; Svedin et al. 1996; Wadsby et al. 1996; Sydsjo et al. 2001; Svedin et al. 2005). For infants living in psychosocial high-risk families professional support may be necessary in order to prevent behavioural problem later in life (Erickson et al. 1985; Renken et al. 1989; Shaw and Vondra 1995; Munson et al. 2001). Short-term programmes to prevent the development of mental and psychosocial symptoms in this context have proved successful (Crowe and Johnson 1991; Wadsby et al. 2001; Brisch et al. 2003).

## **What is stress?**

### ***A friend and a foe***

To our ancestors, the stress response was an essential tool for survival, evolved over many thousands of years living in wild and dangerous environments. When our ancestors faced wild animals they probably had to choose between fight and flight. To us, living in today's technological 21st century, the stress response is often viewed as an ineffective response, which can actively impede us from responding rationally

to a challenge. However, the system is designed to protect us. And even though the stressors today rarely are wild animals we still need the stress response in order to survive. In situations like avoiding a speeding car, running to catch a bus, or caring for a sick infant the stress response may be essential to life (Sapolsky 2004). In contrast to our ancestors the stressors of today are more of the character of daily hassles, performances, or worries (Smyth et al. 1998). Stress related problems occur when the stress response is not turned off; when the stress response is constantly activated (McEwen and Wingfield 2003; Sapolsky 2004).

### ***Stress definitions***

Walter B. Cannon (1871-1945) was the first to use the word "stress" in a biological rather than engineering context. He stated the idea of the "fight or flight" response, which is a general response, based on fundamental emotions and instincts. When we perceive a threat our bodies get ready either for a fight to death or a desperate flight away from the threat (McEwen 2002; Cooper and Dewe 2004; Sapolsky 2004).

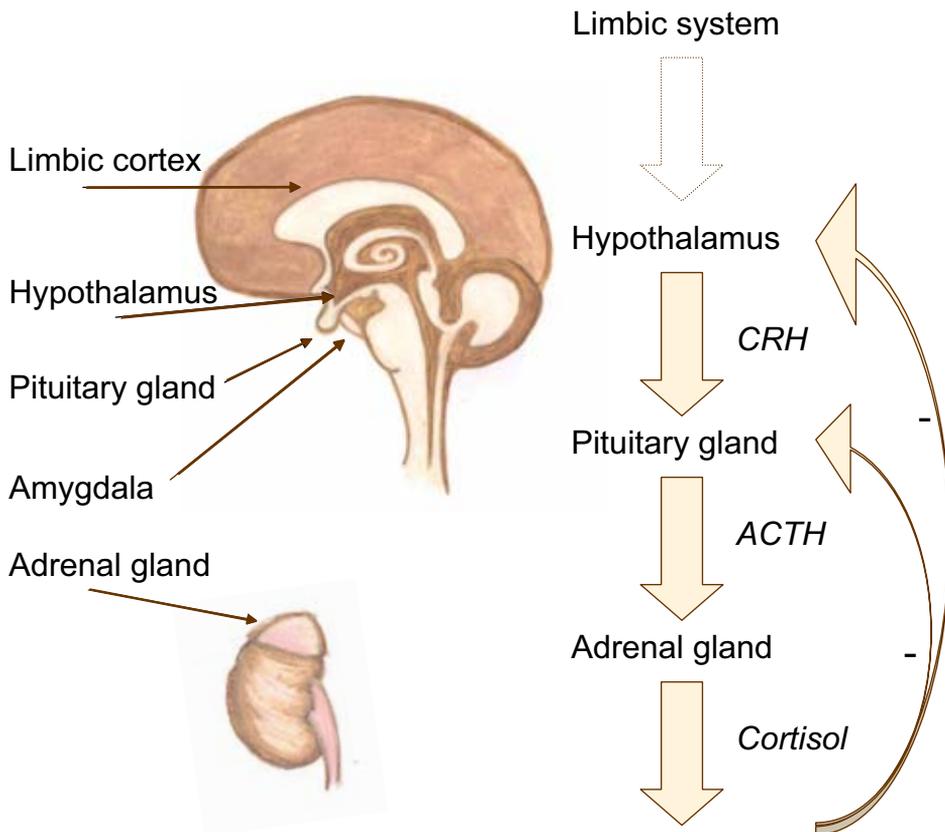
Hans Selye (1907-1982) gave the word stress a biological meaning and explanation. He found the same stereotypical non-specific response of the body to any demand made upon it. Selye called the process, whereby strain influences the body to react in the same way to unrelated or even opposite kinds of stimuli, the general adaptation syndrome (GAS). The GAS theory involves three different phases: 1) alarm i.e. a stressor is noted, an alarm goes off and the brain is alerting danger 2) adaptation, resistance i.e. successful mobilisation of the stress response and re-attainment of homeostasis and 3) exhaustion i.e. prolonged stress, stress related diseases emerge. Selye stated that stress plays a significant role in the development of all types of diseases. The failure to cope with stressors can result in "diseases of adaptation" such as ulcers and high blood pressure (Selye 1974).

Richard Lazarus (1922-2002) and Susan Folkman defined stress as the relationship between the person and the environment. An event is stressful to the individual when the individual appraises the demands as taxing or exceeding his or her resources and endangering his or her well-being. The "appraisal process" links the person and the environment. When a situation has been appraised as stressful, coping processes are initiated to manage the disturbed person-environment relationship (Folkman 1984; Lazarus and Folkman 1984).

Bruce McEwen defines stress as a real or interpreted threat to the physiological or psychological integrity of an individual that results in physiological and/or behavioral responses. In biomedicine, the stress often refers to situations in which adrenal glucocorticoids and catecholamines are elevated because of an experience (McEwen 2001).

### ***The stress response***

The stress response is the set of neural and endocrine adaptations that help re-establish homeostasis (McEwen 2001; McEwen 2002; Sapolsky 2004). In the first line of the stress response there is an activation of the sympathetic-adrenomedullary system (SAM). Catecholamines (adrenaline and noradrenaline) are released from



**Figure 3** This picture shows a simplified figure of the HPA axis (sometimes referred to as the LHPA axis where L stands for limbic system) and the feed-back loop. The picture also shows the locations of structures in the brain involved in the HPA axis along with the adrenal gland at the top of the kidney.

the medulla of the adrenal glands for the fight or flight response. The release of catecholamines is a direct response to sympathetic nerve stimulation. The catecholamines increase the heart rate and respiration sending extra blood and oxygen to the muscles. Extra oxygen is also sent to the brain causing alertness and arousal. Glycogenolysis is stimulated and the release of glucose from the liver increases the blood glucose level. Catecholamines also constrict the peripheral blood vessels that supply the skin and triggers fibrinogen, as a defence against blood loss in case of injury. The brain also releases endorphins, the human body's version of opium, to help the organism handle pain (McEwen 2001; McEwen 2002).

The second line of defence to re-establish homeostasis is the activation of the hypothalamic-pituitary adrenal axis (HPA). Hypothalamus secretes corticotrophin-releasing hormone (CRH). CRH moves through specialised blood vessels to the pituitary. The anterior pituitary releases adrenocorticotrophic hormone (ACTH). ACTH travels through the bloodstream to the cortex of the adrenal glands. The adrenal cortex

secretes glucocorticoids, mainly cortisol, into the circulating blood until the desired levels are met, depending on the magnitude of the stimulus. The circulating glucocorticoids complete a negative feedback loop: glucocorticoid receptors in the brain and pituitary sense the circulating glucocorticoid levels. When the glucocorticoids reach a desired level the brain stops releasing CRH and ACTH (Fig. 3). Once the threat has passed, the organism returns to its normal state. There is a considerable individual variability in the regulation of the HPA axis; a part of this variance may be related to inheritance (Yehuda et al. 2005), rearing conditions during infancy (Levine et al. 1991), and coping abilities (Gunnar 1992).

### ***Physiological stress reactions***

There are four different emotional stress reaction patterns recognised to illustrate how the central nervous system can respond efficiently to various psychosocial challenges (Folkow 1988). 1) The defence reaction is activated whenever the organism is mentally alerted and engaged. It is essential to life and sometimes a matter of life or death. 2) The freezing reaction: is characterised by the intense alertness and attention associated with complete immobility but with high preparedness for sudden action. 3) The emotional depressor reaction/the play-dead-reaction: the opposite to the defence reaction. An activation of nervous vagus causes bradycardia resulting in lowered blood pressure. The breathing discontinues and all motor activity is stopped and the body is totally limp. This reaction is particularly useful in situations with little chance for either flight or fight. 4) The defeat reaction: the reaction of frustration or exhaustion. This reaction is triggered when the individual is overwhelmed with disaster or of emotions as exhaustion, sorrow, and powerless. Severe and prolonged defeat reactions can lead to death (Folkow 1988).

### ***Stressors***

A stressor can be defined in a narrow, physiological sense as any perturbation in the outside world that disrupts homeostasis (Sapolsky 2004). A stressor could be either physiological or psychological, a threat or an actual danger, an injury, excessive exercise, a performance, pain or a certain thought. A stressor could be of intrapersonal, interpersonal or extrapersonal character (Neuman 1995). If a stimulus is considered a stressor involving a stress response or not depends on how the individual evaluates the stimulus and how well the individual may cope with the situation (Lazarus and Folkman 1984).

### ***Homeostasis, Allostasis***

Cannon developed the concept of "homeostasis". Homeostasis is the body's ability to maintain its own consistency; an organism's need to maintain a steady internal state in physiological parameters such as blood oxygen and pH. A normal bodily function requires a steady balance in the function of various organ systems (Cooper and Dewe 2004; Sapolsky 2004).

Allostasis is the process of maintaining stability through a numerous of behavioural and physiological adjustments; the ability to achieve stability through change. The central nervous system, the metabolic- immune- and cardiovascular-systems

go through multiple and complex physiological processes to maintain homeostasis in response to internal and external demands. These systems are most useful when they can be rapidly mobilised and then turned off when not needed (McEwen and Wingfield 2003).

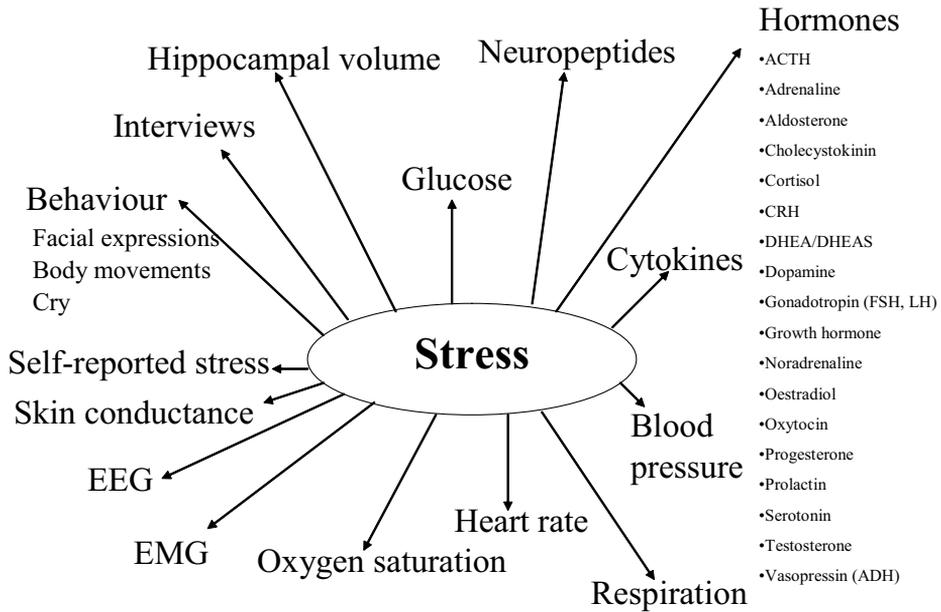
### ***Long-term stress, Allostatic load***

Long-term activation of the stress response to restore homeostasis results in altered functions in virtually all organ systems (McEwen and Wingfield 2003). The wear and tear of long-term adaptation to stressors constitutes the allostatic load of the individual; the stress response becomes destructive and turns against us. Many of the effects of allostatic load are mediated by long-term activation of the HPA axis and the sympathetic nervous system. Prolonged elevation of circulating glucocorticoids has multiple physiological (hyperglycaemia, mobilisation of fat, insulin resistance, increase in heart rate and blood pressure) as well as psychological and cognitive consequences (McEwen 2001; McEwen and Wingfield 2003). There are principally four situations which can lead to allostatic load: frequent stress, failure to habituate to repeated stressors, an inability to shut off allostatic responses, and inadequate allostatic responses that trigger compensatory increase in other allostatic systems (McEwen 2001; McEwen and Wingfield 2003).

### ***Consequences of stress and high levels of cortisol***

The short-term consequence of excessive levels of cortisol is atrophy of dendritic spines in the hippocampus region of the brain (Lombroso and Sapolsky 1998). Long-term stress exposure leads to neuronal loss and decreased connections between the individual neurons. In addition there is less formation of new neurons. Since hippocampus is the centre for several cognitive functions long-term stress may lead to memory, learning, and concentration deficiencies (Lombroso and Sapolsky 1998; McEwen 2001). Low hippocampal volume has been found in primates including baboons and tree shrews in relation to prolonged high stress load (Uno et al. 1989; Czeh et al. 2001; van der Hart et al. 2002). Low hippocampal volume has also been found in adults suffering from Cushing's syndrome (a disease caused by excess of cortisol production or excessive use of glucocorticoids), post traumatic stress syndrome (PTSD), and untreated endogenous depression (Sapolsky 1996; 2000; 2001; Sheline et al. 2003). If the lower hippocampal volume is an effect of the disease, or if the person is predisposed to get the disease because of the lower hippocampal volume is not clarified. However, after treatment for Cushing's syndrome the volume of the hippocampus starts to increase, which supports the theory that high levels of cortisol decrease the hippocampal volume (Starkman et al. 1999). In contrast to adults, children with PTSD are recently reported to have a significantly larger hippocampal volume as compared to control children (Tupler and De Bellis 2006).

Longitudinal research in animal models, indicates that stress in early life can have effects on the HPA axis reactivity that may persist into adulthood (Plotsky and Mea-



**Figure 4** A simplified graph of possible methods, markers, and variables that could be used to measure stress.

ney 1993; Anisman et al. 1998; Ladd et al. 2000). Altered diurnal rhythm of cortisol has been found in children exposed to maltreatment and social deprivation (Carlson and Earls 1997; Bugental et al. 2003). It is also shown that maternal prenatal stress and maternal depression during a child’s first two years of life, may predict elevated baseline cortisol levels in the offspring later in life (Ashman et al. 2002; Gutteling et al. 2004).

**To measure stress**

Since acute stress is a multifactorial phenomenon it can be measured in several different ways, both subjectively and objectively. It is possible to measure stress physiologically, psychologically, and biologically using observations, self-reports, interviews, physiological parameters, or chemical analyses (Fig. 4). Several different biological stress markers, including hormones, neuropeptides, and cytokines have been used in studies on the effects and mechanisms of acute and long-term stress. The most commonly measured neurotransmitters and hormones in relation to acute stress are adrenaline, noradrenaline, cortisol, and ACTH reflecting both SAM and the HPA system.

**Cortisol**

Cortisol, the main glucocorticoid, is a lipophilic molecule. It is synthesised from cholesterol through several enzymatic steps under the regulation from ACTH (Fig 3). During basal conditions cortisol concentrations show a diurnal variation with a cortisol peak level in the morning, a decrease throughout the day, resulting

in the lowest values around bedtime (Gallagher et al. 1973). A diurnal variation of cortisol similar to that in adults is described around the age of two to nine months in infants, (Onishi et al. 1983; Price et al. 1983; Kiess et al. 1995; Santiago et al. 1996; Larson et al. 1998; Antonini et al. 2000), highly depending on the choice of analytical method and the infants' own variability (de Weerth et al. 2003). Prior to the adult circadian rhythm, infants appear to have two daily peaks with 12 hours apart. These two peaks are random with respect of time of the day, and are not synchronised to the daylight cycle (Francis et al. 1987). Cortisol is secreted in response to a stressor. However, caffeine and food intake (Quigley and Yen 1979; Pincomb et al. 1987; Lane et al. 1990), smoking (Wilkins et al. 1982) or physical activity until exhaustion or on a high level of the maximal aerobic capacity of the individual (O'Connor and Corrigan 1987) result in transient cortisol elevations diverging from the basal diurnal rhythm. Cortisol concentrations may also be influenced by the person's posture; in a study by Hennig and co-workers an upright position was related to an increased cortisol concentration while sitting or lying were not (Hennig et al. 2000).

Approximately 95% of cortisol in the blood is bound to protein, mainly transcortin. Around 5% of total plasma cortisol circulates unbound. The unbound cortisol represents the biologically active, free fraction, directly available for action (Gunnar 1992). Cortisol enters the cell by passive diffusion and binds to the intracellular glucocorticoid receptor. Since all cells express glucocorticoid receptors the physiological effects of cortisol are multisystemic (McEwen 2001).

Thus, cortisol as measured in serum or plasma represents total cortisol (protein bound and unbound) whereas cortisol in urine exists only as unbound. Renal secretion, however, is dependent on glomerular and tubular function and the measured daily secretion rate depends on a correct 24-hour collection of urine. Cortisol may by favour be analysed in saliva. Salivary cortisol reflects the free non-protein bound fraction and correlates strongly with cortisol in blood (Riad-Fahmy et al. 1982; Vining et al. 1983a; Gunnar et al. 1989; Aardal and Holm 1995; Calixto et al. 2002). The transfer from serum to saliva occurs by free diffusion of unbound cortisol through the salivary glands (Vining et al. 1983b). The salivary cortisol concentration is not dependent of salivary flow rate (Hiramatsu 1981; Vining et al. 1983b). Cortisol levels in saliva reach a peak 20 to 30 minutes after exposure to a stressor (Gunnar et al. 1989; Gunnar 1992).

### ***Cortisol in infants***

Although it was once believed that the newborn HPA system was relatively unresponsive to stress, this is not the case (Cathro et al. 1969). Fetuses as young as 20 weeks have demonstrated typically large plasma cortisol responses to painful transfusions via the intrahepatic vein (Gitau et al. 2001).

Full-term infants' salivary cortisol responses to different procedures during the first four months of life have been investigated and are displayed in Table 3. The major stressors investigated are painful procedures such as heel stick, immunisation, and circumcision. But, also mild stressors such as physical examinations and emotional

**Table 3** Studies of salivary cortisol responses in infants (0 - 4 months).

Authors, year	Age	n	Intervention	Cortisol response	Comment
Gunnar et al. 1989	Newborns	49	Physical exam	Increased in cortisol 1 <sup>st</sup> exam but not 2 <sup>nd</sup> exam	Saliva stimulants
Lewis & Thomas 1990	2 and 4 months	45	Immunisation	Increased in cortisol	Saliva stimulants
Gunnar et al. 1991	Newborns with optimal birth condition	22	Physical exam	Increased in cortisol 1 <sup>st</sup> exam but not 2 <sup>nd</sup> exam	Saliva stimulants
	Newborns with optimal birth condition	18	Heel stick	Increased in cortisol 1 <sup>st</sup> and 2 <sup>nd</sup> time	Saliva stimulants
	Newborns with non optimal birth condition	40	Physical exam	Increased in cortisol 1 <sup>st</sup> and 2 <sup>nd</sup> exam	Saliva stimulants 16 infants were excluded due to insufficient amount of saliva at baseline
	Newborns with non optimal birth condition	12	Heel stick	Increased in cortisol 1 <sup>st</sup> and 2 <sup>nd</sup> time	Saliva stimulants
Spangler & Schubeck 1993	Newborns with high and low orientation	42	NBAS without HR assessment	Increased cortisol in infants with low orientation. Increased cortisol in infants with high orientation when HR also was assessed	Saliva stimulants Successful saliva sampling in 70 %

Spangler et al. 1994	3 months	41	Play	More increased cortisol in infants of insensitive mothers	Successful saliva sampling in 70 %
Ramsay & Lewis 1994	2 months	40	Immunisation	Increased in cortisol	Saliva stimulants
Lewis & Ramsey 1995	2 and 4 months	141	Physical exam + 2 immunisations at 2 occasions.	Increased cortisol at 2 month for exam Increased cortisol at both ages immunisation	Saliva stimulants
Ramsay & Lewis 1995	2 and 4 months	64	Immunisation	Newborns with optimal birth condition increased at 2 months. Newborns with non-optimal birth condition increased at 4 months	Saliva stimulants
Davis & Emory 1995	Newborns	36	NBAS	Increased cortisol in male infants	Saliva stimulants
Gunnar et al. 1995	Newborns	50	Heel stick	Increased cortisol	Saliva stimulants
Gunnar et al. 1996	2 and 4 months	83	Physical exam + 2 immunisations	Increased cortisol at both ages	Saliva stimulants
	2 and 4 months	18	Physical exam	Increased cortisol at 2 months	Saliva stimulants
Kurihara et al. 1996	Newborns Japanese drum Maternal heartbeat No treatment	131	Heel stick	Less cortisol increase in infants listening to recorded maternal heart beat	Saliva stimulants
Larson et al. 1998	7 - 15 weeks	78	Physical exam	Increased cortisol in infants < 11 weeks but not > 11 weeks	

Kurtis et al. 1999	Newborns	48	Circumcision 4 groups: Mogen clamp with DPNB Mogen clamp with- out DPNB Gomco clamp with DPNB Gomco clamp without DPNB	n.s. between groups	Saliva stimulants
Taylor et al. 2000	2 months, 3 groups: P.N Assisted Cesarean section	76	Immunisation	The increase in cortisol was greatest in the assisted group and least in the cesarean section group	
White et al. 2000	2 months old with and without colic	40	Physical exam	Increased cortisol in both groups	Possible milk contamination
Joyce et al. 2001	Newborns < 24 hours	23	Circumcision	n.s.	Successful saliva sampling in 50 %
Nelson et al. 2001	Newborns	11	Heel stick	Increased cortisol	No saliva stimulants Successful saliva sampling in 95 %
Keenan et al. 2002	Newborns < 48 hours	100	2 occasions: NBAS Heel stick	Increased cortisol in response to NBAS and heel stick	No saliva stimulants Successful saliva sampling in 62 and 64 %

Diego et al. 2002	3 months Infants of depressed mothers; Intrusive vs. Withdrawn	27	Facial expressions	Increased cortisol in infants of intrusive mothers	No saliva stimulants
Greenberg 2002	Newborns 4 groups: Sugar-pacifier Water-pacifier Sucrose No intervention.	84	Heel stick	n.s. between groups	Saliva stimulants
Wilson et al. 2003	2 and 4 months	54	Immunisation	Increased cortisol both ages	Saliva stimulants
Buske-Kirschbaum et al. 2004	Newborns with or without atopic disposition	51	Heel stick	Increased cortisol in both groups, larger response in girls as compared to boys	Saliva stimulants
Miller et al. 2005	2 months 4 groups: P.N Assisted Emergency cesarean section Elective cesarean section	79	Immunisation 2 injections	n.s between groups Infants with highest and lowest cord arterial cortisol had different responses at 2 months and these two groups had different modes of delivery. Low cortisol response in infants born by cesarean section	
South et al. 2005	Newborns NNS vs. control	44	Circumcision	Decreased cortisol with NNS	Successful saliva sampling in 28 - 47 %

**Table 4** Studies of salivary cortisol responses in preterm infants.

Authors, year	GA, weeks	Postnatal age, days	Study design	n	Intervention	Cortisol response	Comment
Magnano et al. 1992	30 - 37	5 - 53	Control vs. Cocaine exposed infants	47 + 11	Physical exam and heel stick	Lower reactivity in cocaine exposed infants	Successful saliva sampling in 80 %
Gitau et al. 2002	26 - 37	< 28	Baseline-response Intervention (massage or SSC) vs. No intervention	15 + 14	Massage or SSC	Decreased cortisol after SSC Diverging results after massage	Saliva stimulants Successful saliva sampling in 83 %
Boyer et al. 2004	< 31	1-7	RCT Baseline-response Placebo vs. Sucrose for one week	105	Different painful procedures	No difference between infants receiving sucrose and water	Saliva available from, at least one day for 57 (54 %) infants

Davis et al. 2004	33 - 34	3 - 6	Baseline-response	9 + 9	Heel stick Physical exam	Infants with antenatal Beta-methasone decreased in cortisol in response to heel stick	Estimated missing saliva samples Statistical method: ANOVA
Herrington et al. 2004	30 - 36	5 - 24	Baseline-response Antenatal Betamethasone vs. No antenatal Beta-methasone	8	Heel stick	Not reported	Successful saliva sampling in 46 %
Harrison et al. 2005	23 - 42	2 - 140	Serial samples to test method and relationship to pain behaviours A prospective observational cohort to study the utility of salivary cortisol as an objective measure of stress in sick infants	144	No intervention	Higher mean cortisol in enterally fed infants	Successful saliva sampling in 35 %
Catelin et al. 2005	≤ 32 32 - 37 ≥ 37	< 7	Randomised cross-over 3 groups depending on age NIDCAP® vs. Conventional treatment	15 + 15 + 15	Weighing	Not reported	No cortisol data presented due to lack of significance

strains may be sufficient to activate the adrenocortical system in neonates. The findings are rather consistent. Extremely healthy infants show habituation to moderate stressors and sensitisation to painful stressors. Infants with nonoptimal birth condition show neither habituation nor sensitisation to repeated handling or nociceptive stimulation (Table 3).

Preterm infants' salivary cortisol responses to different procedures have also been investigated and are displayed in Table 4. In two studies increased salivary cortisol levels in response to heel stick were found (Magnano et al. 1992; Davis et al. 2004). A decreased salivary cortisol has been found in response to skin-to-skin care (Gitau et al. 2002). In other studies the salivary cortisol responses have been diverging, including both increases and decreases in response to heel stick and massage (Boyer et al. 2004; Herrington et al. 2004; Catelin et al. 2005; Harrison 2005).

## **Aspects on mechanisms of relevance for stress**

### ***The adrenal gland of the infant***

The fetal adrenal gland consists of an inner fetal zone and an outer definitive zone. By term the fetal adrenal gland produces 100 to 200 mg of steroids a day. The main steroids produced are cortisol, aldosterone, and the biologically inactive dehydroepiandrosterone (DHA). After birth a rapid involution occurs. In the first four days the adrenal gland loses 25 % of its mass and by one month the size is reduced to half the fetal size. During the gradual reduction in size the fetal zone of the adrenal cortex involutes and zona fasciculata and zona reticularis develop. As the adrenal cortex continues to involute there is a corresponding reduction in steroid secretion (Winter 1998).

### ***Stress hypo-responsive period***

Several authors describe a stress hypo-responsive period (SHRP) in the neonatal period of rodents (postnatal day 4 - 14); the rodent developing brain is protected from the catabolic and other effects of glucocorticoids due to a reduced sensitivity of the HPA axis activation (Sapolsky and Meaney 1986; Walker et al. 1986). The SHRP may not be absolute though, it is possible that strong psychological or physical stressors may circumvent the hypo-responsiveness (Plotsky et al. 2000).

### ***Habituation***

Habituation is a decreased response to a given, repeated stimulus. Habituation involves 9 characteristic phenomena: 1) Repeated exposure to a stimulus results in a decreased response. 2) If the stimulus is withheld, the response tends to recover over time (spontaneous recovery). 3) If repeated series of habituation and spontaneous recovery are presented, habituation becomes successfully more rapid. 4) The more rapid the frequency of stimulation, the more rapid is habituation. 5) Strong stimuli may not yield any habituation. 6) If exposure to the stimulus is continued, the response will eventually disappear, and spontaneous recovery will be much slower. 7) Habituation of responses to a given stimulus exhibits stimulus generalisation to other stimuli. 8) Presentation of another (usually stronger) stimulus results in reco-

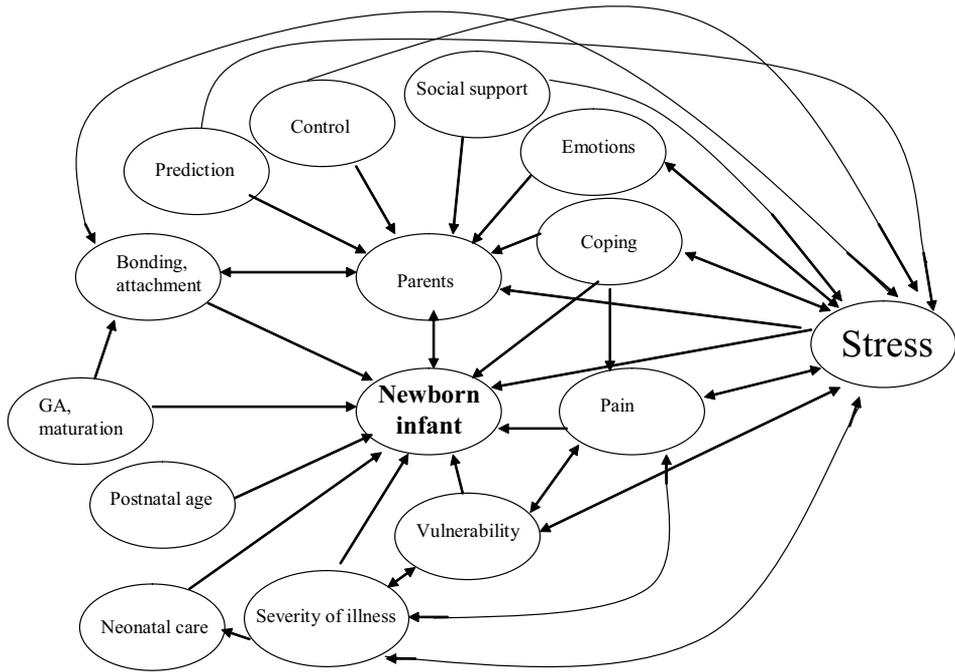
very of the habituated response (dishabituation). 9) Upon repeated exposure of the dishabituated stimulus, the amount of dishabituation produced habituates (habituation of dishabituation) (Thompson and Spencer 1966).

### ***Appraisal and coping***

Hippocampus and amygdala are limbic brain structures that process experiences by interfacing with lower vegetative brain structures and higher cortical centres. Hippocampus and amygdala help to interpret whether an event is threatening or otherwise stressful and to determine the behavioural, neuroendocrine, and autonomic responses (McEwen 2001; Sapolsky 2004). Through primary appraisal a person judges, based on current and past experiences, whether a situation is irrelevant, positive, or stressful. In secondary appraisal the person evaluates coping resources and options (Lazarus and Folkman 1984; Folkman et al. 1986).

Coping is cognitive and behavioural efforts to master, reduce, or tolerate demands created by stressful situations. Coping may, according to Folkman & Lazarus focus either on the regulation of distressing emotions (emotion-focused coping) or on the problem causing the distress (problem-focused coping) (Folkman 1984; Folkman et al. 1986). When facing a stressor a person may experience different emotions. The first emotion may be anxiety, after a few minutes anger, then guilt, then love and joy (Folkman and Lazarus 1985). Coping acts as a mediator of emotional outcomes; coping shapes emotion by influencing the person-environment relationship and how it is appraised (Lazarus 1993).

Studies on animals show that the perception of psychological aspects as predictability, control, and social support are negatively correlated with an activation of the HPA axis in a stressful situation (Hanson et al. 1976; Vogt et al. 1981; Wiener et al. 1990). In humans the same aspects have been found to be of importance for the appraisal of stress. For instance, a threatening situation is more anxiety provoking if the person has no control (Houston 1972). Mothers of hospitalised children have been found to experience less anxiety if they can predict what event to expect next (Schepp 1991). Lower cortisol levels have been related with high controllability (Lundberg and Frankenhaeuser 1978; Hyyppa 1987) whereas increased cortisol levels have been found in relation to new and unfamiliar stressors (Fishman et al. 1962; Davis et al. 1981; Hubert and de Jong-Meyer 1989). High psychological demands in combination with low freedom of decision at work has been associated with several different stress related symptoms and low support has been associated to higher prevalence of sick leave (Theorell 1997; Oxenstierna et al. 2005). The need of continuity of personal relationships when individuals are involved in crisis situations is of importance for an individual's ability to cope with stress (Hamburg and Adams 1967). However, if a person should seek social support as a coping strategy during a stressful encounter highly depends on the social context (Lazarus 1993) (Fig. 5).



**Figure 5** This diagram represent a much simplified scheme of stress related factors that may affect the newborn infant. Several of these factors also affect stress, directly or indirectly. The diagram should not be considered comprehensive or complete and may include several additional factors and inter-relations.

**Mood**

Human mental function is commonly divided into three basic categories: cognition (thinking), conation (willing), and affect (feeling) (Parkinson 1996). The psychological concept of mood falls mainly into the affect category even though mood influence and is influenced by thinking. And, the state of mood may also have direct consequences on motivation and action (willing). According to Parkinson and colleagues “Mood reflects changing non-specific psychological dispositions to evaluate, interpret, and act on past, current, or future concerns in certain patterned ways” (Parkinson 1996, page 216). Mood refers to an affective state that feels pleasant or unpleasant; it provides information about current state of self. Mood reflects and affects evaluations of what is happening. Mood has a relatively long-term but limited duration, which is longer than the duration of emotion. Moods change over time in quality and intensity. People show different characteristic patterns of mood; there are individual differences in dispositions to experience particular moods, and in the variability in mood over time (Nowlis and Nowlis 1956; Parkinson 1996). Nowlis and Nowlis postulated four dimensions of mood: level of activation, social orientation, level of control, and hedonic tone (Nowlis and Nowlis 1956).

# Aims

## General aims

The general aims with the present thesis were twofold. The first aim was to investigate the acute stress response, as measured by salivary cortisol and behaviour, in preterm infants, healthy infants, and infants at high psychosocial risk in response to certain defined handling procedures. The second aim was to investigate the stress response, as assessed by salivary cortisol and psychometric measures, in parents present during the handling procedure of the infant. The intention was also to do all investigations in an as naturally occurring situation as possible, which means that the studied procedures would have been performed irrespective of research projects.

## Specific aims

The specific aims of each paper included in the thesis were:

### Paper I

- To lower the detection limit of the cortisol radioimmunoassay and reduce the volume of saliva needed for cortisol analysis.
- To investigate whether oral glucose in the amounts used to reduce procedural pain in infants interferes with the cortisol radioimmunoassay analytical method.

### Paper II

- To investigate how skin-to-skin contact influences indicators of stress in the mother and her baby.
- To investigate whether there is a difference in the stress response between the first and a later skin-to-skin contact, when the mother and the baby may have adapted to the situation.

### Paper III

- To investigate whether infants in neonatal intensive care have a different pattern of cortisol and pain response compared to full-term healthy infants when challenged with an everyday care handling procedure, in this case a standardised nappy change.
- To investigate if there is a difference in the cortisol and pain response between the first and second week of life.

### Paper IV

- To investigate the salivary cortisol response during an everyday care handling procedure in a group of psychosocial high-risk infant-mother pairs.

- To investigate if there is a difference in the stress response between the first and last week of treatment in a day-care programme to improve infant-mother interaction.

**Paper V**

- To investigate the salivary cortisol and cry response in relation to immunisation in three-month-old infants when given oral glucose in combination with a pacifier.
- To investigate parents' stress response in relation to their infants' routine three-month immunisation.

**Paper VI**

- To investigate if cotton buds made of wooden or plastic sticks could be left uncentrifuged in room temperature for one or two days prior to cortisol analysis and if there were differences in recovery of salivary cortisol between the buds containing wooden or plastic sticks.

# Subjects and methods

## Baseline-response design

The overall design used in papers II-V is a baseline-response design. Baseline is defined as the state before the intervention is introduced. The response refers to the stress response in relation to the intervention introduced. In papers II - IV the study intervention has been performed twice, in these cases all individuals act as their own controls between first and second study intervention.

## Subjects and interventions

All studies were conducted in Linköping, Sweden. Infants in intensive care (paper I, II and III) were recruited from the neonatal intensive care unit at the University Hospital. Healthy full-term infants (paper I and II) were recruited from the maternity ward. Mother-infant dyads participating in paper IV were recruited from Hagadal day-care clinic. Parent-infant dyads participating in paper V were recruited from one primary health care centre (well-baby clinic). Finally, healthy voluntary adults were recruited from the nursing staff at the University Hospital for participation in paper VI. Table 5 displays the interventions, design, numbers of subjects included, and the outcome measurements used in studies II - V.

## Biological marker, salivary cortisol

### *General principles of cortisol radioimmunoassay*

Salivary cortisol may be analysed with enzyme-linked immunosorbent assay (ELISA) (Shimada et al. 1995), fluorescence immunoassay (Klug et al. 2000), and radioimmunoassay (RIA) (Berson and Yalow 1968). RIA is today the most commonly used method to determine saliva concentrations of cortisol. The general principles of a cortisol RIA includes several steps: 1) A sample containing the cortisol to be measured, a specific antibody raised against cortisol, and a known amount of radioactive labelled cortisol (tracer) are mixed. 2) An incubation period follows, when both sample cortisol and tracer cortisol can bind to the antibody. 3) A secondary antibody specifically directed against the primary is added, forming an insoluble complex. 4) Following centrifugation the supernatant containing the unbound cortisol is removed and the amount of tracer associated with the bound fraction can be quantified. If the concentration of sample cortisol is low, then the amount of bound tracer is high, and conversely, if the amount of sample cortisol is high, then only a low amount of tracer will be bound. By incorporating samples with known quantities of cortisol in an assay set-up, a standard curve can be constructed against which unknown samples can be compared.

There are kits commercially available on the market for analyses of salivary cortisol by RIA in adults. These kits usually require 100 - 200  $\mu\text{L}$  of saliva for a single analysis. Previously, it has been difficult to collect sufficient amounts of saliva from infants for analysis of cortisol. The methods to collect saliva with dental rolls (Gunnar

**Table 5** Interventions, design, and measurements for study II-V.

Study	Intervention	Design	Infants n	Parents n	Biological marker	Behavioural measure	Psychometric self- report measure
II	SSC	Paired 1 <sup>st</sup> and 4 <sup>th</sup> SSC	17	17	Salivary cortisol	PIPP NIPS	VAS Mood scale
III	Nappy change	Case-control Paired 1 <sup>st</sup> and 2 <sup>nd</sup> week	39 + 30		Salivary cortisol	PIPP NIPS	
IV	Nappy change	Paired first and last week of treatment at Hagadal	22	22	Salivary cortisol	NIPS Crying-time Brazelton state Ainsworth	
V	Immunisation	RCT 4 groups: Glucose & pacifier Water & pacifier Glucose Water	98	98	Salivary cortisol	Crying-time	VAS

et al. 1989) or aspiration of saliva with a catheter (Davis and Emory 1995) have not been completely successful. Therefore several authors have used citric acid in order to stimulate salivary excretion (see Table 3). Citric acid is, however, known to have the potential to affect the cortisol results by lowering the sample pH, increasing sample osmolality, and interfering with the antibody binding (Schwartz et al. 1998). In 2001 Nelson and colleagues published a paper where they described a modified version of the commercially available kit from Orion Diagnostica (Turku, Finland) (Nelson et al. 2001). The modification, by dilution of the ingredients, enabled RIA analysis to be performed with 25  $\mu\text{L}$  of saliva for single sample analysis with a detection limit of 1.0 nmol/L. The lower amount of saliva needed made it possible to analyse salivary cortisol in infants without the use of citric acid (Nelson et al. 2001). However, Table 4 demonstrates that the problem to collect sufficient amounts of saliva from preterm infants remains.

### ***Saliva sampling method***

The method to collect saliva was developed at the University Hospital in Linköping, Sweden, and is described in paper I. In paper I-V saliva was collected using cotton buds with wooden sticks (SelefaTrade, Spånga, Sweden). Two cotton buds were held together with a sewing thread. The sewing thread was wined around the wooden sticks and held in place with soft surgical tape (Micropore<sup>TM</sup>, 3M, Sollentuna, Sweden). The cotton buds were lightly moved around in the mouth in order to collect saliva (Picture 4). The time for the cotton buds to be fully saturated with saliva is highly individual and probably depending on several different factors, among one is stress (Queiroz et al. 2002). When the cotton buds were moistened they were placed in a polypropylene tube (Sarstedt<sup>®</sup>, Landskrona, Sweden) with the sewing thread on the outside. The cap was tightly screwed on and the sample was centrifuged for 5 minutes (1500G). After centrifugation the cotton buds were removed using the sewing thread. The cap was again screwed on tightly and the saliva sample was stored in the freezer until analysis. The samples were stored at  $-22^{\circ}\text{C}$  for a maximum of three months, in case of longer storage the samples were transferred on ice to a  $-70^{\circ}\text{C}$  freezer.

The same saliva collecting method as described above was used also in paper VI. One difference was, however, the use of cotton buds with plastic sticks as well as wooden sticks (Johnson's<sup>®</sup>, Johnson & Johnson) since the purpose of paper VI was to test possible influences on the cortisol analysis of the two materials.

### ***Salivary cortisol radioimmunoassay***

The method to analyse salivary cortisol was developed at the University Hospital in Linköping, Sweden, and is described in paper I. The method is a further modification from the previously described modified method (Nelson et al. 2001). Using the present method makes it possible to analyse cortisol with RIA using 10  $\mu\text{L}$  of saliva for single analysis. The detection limit is 0.5 nmol/L.



**Picture 4** *Saliva sampling with cotton buds.*

### **Salivary cortisol radioimmunoassay preparation**

Cortisol antiserum (Orion Diagnostica, Turku, Finland) and radioligand [ $^{125}$ I]-cortisol (Orion Diagnostica, Turku, Finland) were both diluted with RIA buffer (0.1 mol/L phosphate buffer containing 0.02% bovine serum albumin (BSA) and 0.01 % triton X-100, pH of 7.4) in the ratio 1:40 respectively until 100  $\mu$ L represented 3000 CPM. Cortisol control 150 nmol/L was serially diluted until 0.07 nmol/L with the same RIA buffer, in order to generate the standard curve.

Two plain polystyrene tubes were labelled T (total), two NSB (non-specific binding), and two 0 (0-reference). Twenty-four tubes were labelled 0.07, 0.14, 0.29, 0.58, 1.17, 2.34, 4.69, 9.38, 18.75, 37.5, 75, 150 respectively, in duplicates for the twelve diluted calibrators representing the standard curve. Additional tubes were labelled in duplicates for the saliva samples and intraassay variation samples. The assay was completed with two 0-references.

### **Salivary cortisol radioimmunoassay procedure**

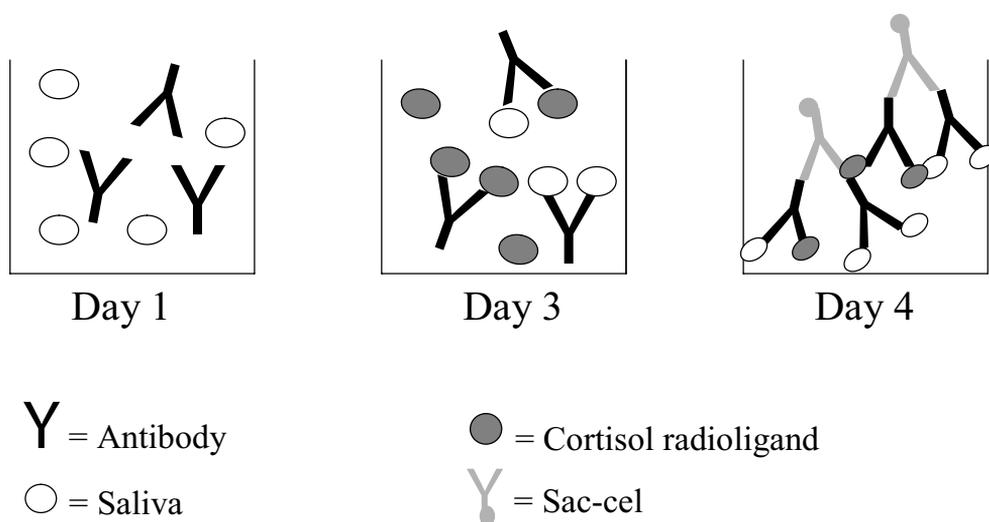
1) Cortisol antiserum (100  $\mu$ L) was added to all tubes except the totals and NSB:s. RIA buffer was added to the NSB tubes (110  $\mu$ L) and to the 0-reference tubes (10  $\mu$ L). Saliva samples and calibrators were added to their respective tubes in volumes of 10  $\mu$ L each. 2) An incubation period followed at +4°C for 48 hours. 3) 100  $\mu$ L tracer (3000 CPM) was added to each tube. 4) A second incubation period followed at +4°C for 24 hours. 5) A specific anti-rabbit antibody (50  $\mu$ L solid phase second antibody coated cellulose suspension (SAC-CEL), Boldon, England) was added to all tubes except the totals and allowed to incubate at room temperature for 30 mi-

minutes. 6) One mL of distilled water was added to all tubes except the totals. The samples were centrifuged at 3000G and +4°C for 15 minutes before decantation. 7) The assay was analysed in a gamma counter 1277 from Wallac (Turku, Finland) (Fig. 6).

All saliva samples were collected from infants older than 48 hours to minimise the risk of influences from birth (Stahl et al. 1979; Gitau et al. 1998). All samples from each individual were run together in duplicates. The intraassay variation of coefficient was 12 % and 6 % for 2.0 and 10.0 nmol/L, respectively. The interassay variation of coefficient was 10.0 % and 5.2 % for 5.0 and 12.5 nmol/L, respectively. In case of extreme values the saliva samples were checked for possible blood contamination and in case of such excluded from further analyses (HemoCue® for b-hemoglobin, Ängelholm, Sweden).

## Behavioural measures

There are several tools to assess pain in infants. However, far from all of the tools are tested for validity and reliability, and even fewer have been tested for clinical utility and feasibility (Abu-Saad et al. 1998; Duhn and Medves 2004). The difficulty with evaluation of pain tools in neonates is the lack of a gold standard. Some of the tools are unidimensional while some are combining both physiological and behavioural cues. A few of the tools also comprise contextual variables such as GA and state of arousal (Stevens et al. 1996). One reason for using contextual variables in a pain instrument is that preterm infants and sleeping infants have more difficulties to express pain (Grunau and Craig 1987; Craig et al. 1993; Johnston et al. 1995)



**Figure 6** The main analytical steps of the cortisol radioimmunoassay presented in paper I. Day 1: saliva containing unknown amount of cortisol is mixed with a antibody. Day 2: a known amount of cortisol radioligand (tracer) is added. Day 3: a secondary antibody, Sac-cel, is added to the sample.

### ***Premature infant pain profile***

The Premature infant pain profile (PIPP) is a multidimensional instrument designed to assess acute pain in neonates. PIPP comprises three behavioural variables (time of brow bulge, eye squeeze, and nasolabial furrow), two physiological variables (changes in heart rate and oxygen saturation (SaO<sub>2</sub>)), and two contextual variables (gestational age and state of arousal). Each variable is scored on a scale from 0 to 3. The minimum PIPP score is 0 (no pain) and the maximum score 21 for infants of lower GA and 18 for infants  $\geq 36$  weeks GA. A total score of  $\leq 6$  is considered to be no or minimal pain while  $\geq 12$  is moderate to severe pain. PIPP has documented reliability and validity for full-term and preterm infants (Stevens et al. 1996; Ballantyne et al. 1999).

### ***Neonatal infant pain scale***

The neonatal infant pain scale (NIPS) is a multidimensional instrument designed to assess acute pain in neonates. NIPS comprises five behavioural variables (facial expression, cry, arm movements, leg movements, and state of arousal) and one physiological variable (breathing pattern). Each variable is scored on a scale from 0 to 1 or (for cry) 2. The minimum NIPS score is 0 (no pain) and the maximum 7. NIPS has documented reliability and validity for full-term and preterm infants (Lawrence et al. 1993).

### ***Crying-time***

Infant cry constitutes four phases; inspiration, exhalation/expiration, pause, and then a quick inspiratory gasp that precedes the next cry (Ludington-Hoe et al. 2002). Crying-time may be used as a measure of pain in full-term and preterm infants when the cause of pain is known (Johnston et al. 1995; Runefors et al. 2000). A cry takes a great deal of energy though. Therefore, preterm and/or sick infants exhibit weaker and shorter cries than older, healthier infants (Johnston et al. 1993; Dimitriou et al. 2000).

Crying-time was measured in paper IV and V. In paper IV crying-time was clocked from onset to end of crying during a nappy change. In paper V crying before immunisation was dichotomised into yes or no. Total crying-time in response to immunisation was also clocked from onset to end with a maximum of three minutes and calculated as percentage of time the infant spent crying during the three minutes period.

### ***Brazelton state***

Brazelton state was used to assess the infant's state of arousal at the start of the nappy change in paper IV. Brazelton state is scored from 1 to 6 and comprises two sleep states (1 = deep sleep, 2 = light sleep) and four awake states (3 = drowsy, 4 = alert with bright look, 5 = eyes open; considerable motor activity; brief fussy vocalizations, and 6 = crying) (Als et al. 1977; Brazelton 1984)

### ***Ainsworth's sensitivity scale***

In paper IV the mother's sensitivity towards her infant's signals was measured using a scale from the Baltimore Study of Mary Ainsworth (Ainsworth et al. 1974; 1978). Ainsworth's sensitivity scale is a nine-point bidimensional scale with five anchor points: 1 = highly insensitive, 3 = insensitive, 5 = inconsistently sensitive, 7 = sensitive, 9 = highly sensitive (Ainsworth et al. 1974; 1978). A highly sensitive mother (9 points) is exquisitely attuned to her baby's signals, and responds to them promptly and appropriately. She reads her baby's signals and communication skilfully. A sensitive mother (7 points) also responds to her baby's signals prompt and appropriate but with less sensitivity and consistency than the highly sensitive mother. An inconsistently sensitive mother (5 points) can be quite sensitive on occasion but there are periods when she is insensitive to her baby's signals. However, she is more frequently sensitive than insensitive. An insensitive mother (3 points) frequently fails to respond to her baby's communication appropriately and/or promptly. Her insensitivity seems linked to inability to see things from the baby's point of view. A highly insensitive (1 point) mother's interventions and initiations of interaction are prompted or shaped largely by signals within herself. The highly insensitive mother seems geared almost exclusively to her own wishes, moods, and activities (Ainsworth et al. 1974). Seven or above is usually recognised as a well-functioning interaction. Ainsworth's sensitivity scale has previously been used in several studies of mother's sensitivity toward her baby's signals (Goldberg et al. 1986; Pederson et al. 1990; Pederson and Moran 1996; De Wolff and van Ijzendoorn 1997).

## **Psychometric self-report measures**

### ***The visual analogue scale***

The visual analogue scale (VAS) is an internationally used method to measure subjective experiences and appraisals (Luria 1975). In the present thesis VAS was used to let the parents grade their emotional stress before, during, and after skin-to-skin care (paper II) and before and after immunisation of their infant (paper V). Parents graded their emotional stress by placing a marker somewhere between "no stress at all" and "worst stress imaginable". The opposite side of the scale (only seen by the researcher) is marked with numbers from 0 (no stress at all) to 100 (worst stress imaginable).

### ***Mood scale***

The mood scale consists of 71 adjectives measuring 6 bipolar dimensions of mood: pleasantness, activation, calmness, social orientation, extraversion, and control (Svensson 1977; Sjoberg et al. 1979). Pleasantness, activation, and calmness are considered to measure the basic bipolar dimension of mood. Each adjective is measured on a four-point scale (1 = it definitely disagrees with what I feel right now, 2 = it disagrees with what I feel right now, 3 = it agrees somewhat with what I feel right now, 4 = it definitely agrees with what I feel right now). The adjectives are for instance: happy, concentrated, friendly, relaxed, attached, and secure. The dimensions may be analysed separately as well as total sum mean score with a min-max of

12 - 47, higher values indicating a better mood (Svensson 1977; Sjoberg et al. 1979). The mood scale is a self-administered instrument also available as a computerised version. The mood scale has been used previously in several intervention studies (Persson et al. 1980; Svensson et al. 1980; Sandberg et al. 2002).

## **Physiological measure**

### ***Heart rate***

Parents' heart rate was measured in paper II by palpating the radial pulse for one minute. Heart rate and SaO<sub>2</sub> for intensive care treated infants (paper II and III) were measured with a cardiorespiratory monitor (Hewlett Packard, Böblingen, Germany). Heart rate and SaO<sub>2</sub> for full-term healthy infants (paper III) were measured with a transportable monitor (Nellcore Puritan Bennett, NPB-40, Pleasanton, CA, USA).

## **Statistics**

Friedman test and Wilcoxon signed ranks test were used to analyse differences between paired data. Kruskal Wallis test and Mann-Whitney U-test were used to analyse differences between independent samples. A binominal test was used to calculate significant differences in nominal data within a group. Chi-square test and Fisher's exact test were used to calculate differences for nominal data between groups. Analyses of variance (ANCOVA and ANOVA) were used to analyse different factors effect on a dependent variable. Logistic regression was used to calculate odds ratio in paper V. Spearman's rho was used for analyses of correlations. Interclass correlation coefficient was used to test interrater reliability between observers using pain scales. Coefficient of variation was used to calculate intraassay and interassay variation of the cortisol radioimmunoassay method. A statistical significance was considered if  $p < 0.05$ .

## **Ethical considerations**

As mentioned earlier infants in intensive care are undergoing several painful as well as handling procedures every day (Murdock 1984; Barker and Rutter 1995; Simons et al. 2003a). It is extremely difficult to add more procedures to these already vulnerable infants in order to do research. On the other hand, there is still a lot to learn about preterm and/or sick infants and about possible side effects of the procedures they are succumbed to. Currently, there is limited research evidence for many of the treatments and technological innovations that are constantly being introduced in neonatal care (Franck 2005). So, one could consider if it is ethical not to do important clinical research on newborn infants (McIntosh et al. 2000), as long as the ethical considerations of the declaration of Helsinki is considered (<http://www.fda.gov/oc/health/helsinki89.html>). When working with infants who cannot give their consent to participation in research we have to rely on the parents. Unfortunately parents sometimes are given valid consent to participate or refuse to participate without having received full information or comprehension (Mason and Allmark 2000). Therefore, it is important that oral and written information is clear so that parents understand what it means to enrol their infant in a clinical trial (Franck 2005).

Previously, mothers of hospitalised children have expressed their appreciation for the opportunity to participate in an interview study during a stressful time, since the interview helped them to sort things out (Schepp 1991). The research in the present thesis has been performed during procedures that would have taken place with or without research involved and the outcome measures of the infants have been observational and not painful. In the papers presented researchers from several different disciplines have worked together for the beneficial of infants and parents.



# Results

## Paper I

The detection limit of the salivary cortisol radioimmunoassay was lowered from 1.0 to 0.5 nmol/L. The sample volume was decreased from 25 to 10  $\mu$ L of saliva. The method to collect saliva with cotton buds was found to be reliable, 97 % of the collected saliva samples contained sufficient amounts of saliva for analyse. We also found that it is safe to administer oral glucose as a pain reliever, in recommended doses, prior to a painful procedure and still collect saliva 30 minutes later and analyse cortisol without glucose interference. The percentages of samples containing sufficient amounts of saliva for analyse with radioimmunoassay according to the method described in paper 1 are displayed for each study in Table 6.

## Paper II

### Infants

There were no significant changes in salivary cortisol levels during and after skin-to-skin care (SSC) as compared to baseline (in the incubator), neither at first nor second SSC (= fourth SSC with the mother, second intervention). During first SSC 38 % of the infants increased in salivary cortisol levels and 62 % decreased or remained unchanged. During second SSC 64 % of the infants increased in salivary cortisol levels and 36 % decreased or remained unchanged (Table 7). Baseline salivary cortisol levels were significantly lower at the second SSC as compared to the first SSC ( $p < 0.01$ ). Baseline and response salivary cortisol levels are shown in Table 8. NIPS decreased significantly during both first and second SSC (Table 7). After SSC NIPS was still lower than baseline. The heart rate decreased significantly during both first and second SSC but remained within normal limits (Table 7).

### Mothers

Mothers rated more stress on VAS before the first SSC as compared to the second SSC ( $p < 0.05$ ). Mood (control, calmness, and pleasantness) and VAS improved during first SSC ( $p < 0.01$  and  $p < 0.01$ , respectively). During second SSC VAS improved ( $p < 0.01$ ) while mood remained unchanged. Salivary cortisol and heart rate decreased after the first SSC, but not during first SSC, as compared to baseline ( $p < 0.01$  and  $p < 0.05$ , respectively). However, at the second SSC salivary cortisol and heart rate decreased significantly during SSC as compared to baseline ( $p = 0.01$  and  $p < 0.01$ , respectively) (Table 9).

## Paper III

There was no significant difference in salivary cortisol in response to a standardised nappy change, neither for NICU infants nor for healthy full-term infants, during the first postnatal week. During the second week, NICU infants decreased significantly in salivary cortisol in response to the nappy change ( $p = 0.01$ ). There were salivary cortisol ‘increasers’ as well as ‘decreasers’ among both NICU infants and control infants at both nappy changes (Table 7). NICU infants had higher median

**Table 6** Percent of successful saliva sampling from infants in paper I-V and adults in paper VI.

Study	Intervention	%
I	Methodological	97
II	1 <sup>st</sup> Skin-to-skin care	84
	2 <sup>nd</sup> Skin-to-skin care	95
III	1 <sup>st</sup> Nappy change	91
	2 <sup>nd</sup> Nappy change	89
IV	1 <sup>st</sup> Nappy change	100
	2 <sup>nd</sup> Nappy change	100
V	Immunisation	92
VI	Methodological	100

baseline salivary cortisol levels as compared to full-term newborns on both occasions, infants < 30 weeks showing the highest levels. In both groups, baseline salivary cortisol levels were significantly lower at the second nappy change as compared to the first nappy change (NICU infants:  $p < 0.01$  controls:  $p < 0.01$ ). Baseline and response salivary cortisol levels are shown in Table 8.

PIPP and NIPS increased for both groups during both nappy changes. PIPP and NIPS remained significantly higher in NICU infants until the 3 minutes measure point during first nappy change and until the 3 minutes (PIPP) and 30 minutes (NIPS) measure point at the second nappy change. NICU infants had higher PIPP

scores during both nappy changes as compared to full-term newborns ( $p < 0.001$  for both occasions). Infants with gestational age < 30 weeks had higher scores on PIPP and NIPS as compared to infants with gestational age  $\geq 30$  weeks.

## Paper IV

### Infants

Infants younger than three months increased significantly in salivary cortisol level in response to a nappy change during the first but not the last week of treatment in the Hagadal day-care programme ( $p < 0.05$ ). This increased salivary cortisol level was not found in infants three months or older. Baseline and response salivary cortisol levels are shown in Table 8.

However, among all infants there were significantly fewer salivary cortisol 'increasers' in response to the nappy change during the last week of treatment in the Hagadal day-care programme as compared to the first week ( $p < 0.05$ ) (Table 7).

### Mothers

Mothers' sensitivity towards their infants' signals improved significantly from first to last week of treatment ( $p < 0.001$ ). There was a positive correlation between Ainsworth's sensitivity scale and the child's age during the first week

**Table 7 Main results for infants in study II - V.**

Study	Intervention	Salivary cortisol	Cortisol "increasers" %	PIPP	NIPS	State of arousal
II	1 <sup>st</sup> SSC	n.s.	38	n.s.	Decreased	Deeper sleep during SSC n.s.
	2 <sup>nd</sup> SSC	n.s.	64	n.s.	Decreased	n.s.
III	1 <sup>st</sup> nappy change	NICU: n.s. Control: n.s.	NICU: 39 Control: 31	Increased in both groups	Increased in both groups	
	2 <sup>nd</sup> nappy change	NICU: decreased Control: n.s.	NICU: 12 Control: 46	Increased in both groups	Increased in both groups	
IV	1 <sup>st</sup> week	Increased in infants < 3 months	< 3 mon: 73 ≥ 3 mon: 57			
	Last week	n.s.	< 3 mon: 33 ≥ 3 mon: 57		n.s. from 1 <sup>st</sup> week	n.s. from 1 <sup>st</sup> week
V	Immunisation	The change in cortisol was related to type of intervention and cortisol increased more if the infant cried before the procedure	Glucose & pacifier: 35 Water & pacifier: 67 Glucose: 61 Water: 57			Infants cried longer if they cried before the procedure

**Table 8** Infants' median baseline and response salivary cortisol in study II - V.

<b>Study</b>	<b>Intervention</b>	<b>Subjects</b>	<b>Median (q1 - q3) baseline salivary cortisol, nmol/L</b>	<b>Median (q1 - q3) response salivary cortisol, nmol/L</b>
II	1 <sup>st</sup> SSC	NICU-infants	23.5 (11.1 - 41.1)	41.5 (11.0 - 61.0)
	2 <sup>nd</sup> SSC	NICU-infants	14.7 (6.9 - 41.9)	19.5 (9.4 - 43.2)
III	1 <sup>st</sup> nappy change	NICU-infants < 30 weeks GA	20.2 (14.7 - 40.3)	18.6 (11.7 - 36.2)
		NICU-infants ≥ 30 weeks GA	5.8 (3.9 - 15.9)	4.4 (3.3 - 12.0)
		Control infants	6.2 (4.1 - 11.1)	7.7 (3.7 - 13.1)
	2 <sup>nd</sup> nappy change	NICU-infants < 30 weeks GA	10.6 (5.1 - 16.8)	6.9 (3.8 - 15.2)
		NICU-infants ≥ 30 weeks GA	3.6 (2.1 - 8.4)	3.2 (2.5 - 7.1)
		Control infants	2.4 (1.8 - 4.5)	4.2 (2.0 - 11.0)
IV	1 <sup>st</sup> nappy change	Psychosocial high-risk infants < 3 months	1.4 (1.2- 3.1)	3.8 (1.9 - 5.6)
		Psychosocial high-risk infants ≥ 3 months	4.1 (1.8 - 4.9)	3.1 (1.8 - 6.7)
	2 <sup>nd</sup> nappy change	Psychosocial high-risk infants < 3 months	3.6 (1.4 - 7.8)	2.9 (1.8 - 6.2)
		Psychosocial high-risk infants ≥ 3 months	2.6 (1.8 - 3.0)	4.4 (2.8 - 13.1)
V	Immunisation	3 months old infants (n=98)	4.0 (2.6 - 7.3)	4.8 (3.2 - 7.4)

( $r = 0.435$ ,  $p < 0.05$ ), but not during the last week. Mothers' median salivary cortisol decreased after first and second nappy change ( $p < 0.01$  and  $p = 0.001$ , respectively) (Table 9).

## Paper V

### Infants

ANCOVA revealed significant main effect of intervention ( $F_{3,72} = 3.1$ ,  $p < 0.05$ ) for the percent of change of infants' cortisol in response to the three-month immunisation. Infants randomised to pacifier and glucose decreased 33 % in median cortisol level while infants randomised to pacifier and water increased 50 % in median cortisol level. Delta salivary cortisol in percent ( $\Delta$  cortisol %) was significantly lower in infants randomised to pacifier and glucose as compared to infants randomised to pacifier and water ( $p < 0.05$ ). There were no significant differences in  $\Delta$  cortisol % between the group of infants who received oral glucose and the group of infants who received water within the non-pacifier group; median cortisol levels increased 42 and 8 %, respectively. Baseline and response salivary cortisol levels for all randomisation groups together are shown in Table 8.

In addition, if the infant was crying before the immunisation had a significant main effect on the infant's cortisol response ( $F_{1,72} = 8.2$ ,  $p < 0.01$ ). Infants crying before immunisation increased more in cortisol and cried longer during immunisation as compared to infants who did not cry before start of the procedure ( $p < 0.01$  and  $p < 0.01$ , respectively) (Table 7). Glucose and pacifier had no effect on crying-time.

### Parents

Parents scored higher on self-rated emotional stress on VAS before immunisation if they were primipara as compared to multipara parents ( $p < 0.01$ ). All parents rated significantly higher on VAS after immunisation as compared to before immunisation ( $p < 0.0001$ ). Parents decreased significantly in salivary cortisol concentration after immunisation as compared to before immunisation ( $p < 0.01$ ). We also found that infants' crying-time had a significant main effect for parents'  $\Delta$  cortisol % ( $p < 0.05$ ) and change in VAS ( $p < 0.05$ ).

### Infants and parents

There was a positive correlation between parents'  $\Delta$  cortisol % and infants' crying-time (Spearman's  $\rho = 0.24$ ,  $p < 0.05$ ) and between parents' change in VAS and infants crying-time (Spearman's  $\rho = 0.28$ ,  $p < 0.01$ ). There was a significant correlation between parents'  $\Delta$  cortisol % and the infants'  $\Delta$  cortisol % ( $r = 0.22$ ,  $p < 0.05$ ). There was also a significant correlation between parents' baseline cortisol and infants' baseline cortisol ( $r = 0.22$ ,  $p < 0.05$ ).

## Paper VI

We found that cotton buds with plastic sticks, but not wooden sticks are reliable to use when centrifugation is not possible in close connection to the sampling. The concentration of cortisol changed when cotton buds with wooden sticks were left uncentrifuged for 24 ( $p < 0.001$ ) or 48 hours ( $p < 0.001$ ).

**Table 9** Main results for parents. Arrows indicate significant differences (increase or decrease) from baseline.

Study	Intervention	Salivary cortisol	Mood scale	VAS	Heart rate	Ainsworth
II	During 1 <sup>st</sup> SSC	n.s	↑	↓	n.s	
	After 1 <sup>st</sup> SSC	↓	↑	↓	↓	
	During 2 <sup>nd</sup> SSC	↓	n.s	↓	↓	
	After 2 <sup>nd</sup> SSC	↓	n.s	↓	↓	
IV	1 <sup>st</sup> week	↓				
	Last week	↓				Improved from 1 <sup>st</sup> week
V	Immunisation	↓		↑		

# Discussion

## Infants

The results of the studies included in the present thesis show that it is feasible to collect sufficient amounts of unstimulated saliva from infants by means of common cotton buds to be analysed for salivary cortisol using radioimmunoassay as described in paper I. However, if the cotton buds for practical reasons cannot be centrifuged within 24 hours cotton buds with plastic sticks should be used instead of cotton buds with wooden sticks for optimal recovery of salivary cortisol (paper VI).

### ***Baseline salivary cortisol***

We have found that infants in a NICU and healthy newborns have higher salivary cortisol baseline at younger postnatal age. This is confirmed by previous studies of salivary cortisol in full-term infants (Price et al. 1983; Spangler and Scheubeck 1993) and plasma cortisol in preterm infants (Scott and Watterberg 1995). Probably, this can be explained by the normal development and maturation of the adrenal gland; as the adrenal gland reduces in size after birth there is a corresponding reduction in steroid secretion (Winter 1998). Other studies indicate that cortisol levels increase in relation to delivery but stabilises after birth (Stahl et al. 1979; Gitau et al. 1998).

Infants in NICU were found to have a higher salivary cortisol baseline as compared to healthy full-term infants. Maybe, the higher levels in NICU infants also can be an effect of age and the involution of the adrenal gland in combination with preterm birth (Winter 1998). However, higher baseline salivary cortisol has recently been reported in preterm infants at eight months corrected age as compared with control infants born at full-term (Grunau et al. 2004). Thus, the higher salivary cortisol, found in the present thesis, could be an early sign of a disturbance in the HPA system related to stress of preterm birth and/or intensive care. It has previously been shown in animal models that stress early in life can have a long-term effect on the HPA axis activity (Plotsky and Meaney 1993; Anisman et al. 1998; Ladd et al. 2000). Maternal separations have for instance caused increased cortisol levels in rhesus monkey offsprings (Sanchez et al. 2005). Moreover, stress at birth has been suggested to influence the HPA axis of the infant for up to four months (Gunnar et al. 1991; Ramsay and Lewis 1995; Taylor et al. 2000; Miller et al. 2005).

Within the group of psychosocial high-risk infants the older infants ( $\geq 3$  months) had a significantly higher salivary cortisol baseline as compared to the infants  $< 3$  months during the first week of treatment in the Hagadal day-care programme. This is in contrast to the hypothesis of age and normal development of the adrenal gland and more likely to be a complication of stress. Social deprivation, maltreatment, and maternal depression have all been shown to cause altered cortisol diurnal rhythms and elevated cortisol levels in infants (Carlson and Earls 1997; Ashman et al. 2002; Bugental et al. 2003). Thus, the explanation for the older infants' higher baseline may be altered cortisol levels due to stress from the psychosocial milieu these in-

fants have been living in since birth.

It is important to identify and treat/support the groups of infants with higher cortisol levels in order to prevent allostatic load and long-term consequences as cognitive problems, high blood pressure, and development of the metabolic syndrome (Sapolsky 1996; Lombroso and Sapolsky 1998; Sapolsky 2000; McEwen 2001; Sapolsky 2001; Sheline et al. 2003).

### ***Infants' salivary cortisol response***

The infants in the NICU showed variable stress reactivity in relation to both skin-to-skin care (paper I) and nappy change (paper II). Unexpectedly more NICU infants responded with a cortisol increase during the second SSC as compared to the first SSC. This is contradictory to the hypothesis of habituation and more in agreement with the hypothesis of sensitisation, meaning that NICU infants have become more sensitised to handling (Thompson and Spencer 1966; Natelson et al. 1988; Gunnar et al. 1989).

On the other hand, more NICU infants decreased in cortisol in response to the nappy change in their second week of life as compared to first week, which is in agreement with the rules of habituation (Thompson and Spencer 1966). Even if it was a nappy change in their first week it was not their first nappy change ever, which means they normally should have habituated earlier. It is, however, possible that those preterm and/or sick NICU infants need more time and more exposure to the stimulus in order to habituate (Gunnar et al. 1991). It is also possible to dishabituate when a stronger stimulus is presented (Thompson and Spencer 1966) which is commonly happening in neonatal intensive care. However, activation and regulation of the cortisol response can probably not be explained solely by the novelty/familiarity of the stressor.

The law of initial value could potentially be another explanation to these inconsistent salivary cortisol responses (Wilder 1957). However, it is only likely to be an explanation for the SSC procedure since more infants increased in cortisol during second SSC when the baseline values were lower while the opposite was evident for the nappy change; more infants decreased in salivary cortisol during the second nappy change when the baselines were lower.

The inconsistency of the NICU infants' salivary cortisol response may instead be an effect of immaturity of the HPA system (Rosenfeld et al. 1992; Hanna et al. 1993; Suchecki et al. 1993; Korte et al. 1996). Anand and colleagues have previously reported that the glucocorticoid response in preterm infants is less robust, in relation to surgical procedures, as compared to term infants (Anand and Hickey 1987; Anand et al. 1988). It has also been shown that adults with normal cortisol cycles respond with increased cortisol to stressors while adults with inconsistent cortisol cycles respond with a decreased or unchanged cortisol (Smyth et al. 1998). The cortisol cycles have not been investigated in the present thesis. However, young infants are known to have an absence of cortisol diurnal rhythm which could be compareable to an inconsistent cycle (Onishi et al. 1983; Price et al. 1983; Kiess et al. 1995; Santiago

et al. 1996; Larson et al. 1998; Antonini et al. 2000).

The increased salivary cortisol response seen in psychosocial high-risk infants younger than three months, after a nappy change performed by their mothers (paper IV), may indicate that infants do not feel safe in the presence of the mother because of an insufficiency in the mother-infant relationship. A situation that is supposed to be a nice moment for both mother and infant, a procedure with opportunity for interaction and pleasantness turns out to be threatening and overwhelming (Stansbury and Gunnar 1994). In this case the nappy change is a strong stimulus and strong stimuli do not usually yield habituation (Thompson and Spencer 1966). An increased cortisol response has previously been found in infants expressing sadness during goal blocking (Lewis and Ramsay 2005), in infants of insensitive mothers during play situations (Spangler et al. 1994), and in infants of depressive intrusive mothers during interaction (Diego et al. 2002). Moreover, disorganised infants have previously been shown to lack appropriate coping strategies (Spangler and Grossmann 1993).

Infants three months and older did not increase in cortisol in response to the nappy change at Hagadal, neither the first nor the last week of treatment. It is possible that a nappy change is a too mild a stressor for these more mature infants. For instance, older infants have more social potential to communicate and initiate interaction (Blehar et al. 1977; Green et al. 1980; Crain 2005). An organised infant also has the cognitive ability to attenuate and process inputs (Als et al. 1986). Several studies have shown that responsivity to novel and painful stressors decreases markedly between the age of two and six months, and this low reactivity continues into the second year of life (Lewis and Ramsay 1995; Gunnar et al. 1996; Larson et al. 1998).

An increased salivary cortisol response in relation to immunisation has previously been demonstrated in infants four months and younger (Lewis and Thomas 1990; Ramsay and Lewis 1994; Lewis and Ramsay 1995; Ramsay and Lewis 1995; Gunnar et al. 1996; Wilson et al. 2003). An absence of cortisol response was reached when the infants in our study (paper V) received oral glucose in combination with a pacifier. The odds ratio for an increased cortisol response was significant if the infant received water in combination with pacifier, only water or only glucose.

So, is an absence of a cortisol response in relation to a stressor, as found in infants receiving pacifier and glucose during immunisation, or a significantly decreased cortisol response, as found in NICU infants during the second nappy change, equal to no stress? There is a lot of evidence that an increased cortisol level in response to a stressor is a sign of stress (Table 3), in that case the opposite (decreased or unchanged cortisol level in response to a stressor) may be a sign of a lower degree or absence of stress (Acolet et al. 1993; Smyth et al. 1998; Gitau et al. 2002). On the other hand, it may also be an effect of suppressed adrenal activity due to long-standing and/or repetitive high stress load i.e exhaustion or allostatic load (McEwen and Wingfield 2003; Cooper and Dewe 2004). A dampened cortisol response in healthy infants receiving pacifier and glucose during immunisation is likely to reflect an

absence or lower degree of stress. A decreased cortisol response in NICU infants is, on the other hand, more plausible to reflect a high stress load or an immaturity in the HPA system. However, stress is a multifaceted phenomenon which has to be studied by several different approaches, among which cortisol is just one.

### ***Aspects on behaviour and heart rate***

We have used two validated pain instruments (NIPS and PIPP) to assess infants' behaviour during SSC (paper II) and during nappy change (paper III). The instruments are designed to measure pain during painful procedures in preterm and full-term infants (Lawrence et al. 1993; Stevens et al. 1996). One reason for using the instruments in non-painful handling situations was to measure potential stress behaviour. Stress and pain are difficult to distinguish from each other in the neonatal period and many behavioural cues are probably the same. However, the interpretation of the results becomes more difficult. In paper III, NICU infants and healthy full-term infants increased significantly in pain scores but not in cortisol during both nappy changes as compared to baseline. So, is a nappy change painful to neonates? Or, is it stressful? If we should rely on the salivary cortisol responses we would say that the infants are not stressed by the nappy change. On the other hand, if we rely on the pain scales we would say that the nappy change is painful. It is clear that pain can be stressful, but can stress be painful? For a sick infant a nappy change involving repositioning, handling, and disturbance may be a stressful procedure or potentially painful for some sensitised infants. However, since even the full-term healthy infants had a smaller but still significant increase in pain scores from baseline it has to be considered as a behaviour more likely to arise from stress than from pain. Thus, the pain instruments used may not measure pain exclusively but also stress.

Heart rate is one variable included in the PIPP scale. The more the heart rate increases from baseline the higher the pain score (Stevens et al. 1996). Vulnerable, neurobehavioural disorganised preterm infants do, however, often increase in heart rate as soon as they are disturbed. Clinically, disturbance of a vulnerable preterm infant may as well result in a vagally mediated bradycardia, preceded or not by a rise in heart rate. This reaction is very similar to the emotional depressor reaction (play dead reaction) (Folkow 1988). The emotional depressor reaction may be the only option when neither flight nor fight is possible, which is commonly the case for infants (Folkow 1988). However, in its previous form the PIPP scale does not take a bradycardia into consideration, which may cause falsely low scores on the PIPP, unless the bradycardia is preceded by a rise in heart rate. The heart rate may also rule under the law of the initial value (Wilder 1957), i.e. the higher baseline the least increase in heart rate during handling/pain, which is neither considered in the PIPP. Also, does an infant whose heart rate increases from 150 to 190 have more pain than an infant increasing from 180 to 190?

Another difficulty in the interpretation of the heart rate in neonates is the wide range of normality. For instance, infants in paper II decrease in heart rate during SSC as compared to before SSC. This result was interpreted in favour for the infant since it was accompanied with a deeper sleep state, which presumably lowers the energy

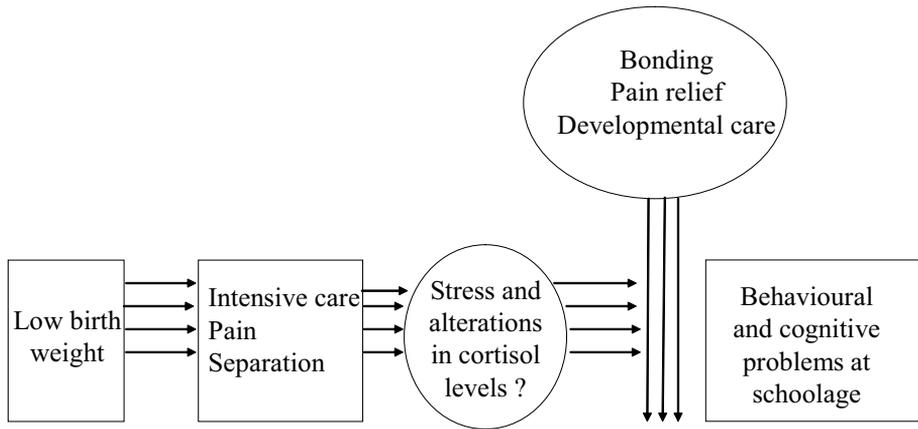
expenditure. However, several other studies report an increased heart rate during skin-to-skin care (Acolet et al. 1989; Ludington-Hoe et al. 1991; Ludington-Hoe et al. 1992; Fohe et al. 2000). The increased heart rates in those studies were not interpreted as stress or pain but rather seen as a sign of increased gas exchange and have thereby also been interpreted in favour for the infant. Even though the heart rate changed it remained within clinically normal, acceptable limits in paper II as well as in the papers reporting increased heart rate, speaking in favour of incorporating the context in the evaluation of data.

Today PIPP is the most used instrument to measure pain in preterm infants. It is a composite tool that includes contextual variables such as gestational age and state of arousal (Abu-Saad et al. 1998; Duhn and Medves 2004). Still, new pain instruments continue to pop-up, which may be a sign of dissatisfaction of the current pain instruments available, and/or a sign of how difficult it is to assess pain in infants. Nevertheless, to assess infants' behaviour with structured instruments is important in order to treat and support them accordingly and to continue to develop the neonatal care. Currently, we have to rely on the pain and behavioural instruments available. For the future, additional research is needed to further differentiate what the infant's signals really stand for.

In paper V it was found that infants who cried before the immunisation cried longer during immunisation and increased more in cortisol in response to the immunisation. The infant reacts more negatively to the immunisation if he/she is upset at the start of the procedure. This clearly indicates that exposure to a painful procedure should be avoided to a crying infant and speaks in favour of calming the baby before initiating a potentially painful or stressful procedure.

### ***Associations between salivary cortisol and behavioural state***

There was a weak but significant correlation between crying-time during immunisation and the change in cortisol in paper V, which is in line with previous findings of serum cortisol and cry during circumcision (Gunnar et al. 1981; Gunnar et al. 1988). However, there were no correlations between pain scales and changes in salivary cortisol in paper II and III. Previously, Larson et al. found a correlation between behavioural state and salivary cortisol during stressful conditions but not during conditions that did not produce elevations of cortisol levels, which is in agreement with our findings (Larson et al. 1998). Nonetheless, past research has consistently reported only a minor to moderate cortisol-behaviour relation in infants (Grunau and Craig 1987; Lewis and Thomas 1990; Johnston et al. 1993; Ramsay and Lewis 1994; Keenan et al. 2002; Herrington et al. 2004). Moreover, Lewis and Ramsay found that infants who expressed sadness during goal blocking increased in salivary cortisol while infants who expressed anger did not (Lewis and Ramsay 2005). Thus, it is clear that cortisol and behavioural measures provide relatively independent indices of infant stress reactivity. Since stress is a multifaceted phenomenon it is possible that in certain situations one facet is superior and more exaggerated to another and vice versa.

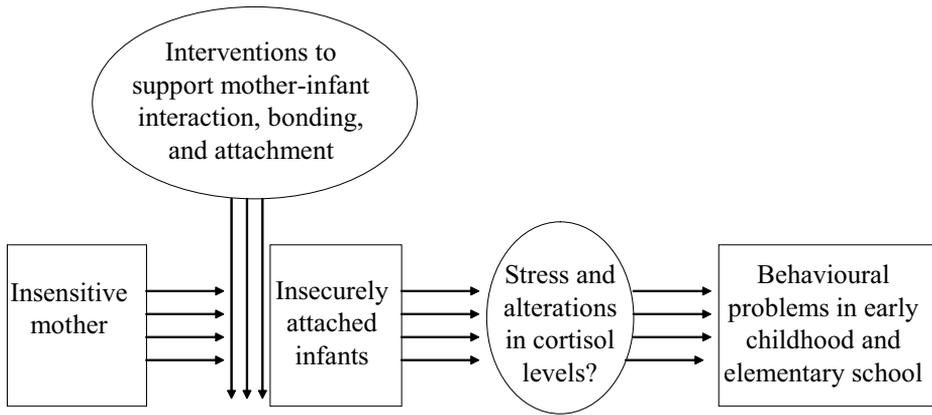


**Figure 7** This figure includes the possible "missing link" between low birthweight and behavioural problems later in life, from figure 1. High stress load as a result of intensive care, pain, and parent-infant separation may cause altered cortisol levels with subsequent effects on hippocampus leading to behavioural and cognitive problems. However, support to the infant in terms of SSC (bonding), pain relief, and developmental care may prevent possible behavioural problems later in life.

### **Neonatal intensive care**

Clinically, handling procedures in neonatal intensive care, as SSC and nappy changes, may be stressful for some infants while beneficial for others depending on context. However, we have to continue to change nappies and previous results show a lot of benefits with SSC (see Table 2). Therefore, it is valuable to find the unique needs of each individual infant. More research is needed in order to find the best ways to assess each infant's status and needs before initiating SSC to be sure that the procedure will be beneficial for each infant. Moreover, it is important to provide the infant with opportunities for rest and recovery. It may also be wise to individualise, and in certain cases reduce, the numbers of nappy changes according to the infant's need rather than to a predetermined schedule. Thus, we address a future need for research concerning individualised care and measures to fulfil best possible routines in neonatal care.

It is not possible to ignore the well substantiated findings of complications related to preterm birth and neonatal intensive care (Anand 1998). However, infants may show an impressive ability to recover from extreme traumas including being severely ill in combination with exposure to numerous invasive and handling procedures as a part of the neonatal intensive care (Murdock 1984; Barker and Rutter 1995; Simons et al. 2003a; Stevens et al. 2003). Even if to high levels of circulating glucocorticoids are harmful to the brain and there is a risk of pain and stress causing high blood pressure and IVH (Korte et al. 1996; Anand 1998) surprisingly few preterm infants develop long-term disabilities (Bylund et al. 1998; Finnstrom et al. 2003; Leijon et al. 2003) How can this be possible? Are there protective factors within the infant or is it the care we provide, which contribute to protection and/or recovery? Figure 7 displays a flowchart showing possible mediating factors or "missing links"



**Figure 8** This figure includes one possible "missing link" between insecurely attached infants and behavioural problems later in life from figure 2. High stress load as a result of insensitive mothers and insecure attachment may cause altered cortisol levels with subsequent effects on hippocampus leading to behavioural problems. However, support to the mother-infant dyad with the aim of improving interaction may prevent possible behavioural problems for the infant later in life.

between low birthweight and behavioural problems later in life. Probably the ability to recover can refer to several different internal and external aspects, single or combined: 1) the newborn infants' plasticity of the brain (Blows 2003), 2) a stress hypo-responsive period (Sapolsky and Meaney 1986; Walker et al. 1986), 3) recovery of the hippocampus (McEwen 2001), 4) an environmental supportive milieu (Coe et al. 1989; Francis et al. 2002), 5) developmental care and help to self-regulation (Als et al. 1986; Campos 1994; Als 1998), 6) mother-to-infant attachment behaviours (Klaus et al. 1970) and 7) adequate pain relief (Anand et al. 2004).

Development of the HPA response to stressful stimuli is altered by early environmental events. Animals exposed to short periods of infantile stimulation or handling show decreased HPA axis responsivity to stress whereas maternal separation or physical traumas enhance the HPA axis responsiveness to stress (Caldji et al. 1998). Recently Grunau et al. found that elevated salivary cortisol levels in eight-month old preterm infants positively correlated to the amount of experienced pain in the neonatal period (Grunau et al. 2004). Clearly, there are outcome variables to be measured in the future, in relation to neonatal intensive care that has not yet been explored. In addition there are still a lot of interventions commonly performed in the NICU that are not yet evaluated (Franck 2005). Perhaps prospective long-term follow-up studies of infants' salivary cortisol and hippocampal volume will provide more information of infants' developing HPA system and possible consequences of neonatal intensive care.

### ***Psychosocial high-risk infants***

Our findings in paper IV indicate that infants younger than three months benefit from treatment provided to the mother-infant dyad with the aim of improving interaction. As the mother's sensitivity improves the infant's cortisol response to a nappy change disappear. These findings are supported by previous research (Klaus et al. 1970; Ainsworth et al. 1974; Blehar et al. 1977; Nachmias et al. 1996). For instance, it has been shown that insecurely attached children are more likely to have increased cortisol responses to novel situations as compared to securely attached children (Nachmias et al. 1996). Clearly our results lend further support to the importance of early interventions in order to prevent long-term negative consequences (Crowe and Johnson 1991; Wadsby et al. 1996; 2001; Brisch et al. 2003). Figure 8 displays a flowchart showing possible mediating factors and "missing links" between insensitive mothers and behavioural problems in the offspring later in life. Long-term follow up studies of salivary cortisol in psychosocial high-risk children are however needed to learn more about the development of the HPA system and the role of cortisol as a possible mediator of behavioural problems in early childhood and elementary school (Erickson et al. 1985; Renken et al. 1989; Shaw and Vondra 1995; Munson et al. 2001).

### ***Three-month immunisation***

Pain in infancy should be prevented if possible since even the youngest infant is capable of feeling pain, interpret noxious stimuli as painful, and remember pain (Anand and Carr 1989; Taddio et al. 1995; Taddio et al. 1997). In paper V it is shown that oral glucose in combination with a pacifier dampen the cortisol response. Therefore, 1 mL of oral glucose (300 mg/mL) could be recommended to three months old infants during routine immunisation, if they use a pacifier.

The physiological mechanism behind the effectiveness of oral sweet-tasting solution is not clarified (Shide and Blass 1989; Gradin and Schollin 2005) and we do not know enough about any possible long-term consequences of the use of oral sweet-tasting solutions (Eriksson and Finnstrom 2004). Therefore, it is important that oral sweet-tasting solutions are documented as a medication in the infants' medical record (Noerr 2001) also improving the chances of retrospective studies in the future.

## **Parents**

### ***Mothers in neonatal intensive care***

We have found that mothers in neonatal intensive care rate higher stress before the first time the infant is taken out of the incubator to rest skin-to-skin as compared to the second SSC (= fourth SSC with the mother, second intervention). During the first SSC the self-rated stress decrease and mood (control, calmness, and pleasantness) increase but salivary cortisol remain unchanged. However, when the infant is removed and replaced in the incubator the mothers' salivary cortisol levels decrease.

The neonatal intensive care unit is an environment where parents typically have

little control over activities and interventions. Emotion-focused coping is probably more of an option for these parents than problem-focused coping (Folkman 1984; Folkman et al. 1986). The first time when taking the vulnerable infant out of the safe incubator and holding him/her skin-to-skin must evoke a lot of emotions for the mother who has been separated from her infant since she gave birth (Miles et al. 1991; Gale et al. 1993; Ludington-Hoe et al. 1994; Seideman et al. 1997; Neu 1999; Nystrom and Axelsson 2002). The situation is new for both her and the infant. It is not possible for the mother to predict how the situation will proceed or what emotions it will evoke, until it is over. One of the mood dimensions improving during first SSC was control and it improved even further after the SSC session was over, along with salivary cortisol. This is in agreement with previous research. Control and an activation of the HPA axis have been found to correlate negatively, which is the same for predictability and an activation of the HPA axis (Fishman et al. 1962; Hanson et al. 1976; Lundberg and Frankenhaeuser 1978; Davis et al. 1981; Vogt et al. 1981; Hyyppa 1987; Hubert and de Jong-Meyer 1989; Wiener et al. 1990).

The postpartum period is especially important for development of mother-to-infant attachment behaviour (Klaus and Kennell 1970; Klaus et al. 1970). Skin-to-skin contact is one intervention supporting the bonding between the mother and her baby. Since the mothers clearly show high stress in combination with low control before and during the first SSC the nurses can help parents to regain control by providing support. However, further research is needed to find out what kind of support the mothers would best benefit from.

The mood scale used in paper II was constructed in 1979. It comprises 71 different adjectives especially reflecting the mood (Sjoberg et al. 1979). It has been used in several studies and has proved to be sensitive enough for fluctuations of mood on short durations (Persson et al. 1980; Svensson et al. 1980). However, living in the 21st century some of the adjectives used sometimes feel a bit old fashioned. Since mood scale is such a useful instrument future research would certainly benefit from a revision of the mood scale.

### ***Parents and the three-month immunisation***

Predictability is also of importance during the three-month immunisation. In paper V we found that multipara parents rated lower stress on VAS before the immunisation as compared to primipara parents. Thus, parents need support from the nursing staff even when their infants are healthy. Especially primipara parents need support before the first immunisation of their baby. Similar results from studies of parents with hospitalised and/or sick infants have been reported earlier suggesting that the severity of illness is not as much of importance as coping, control, and predictability (Schepp 1991; Davis et al. 1998; Morelius et al. 2002).

After the immunisation the parents rated significantly more stress than before the immunisation. On the other hand, salivary cortisol decreased after the immunisation. It is not unlikely that people are experiencing different emotions when challenged with a stressor (Folkman and Lazarus 1985). Immediately after the immunisation

the parents may have emotions of threat, as worry and fear that reflect the increased VAS. However, the threat passes by fast and is replaced by emotions of relief, reflected by the decreased salivary cortisol (Folkman and Lazarus 1985; Folkman et al. 1986).

### ***Psychosocial high-risk mothers***

Mothers enrolled in the Hagadal day-care programme certainly benefited from treatment aimed to improve interaction between mother and child (paper IV). The mother's sensitivity towards her infant, as measured with Ainsworth's sensitivity scale, improved significantly during the treatment period. On the other hand, we did not find any differences in mothers' cortisol reactivity between first and last week of treatment. The mothers decreased in salivary cortisol levels in response to both nappy changes. The decreased cortisol levels may reflect emotions of benefit as pleasantness and joy (Folkman and Lazarus 1985). The decreased salivary cortisol may also be a sign of security in their parenting role when the caregivers are present. It certainly would have been interesting if mood scale had been included in this study.

### **Infants and parents**

In paper V there was a significant positive correlation between change in parent's cortisol and the change in infant's cortisol. There was also a correlation between parents' and infants' baseline values. There is some evidence of inheritance factors playing a role for cortisol levels. Women who were pregnant during September 11 were included in a study by Yehuda and colleagues (Yehuda et al. 2005). The women who developed PTSD had significantly lower cortisol levels as compared to the women not developing PTSD. The offspring of the women who developed PTSD after the trauma also had lower levels of cortisol at the age of one as compared to the offspring of women who did not develop PTSD (Yehuda et al. 2005).

We found no significant correlations between mothers' changes in cortisol levels and infants' changes in cortisol in paper II and IV. However, if the mother's baseline salivary cortisol was an outlier the infant's baseline salivary cortisol also tended to be an outlier. The absence of correlation could either depend on 1) a small sample size 2) neurochemically immature and/or disorganised infants, 3) lack of attachment in psychosocial high-risk infants (paper IV) and lack of bonding because of separation (paper II) or 4) it is not supposed to be a correlation.

In paper V there was a significant positive correlation between infant's crying-time and the magnitude of change in parent's cortisol and VAS. However, we found no evidence that parents' self-rated stress had an impact on the way the infants reacted in response to the immunisation. These results indicate that parents get affected by infants' way to react during a painful procedure, but not the other way around. The importance of proximity for infant's sense of security has been addressed in previous studies (Bowlby 1953; Ainsworth 1979). It is possible that three months old infants feel secure by the proximity of a parent during the immunisation, but that they are too young to sense parent's possible feeling of stress. In paper IV we found

that the professional support given to the mothers was beneficial especially for the infants younger than three months who changed their cortisol reactivity during the treatment period. This result indicates that it is possible to help the infant by helping the mother (Fig. 8).

## Conclusions

The results of the salivary cortisol responses in the preterm infants include large individual differences; some infants increase and some decrease in cortisol levels, and with small sample sizes it is difficult to draw conclusions. However, in comparison with other studies (Magnano et al. 1992; Gitau et al. 2002; Boyer et al. 2004; Davis et al. 2004; Herrington et al. 2004; Catelin et al. 2005; Harrison 2005) we have managed to collect saliva and analyse cortisol in a high percentage of these very preterm infants without the use of saliva stimulants. The following conclusions can be drawn from the present thesis:

- It is practically feasible to collect saliva even from the smallest neonates using cotton buds.
- It is reliable to analyse cortisol in small volumes of saliva with radioimmunoassay.
- It is possible to administer oral glucose and still analyse cortisol in saliva 30 minutes later without interference.
- The studies lend further support to individual care in neonatal intensive care.
- Infants in neonatal intensive care have higher baseline salivary cortisol than healthy full-term infants.
- Baseline salivary cortisol decreases in the newborn infant by postnatal age.
- Psychosocial high-risk infants three months or older have higher baseline salivary cortisol than psychosocial high-risk infants younger than three months.
- It is important to calm a crying baby before a painful procedure.
- Oral glucose in combination with pacifier during the three-month immunisation dampen the salivary cortisol response.
- Parents need support before and during first SSC in neonatal intensive care.
- Parents need support before the infants' routine three-month immunisation, especially if it is their first child.
- Early interventions are of importance for infants of psychosocial high-risk mothers.
- Psychosocial high-risk mothers can improve their sensitivity toward their infants' signals if they get individual and group support in a day-care programme.

- Saliva for analysis of cortisol should be collected using cotton buds with plastic sticks if not centrifuged within 24 hours.

**The end**

This is the end of this thesis but just the beginning of research on stress in infants.

# Acknowledgement

I wish to express my gratitude to all infants, parents, and others for helping me with this thesis. I would especially like to thank:

Associate professor Nina Nelson, main supervisor: Thank you for introducing me into your research field! Thank you for sharing your never-ending enthusiasm, for always being supportive, for encouragement, patience, advice, and guidance in research. Thank you for fruitful discussions of life and research and for being a good friend and company on congress trips to Australia, Canada, and London. Thank you for a great education. "A teacher affects eternity; he can never tell where his influence stops" (Henry Adams, 1838-1918).

Professor Elvar Theodorsson, associate supervisor: Thank you for introducing and guiding me in the chemical world, for teaching me the radio-immunoassay, how to use a pipette, for letting me use your laboratory (and not throwing me out when I showed you the standard curve of my first test results) and for advising me in scientific thinking and writing.

Christine Rosén: for nappy changes, saliva sampling, and accurate help with map-reading during our trips to different homes for nappy changes on newborn infants. Thank you for your willingness to do a good work.

Anne Sandehed, Lisbeth deJong: for excellent help with data collection at the NICU in Linköping.

Irja Väinämö: for your creativity with the cotton buds.

Lena Blom, Monica Svedbom Magnusson, and the staff members at Hagadal: for helping mothers and infants, and for helping me with data collection.

Meta Philipson and the staff members at the Well Baby Clinic Skogsfrid in Linköping: for excellent help with data collection.

Associate professor Per A Gustafsson: for co-authorship, supervising, and guidance in paper IV.

Associate professor Lena Hellström-Westas, Catarina Carlén, Agneta Kleberg, Ann-Cathrine Berg, and Elisabeth Norman at the NICU in Lund: for co-operation and scientific discussions.

To all the staff members at the NICU, Neo-IVA in Linköping: For taking such good care of all the treasures (newborns) at the neonatal intensive care unit. Keep up the good work!

My coffee-break friends Anneli Jonsson, Anneli Sepa, Felix Koch, Katarina Ekholm Selling, Lena Hanberger, Lotta Samelius, and Stina Bodén: for endless, hilarious and unbelievable discussions about life, science, and "care and abuse". Special thanks for valuable research discussions in our journal-club and for letting me share ups (= Champagne) and downs with you.

Lena Forsén: For believing in me.

Marie Nilsson: for understanding how important this thesis was for me.

Ann-Christine Gillmore-Ellis: For all help and support with administration.

Professor Orvar Finnström and research colleagues at the division of Pediatrics: for comments and critiques over the years, leading to improvements of my research and presentations.

Olle Eriksson and Katarina Ekholm Selling: for statistical help and advice.

Lovisa Holm and Susanne Hilke: for guiding me at the laboratory, helping me with the gamma counter, and for extinguishing the fire I was luckily unaware of.

Inga Warren: for your hospitality in London, for guiding me at the neonatal intensive care unit at St. Mary's, and for great discussions of research.

Elisabeth Lundgren and the staff members at the children's ward 67 (former 93) in Lund: for teaching me how to act as a nurse when I worked with you in my early career.

Annika Persson, department of child and adolescent psychiatry: for videotranscriptions in paper IV.

My friends: for keeping in-touch even though I am only working and never have time to contact you.

My parents Ann-Ruth and Egon Mörelius: For I being I.

Molly, 12 years: for being so reliable, responsible, lovely, and wonderful.

Smilla, 8 years: for your never-ending hugs, kisses, and smiles. And, for saying "it doesn't matter, mummy!" when I have once again failed with home service.

Professor Peter Pålsson: You are my idol! I lurv you!





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