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Cortisol, Behavior, and Heart Rate Reactivity to Immunization Pain at 4 Months Corrected Age in Infants Born Very Preterm

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Abstract

Objective—Pain response may be altered in infants born very preterm owing to repeated exposure to procedures in the neonatal intensive care unit. Findings have been inconsistent in studies of behavioral and cardiac responses to brief pain in preterm versus full-term infants following neonatal intensive care unit discharge. To our knowledge, cortisol reactivity to pain has not been compared in preterm and full-term infants. We examined pain reactivity to immunization in preterm and full-term infants.

Method—Cortisol, facial behavior, and heart rate reactivity before, during, and after immunization were examined in infants born preterm at extremely low gestational age (ELGA 24 to 28 wk), very low gestational age (VLGA 29 to 32 wk), and full-term, at corrected age 4 months.

Results—In all groups, cortisol, behavior, and heart rate increased during immunizations. Cortisol concentrations were lower in preterm ELGA and VLGA boys, compared with full-term boys. In contrast, facial and heart rate responses to immunization did not differ between preterm and full-term infants.

Discussion—Although earlier reports found differences in pain processing in preterm infants earlier and later in development, the present findings indicate that pain responses, indexed by behavior and heart-rate, do not seem to differ in preterm compared with full-term infants at 4 months corrected age. Importantly, however, stress regulation seems altered in preterm male infants. As cortisol impacts development and functioning of the brain, altered stress regulation has important implications beyond pain systems.

Keywords

preterm infant; pain; stress; cortisol; heart rate; behavior; sex

There is growing evidence that pain and tactile processing are altered in preterm infants while they are in the neonatal intensive care unit (NICU),^{1,2} in infancy,³ and later in childhood.⁴⁻⁹ Animal studies have highlighted the importance of timing and extent of neonatal insult in modulating later pain reactivity, and that effects include long-term changes in somatosensory processing.¹⁰⁻¹³

To understand the development of altered pain expression in preterm children, studies are needed in infancy, as pain responses as children develop are modified by social factors such as pain modeling in the family.¹⁴ There are few biobehavioral studies of pain reactivity in preterm compared with full-term infants in the first-year after hospital discharge. In an earlier longitudinal study, using blood collection by finger lance at a “pain-naïve” site, we unexpectedly found no differences in behavioral and heart rate reactivity or recovery at 4 months corrected chronological age (CCA),¹⁵ but significant differences later at 8 months CCA.³ However, finger lance is a relatively mild pain stimulus, involving only a single skin-break. Therefore it is important to evaluate reactivity to a more intense stimulus. Immunization injections are painful stimuli routinely experienced by infants and young children.¹⁶ In full-term infants, immunization induces behavioral reactions characteristic of infant pain, and increased heart rate and cortisol.¹⁷⁻²⁰ Earlier work has shown that the pattern of basal cortisol secretion is altered in preterm infants,^{21,22} and children,²³ and during reactivity to cognitive novelty in infancy,²¹ but not to a social stressor at school age.²³ Cortisol reactivity to physical stressors has not been examined, to our knowledge, in preterm compared with full-term infants.

In the present study we examined reactivity and recovery to pain of immunization injections, in preterm compared with full-term infants at 4 months CCA. We hypothesized that infants born very preterm would show altered behavioral and physiological reactivity to injections during immunization, and that these effects would be especially marked in those born below 29 weeks gestation, as these infants were exposed to the most invasive procedures during neonatal care, in addition to being developmentally the most immature. Furthermore, sex differences in long-term effects of pain have been reported in neonatal rats,^{24,25} with the direction depending on multiple factors. Therefore, we explored sex differences in this study, as a secondary question.

MATERIALS AND METHODS

Participants

A total of 138 infants (99 preterm and 39 full-term) at mean 4.2 months (sd 1.0) CCA participated in a cohort study. Preterm infants were recruited from the neonatal intensive care unit (NICU) at the Children’s and Women’s Health Center of British Columbia, which is the major tertiary neonatal unit in British Columbia, Canada; full-term infants were recruited by advertisement.

During the study period, vaccination protocols changed in British Columbia, affecting the number of injections given. We studied infants during one vaccination between 2 to 6 months corrected age, since earlier studies in full-term infants have shown that this age range provides the maximal cortisol response to immunization injections, with significantly

less response at later ages.^{26,27} Immunizations are scheduled by chronological age, whereas physiological reactivity must be compared by corrected age, thus age was difficult to match in relation to prematurity status. In this study, we included only infants that received 3 injections (excluding 30 infants with 2 injections and 1 with 4 injections). A further 7 infants (3 preterm, 4 controls) were excluded due to feeding within 30 minutes that could affect cortisol assays.²⁸ Infant recruitment and exclusions are shown in Figure 1. The final sample of 100 infants (68 preterm, 32 full-term), comprised 29 infants born preterm at extremely low gestational age (ELGA < 28 wk GA; 16 boys, 13 girls), 39 born preterm at very low gestational age (VLGA 29 to 32 wk; 27 boys, 12 girls) and 32 infants born full-term (39 to 41 wk GA; 20 boys, 12 girls), Table 1. For power of .80 with a Type 1 error rate of 0.05, 29 infants were needed in each group, based on earlier work.³

Measures

Behavior—Using the Neonatal Facial Coding System (NFCS²⁹), the 7 facial actions (brow bulge, eye squeeze, nasolabial furrow, open mouth, vertical mouth stretch, horizontal mouth stretch, taut tongue) most commonly indicative of infant pain were coded from videotapes in 2 second time segments, across 10 epochs from the start of each phase of vaccination (Baseline, Prep, first Injection, third Injection, Recovery), for a total of 20 seconds of facial coding per event. Coders were blinded to all infant and parent information, and trained to achieve interrater reliability above 0.85 using the conservative method of Eckman and Friesen.²⁹

Heart Rate—Heart rate was measured continuously with the Biolog 3992, using 3 leads. Heart rate was averaged for 30-second epochs: Baseline (90 s, 3 epochs), first Injection (30 s, 1 epoch), third Injection (30 s, 1 epoch), and Recovery (180 s, 6 epochs).

Cortisol—Saliva was collected with a sorbette, extracted and stored at −20°C until assayed in duplicate using the Salimetrics High Sensitivity Salivary Cortisol Enzyme Immunoassay Kit (Salimetrics, LCC, Pennsylvania). Intra and interassay coefficients of variation were 3.65% and 6.41%, respectively. Saliva was collected 3 times: Basal (before vaccination), Reactivity (20 min after first Injection), Recovery (30 min after first Injection). The timing of cortisol measures was based on a earlier study of immunization in full-term infants.¹⁹

Medical and Nursing Chart Review—A neonatal research nurse conducted chart review from birth to term equivalent (39 wk + 6 d) including, but not limited to, gestational age, early illness severity using the Score for Neonatal Acute Physiology II (SNAP II) on postnatal day 1, the number of skin-breaking procedures (eg, heel lances, intramuscular injections, central lines, and chest tube insertions), intravenous morphine exposure adjusted for daily weight.

Procedures

This study was approved by the University of British Columbia Clinical Research Ethics Board. A parent provided written informed consent. Infants received their vaccination at home, in one visit by 2 research staff and a registered nurse from the Vaccine Evaluation Center, Child and Family Research Institute who administered the vaccinations. Visits were

9:00 to 11:30 a.m. to avoid diurnal variation affecting cortisol. Infant health status was confirmed by phone interview on the day before the visit.

When the infant was comfortable, the first saliva sample was collected (Basal), the videocameras turned on, and leads attached to the infant's chest for cardiac recording. Three intramuscular injections of 0.5 mL volume were administered in alternate thighs using 25 gauge needles: Injection 1: Diphtheria, Tetanus, acellular Pertussis, inactivated Polio, *Haemophilus influenzae* type b conjugate; Injection 2: recombinant Hepatitis B; Injection 3: pneumococcal conjugate vaccine. Vaccine Evaluation Center nurses used a standard procedure, with the infant on the mother's lap, facing outward to expose the anterior portion of the thighs. In this naturalistic study mothers were free to provide soothing as needed. Data collection, videotape scoring, cardiac data processing and cortisol assays were carried out by staff blinded to the purpose of the study, group status and all clinical information.

Data Analysis

Univariate repeated measures ANOVAs, with the conservative Greenhouse-Geisser correction, was used to examine facial activity (NFCS scores), heart rate, and cortisol across phases of immunization (Baseline, Injections, Recovery), by Group (ELGA, VLGA, Full-term) and Sex (Boys, Girls). Statistical significance for main effects and interaction effects was set at $P < 0.05$. Significance levels for posthoc analyses were adjusted using the Bonferroni correction to reduce type-one error.

RESULTS

Infant Characteristics

As expected, the groups (ELGA, VLGA, Full-term) differed on neonatal variables (Table 1). Since immunizations are scheduled by chronological rather than corrected age, CCA on the test day was significantly lower for the ELGA group (mean 3.6 mo) compared with the VLGA (4.5 mo) and full-term (4.5 mo) infants, but did not differ by sex ($P=0.15$). Therefore CCA was used as a covariate in all ANOVAs.

Facial Behavior

Brief periods of missing data owing to the face out of view of the camera were replaced with the mean for the subgroup by sex, affecting 3% of the facial data. Analyses were repeated with and without replacement for missing data and results were the same. NFCS face actions showed significant changes across Events ($P < 0.0001$, $\eta = 0.122$). Posthoc t tests with Bonferroni correction showed that facial activity did not change from Baseline to Preparation of cleansing with the alcohol swab ($P=0.71$), but increased after the first Injection ($P < 0.0001$), increased further from the first to the third Injection ($P < 0.0001$), decreased during the first Recovery epoch ($P < 0.0001$) and decreased further during the second Recovery epoch ($P < 0.001$). There were no significant differences for the Subgroups, Sex, or CCA (each $P > 0.25$). NFCS scores for the ELGA, VLGA, and Full-term subgroups, adjusted for CCA, are shown across Phases of immunization in Figure 2.

Heart Rate (HR)

Mean HR was compared across 30 second epochs for Events (3 epochs of Baseline, after the first and third Injections, and 6 epochs of Recovery). Brief periods of missing data owing to turbulence in the signal were replaced with the subgroup mean by sex for 7% of the HR data. Data analyses were repeated with and without missing data replaced, and the results were the same. HR changed significantly across Events for all Groups ($P < 0.0001$, $\eta = 0.05$), with post hoc t tests using Bonferroni correction indicating significant increase from the end of Baseline to the first Injection, increase from the first to the third Injection, decrease from the third Injection to the first Recovery epoch, and decline during each epoch of Recovery (every $P < 0.0001$). There were no differences by Subgroup ($P = 0.70$) or Sex ($P = 0.78$), however CCA was significant ($P = 0.023$). Pearson correlations between CCA and each phase of immunization (using Bonferroni corrections to control type-1 error) indicated that lower CCA was associated with high basal HR (Baseline 1 & 2, $r = -0.30$, $P = 0.003$, $r = -0.33$, $P = 0.001$, respectively) but no differences during Reactivity or Recovery. HR for the Subgroups, adjusted for CCA, across the Phases of immunization is shown in Figure 3.

Cortisol

Cortisol values for 3 infants were outliers (outside 3 SD from the mean) and were winsorized.³⁰ Inadequate quantity of saliva affected 18/150 samples (12%). Analyses were repeated with and without replacement for missing data, and the results were the same.

Cortisol levels changed significantly for all Groups across Events ($P = 0.011$, $\eta = 0.063$), with post hoc tests using Bonferroni correction indicating significant increase from Basal level to 20 minutes postinjections ($P = 0.0001$), and significant decrease by 30 minutes postinjections ($P = 0.0001$). There was no main effect for Subgroup or Sex, and CCA was not significant (each $P > 0.49$). Importantly, there was a significant Subgroup X Sex interaction ($P = 0.045$, $\eta = 0.07$). To explore the interaction, one-way ANOVA for boys and girls separately was conducted, adjusted for CCA. The girls did not differ significantly ($P = 0.64$). For boys, after adjusting for age (CCA, $P = 0.36$), planned contrasts indicated lower overall cortisol concentrations in the ELGA and VLGA preterm boys combined compared with the full-term boys ($P = 0.028$), but no difference between the ELGA and VLGA boys. As reactivity was the primary question in this paper, we explored cortisol reactivity (after the injections) using planned contrasts, indicating that both the ELGA and VLGA boys showed lower cortisol reactivity to the injections than the full-term boys ($P = 0.006$, $\eta = 0.132$). Mean cortisol concentrations by Subgroup and Sex, adjusted for CCA are displayed in Figure 4, and for boys only in Figure 5.

Potentially confounding medical factors in the NICU that may have contributed to this sex difference were examined within the preterm sample using t tests. Surprisingly, there were no statistically significant differences between boys and girls in early illness severity (day 1 SNAP II; $P = 0.61$), number of days of mechanical ventilation ($P = 0.85$), duration of other respiratory support ($P = 0.77$), or number of skin-breaking procedures ($P = 0.59$). One ELGA boy and one ELGA girl had an abnormal finding on neonatal cranial ultrasound. When the preterm infants were in the NICU, the number who had surgery did not differ between the boys (25%) and girls (20%), $P = 0.19$. In contrast, however, surgery (eg, hernia repair) was

carried out between term equivalent (ie, 39 wk and 6 d gestational age) and the study day, on 7 boys (4 ELGA, 2 VLGA, 1 full-term), but none of the girls.

Additional Analyses

Data were reanalyzed excluding 3 ELGA infants that had received postnatal dexamethasone in the NICU, with the same results.

DISCUSSION

We examined facial activity, heart rate, and stress hormone (cortisol) responses concurrently to the pain of 3 immunization injections, in infants born extremely preterm (ELGA), and very preterm (VLGA), compared with full-term. Cortisol, the end-product of the hypothalamic-pituitary-adrenal (HPA) axis, and thereby the primary stress hormone in humans, is an important biomarker of overall stress response, whereas facial reactivity (using the NFCS) together with change in heart rate, have been widely used as indicators of infant pain. We compared reactivity of preterm and full-term infants to a relatively prolonged stimulus involving 3 injections, rather than a brief single stimulus of finger lance studied previously.^{3,15}

There were 3 important findings in this study. First, contrary to our hypothesis, regardless of maturity at birth, infants showed no differences in facial and heart rate responses to immunization at 4 months CCA, suggesting comparable pain reactivity and recovery. Second, in contrast to these pain indices, cortisol reactivity differed depending on infant sex and prematurity status. Third, both facial behavior and heart-rate responses increased from the first to the third Injection in all groups, indicating that 3 injections elicited a greater pain response than a single injection. Given that cortisol concentrations do not reach peak levels until at least 15 minutes poststressor, it was not possible to compare cortisol changes to the first versus the third injection as they were administered in quick succession. Similarly, owing to the rapid succession of injections, it was not possible to examine change in facial or heart rate responses from the first to the second injection.

This is the first study, to our knowledge, to compare cortisol reactivity to a physical stressor in preterm and full-term infants. Our earlier studies found preterm infants (ELGA and VLGA) showed significantly lower basal cortisol levels than full-term infants at 3 months CCA,²² no differences at 6 months CCA,²² and a shift to higher basal cortisol levels in ELGA relative to VLGA and full-term infants at 8 and 18 months CCA.^{21,22} In the present study, at 4 months CCA we found that preterm boys (both ELGA and VLGA) had lower cortisol concentrations after the 3 immunization injections compared with full-term boys, whereas girls did not differ by prematurity status. Importantly, in a study of cortisol reactivity to vaccination in preterm infants at 4 months (with no term born controls), higher neonatal cortisol levels in the NICU predicted greater cortisol response at 4 months, but male sex was associated with lower response,³¹ consistent with the present findings. In earlier studies of full-term infants' response to immunization, either no sex difference^{19,32,33} or greater response in boys¹⁸ was reported. In studies comparing pain responses in former preterms and full-terms later in childhood, sex differences have not been examined to our knowledge.⁶⁻⁹ Animal studies, however, reveal complex sex differences in long-term effects

of early pain exposure, depending on multiple factors, including genetics, sex, and experience.^{24,34} In the adult pain literature, women show higher levels of pain and more recurrent pain than men (eg,³⁵). In the present study, we found no sex differences in facial or heart rate reactivity at 4 months CCA, however, lower cortisol concentrations in preterm boys may have more widespread implications for the developing infant, given the importance of cortisol for metabolic and brain function.³⁶ Too high or too low levels of cortisol are associated with difficulties in memory and executive functions subsumed by brain areas (hippocampus, frontal cortex) that are rich in glucocorticoid receptors in humans.³⁷ Boys born preterm show poorer neurodevelopment later in childhood relative to preterm girls,³⁸ however, whether altered cortisol regulation may in part contribute to this is unknown.

Currently, infants in industrialized countries receive as many as 20 immunization injections in the first 18 months of life, with multiple injections at some visits. In this study, we found that both facial behavior and heart-rate responses increased from the first to the third injection in all groups, indicating that 3 injections were demonstrably more “painful” than a single injection. Owing to the rapid succession of injections, the second injection could not be compared. Our findings suggest that consideration be given to the spacing or grouping of injections, particularly for vulnerable infants.

Strengths of this study were that all vaccinations were carried out by experienced research nurses, using a standard protocol for injection administration and infant position, and that we compared responses by degree of prematurity (ie, infants born 28 wk with those born 29 to 32 wk), and by sex. A limitation of this study was the range of age at immunization. However, given that immunizations are administered by chronological age, and infants must be healthy to receive their shots, controlling timing of vaccinations across the full range of very preterm compared with full-term birth does not seem to be possible. Corrected age was not related to the facial or cortisol measures; baseline heart rate was higher in the ELGA group, consistent with earlier findings,³ but did not affect reactivity or recovery. Further limitations were that sex was unbalanced (fewer girls), which may have contributed to our finding of no differences between the groups for girls, and ethically we encouraged mothers to provide soothing that thereby varied. It is a major challenge to examine pain past the neonatal period in preterm infants, as only medically necessary procedures can be studied.

Neonatal invasive procedures are associated with behavioral, physiological, and hormonal changes that impact neurobiological development of both stress systems^{9,21–23} and nociceptive (pain) systems in human infants born very preterm,^{3,5,6} months to years after hospital discharge. However, effects are complex. For example, quantitative sensory testing at 9 to 14 years showed sensitization to prolonged painful stimulation, but hypoalgesia to brief pain, in children who had received neonatal intensive care,⁷ consistent with our earlier findings of hypersensitivity and hyposensitivity in preterm infants at 8 months CCA.³ Animal studies have confirmed the importance of timing and extent of neonatal insult, and that effects are not only local, but include global long-term consequences on somatosensory processing.^{10–13} It is unclear why pain response is not differentiated in preterm infants at 4 months in this study, or previously at similar age with a different sample and stimulus condition.¹⁵ Age 3 to 6 months is a period of reorganization in multiple physiological and

behavioral systems, such as sleep/wake states and cry patterns,³⁹ and the emergence of cyclical diurnal rhythm in cortisol.⁴⁰ Longitudinal studies are needed to understand the developmental trajectory of tactile, nociceptive, and somatosensory processing in infants born very preterm.

Importantly, our findings of altered cortisol regulation in boys born preterm, compared with full-term, may have important implications for understanding greater vulnerability in cognition and behavior in male preterm infants across the developmental spectrum. In contrast, infant pain perception, indexed by facial activity and HR to immunization, seemed indistinguishable among infants born ELGA, VLGA, and full-term at 4 months CCA, unlike previous work at earlier and later ages.

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References

1. Johnston CC, Stevens BJ. Experience in a neonatal intensive care unit affects pain response. *Pediatrics*. 1996; 98:925–930. [PubMed: 8909487]
2. Grunau RE, Holsti L, Haley DW, et al. Neonatal procedural pain exposure predicts lower cortisol and behavioral reactivity in preterm infants in the NICU. *Pain*. 2005; 113:293–300. [PubMed: 15661436]
3. Grunau RE, Oberlander TF, Whitfield MF, et al. Pain reactivity in former extremely low birth weight infants at corrected age 8 months compared with term born controls. *Infant Behavior and Development*. 2001; 24:41–55.
4. Abdulkader HM, Freer Y, Garry EM, et al. Prematurity and neonatal noxious events exert lasting effects on infant pain behaviour. *Early Hum Dev*. 2008; 84:351–355. [PubMed: 17964090]
5. Grunau RV, Whitfield MF, Petrie JH. Pain sensitivity and temperament in extremely low-birth-weight premature toddlers and preterm and full-term controls. *Pain*. 1994; 58:341–346. [PubMed: 7838583]
6. Buskila D, Neumann L, Zmora E, et al. Pain sensitivity in prematurely born adolescents. *Arch Pediatr Adolesc Med*. 2003; 157:1079–1082. [PubMed: 14609898]
7. Hermann C, Hohmeister J, Demirakca S, et al. Long-term alteration of pain sensitivity in school-aged children with early pain experiences. *Pain*. 2006; 125:278–285. [PubMed: 17011707]
8. Schmelzle-Lubiecki BM, Campbell KA, Howard RH, et al. Long-term consequences of early infant injury and trauma upon somatosensory processing. *Eur J Pain*. 2007; 11:799–809. [PubMed: 17320438]
9. Goffaux P, Lafrenaye S, Morin M, et al. Preterm births: can neonatal pain alter the development of endogenous gating systems? *Eur J Pain*. 2008; 12:945–951. [PubMed: 18308597]
10. Anand KJ, Coskun V, Thrivikraman KV, et al. Long-term behavioral effects of repetitive pain in neonatal rat pups. *Physiol Behav*. 1999; 66:627–637. [PubMed: 10386907]

11. Ren K, Anseloni V, Zou SP, et al. Characterization of basal and re-inflammation-associated long-term alteration in pain responsivity following short-lasting neonatal local inflammatory insult. *Pain*. 2004; 110:588–596. [PubMed: 15288399]
12. Ririe DG, Bremner LR, Fitzgerald M. Comparison of the immediate effects of surgical incision on dorsal horn neuronal receptive field size and responses during postnatal development. *Anesthesiology*. 2008; 109:698–706. [PubMed: 18813050]
13. Fitzgerald M. The development of nociceptive circuits. *Nature Reviews Neuroscience*. 2005; 6:507–520. [PubMed: 15995722]
14. Chambers CT. The role of family factors in pediatric pain. *Prog Pain Res Manag*. 2003; 26:99–130.
15. Oberlander TF, Grunau RE, Whitfield MF, et al. Biobehavioral pain responses in former extremely low birth weight infants at four months' corrected age. *Pediatrics*. 2000; 105:e6. [PubMed: 10617743]
16. Schechter NL, Zempsky WT, Cohen LL, et al. Pain reduction during pediatric immunizations: evidence-based review and recommendations. *Pediatrics*. 2007; 119:e1184–e1198. [PubMed: 17473085]
17. Lilley CM, Craig KD, Grunau RE. The expression of pain in infants and toddlers: developmental changes in facial action. *Pain*. 1997; 72:161–170. [PubMed: 9272800]
18. Piira T, Champion GD, Bustos T, et al. Factors associated with infant pain response following an immunization injection. *Early Hum Dev*. 2007; 83:319–326. [PubMed: 16920282]
19. Ramsay D, Lewis M. Reactivity and regulation in cortisol and behavioral responses to stress. *Child Dev*. 2003; 74:456–464. [PubMed: 12705566]
20. Braarud HC, Stormark KM. Maternal soothing and infant stress responses: soothing, crying and adrenocortical activity during inoculation. *Infant Behavior and Development*. 2006; 29:70–79. [PubMed: 17138263]
21. Grunau RE, Weinberg J, Whitfield MF. Neonatal procedural pain and preterm infant cortisol response to novelty at 8 months. *Pediatrics*. 2004; 114:e77–e84. [PubMed: 15231977]
22. Grunau RE, Haley DW, Whitfield MF, et al. Altered basal cortisol levels at 3, 6, 8 and 18 months in infants born at extremely low gestational age. *Journal of Pediatrics*. 2007; 150:151–156. [PubMed: 17236892]
23. Buske-Kirschbaum A, Krieger S, Wilkes C, et al. Hypothalamic-pituitary-adrenal axis function and the cellular immune response in former preterm children. *J Clin Endocrinol Metab*. 2007; 92:3429–3435. [PubMed: 17566098]
24. LaPrairie JL, Murphy AZ. Female rats are more vulnerable to the long-term consequences of neonatal inflammatory injury. *Pain*. 2007; 132(suppl 1):S124–S133. [PubMed: 17904745]
25. Page GG, Blakely WP, Kim M. The impact of early repeated pain experiences on stress responsiveness and emotionality at maturity in rats. *Brain Behav Immun*. 2005; 19:78–87. [PubMed: 15581741]
26. Gunnar MR. Quality of early care and buffering of neuroendocrine stress reactions: potential effects on the developing human brain. *Prev Med*. 1998; 27:208–211. [PubMed: 9578997]
27. Davis EP, Granger DA. Developmental differences in infant salivary alpha-amylase and cortisol responses to stress. *Psychoneuroendocrinology*. 2009; 34:795–804. [PubMed: 19268476]
28. Hanrahan K, McCarthy AM, Kleiber C, et al. Strategies for salivary cortisol collection and analysis in research with children. *Appl Nurs Res*. 2006; 19:95–101. [PubMed: 16728293]
29. Grunau R, Craig K. Pain expression in neonates: facial action and cry. *Pain*. 1987; 28:395–410. [PubMed: 3574966]
30. Tukey, JW. *Exploratory Data Analysis*. Don Mills, ON: Addison-Wesley; 1977.
31. Glover V, Miles R, Matta S, et al. Glucocorticoid exposure in preterm babies predicts saliva cortisol response to immunization at 4 months. *Pediatr Res*. 2005; 58:1233–1237. [PubMed: 16306199]
32. Lewis M, Thomas D. Cortisol release in infants in response to inoculation. *Child Dev*. 1990; 61:50–59. [PubMed: 2307046]
33. Ipp M, Taddio A, Goldbach M, et al. Effects of age, gender and holding on pain response during infant immunization. *Can J Clin Pharmacol*. 2004; 11:e2–e7. [PubMed: 15226521]

34. Stephan M, Helfritz F, Pabst R, et al. Postnatally induced differences in adult pain sensitivity depend on genetics, gender and specific experiences: reversal of maternal deprivation effects by additional postnatal tactile stimulation or chronic imipramine treatment. *Behav Brain Res.* 2002; 133:149–158. [PubMed: 12110448]
35. Unruh AM. Gender variations in clinical pain experience. *Pain.* 1996; 65:123–167. [PubMed: 8826503]
36. Matthews SG. Early programming of the hypothalamo-pituitary-adrenal axis. *Trends Endocrinol Metab.* 2002; 13:373–380. [PubMed: 12367818]
37. Pryce CR. Postnatal ontogeny of expression of the corticosteroid receptor genes in mammalian brains: inter-species and intra-species differences. *Brain Res Rev.* 2008; 57:596–605. [PubMed: 17916381]
38. Whitfield M, Grunau R, Holsti L. Extremely premature (≤ 800 g) schoolchildren: multiple areas of hidden disability. *Arch Dis Child Fetal Neonatal Ed.* 1997; 77:F85–F90. [PubMed: 9377151]
39. White BP, Gunnar MR, Larson MC, et al. Behavioral and physiological responsivity, sleep, and patterns of daily cortisol production in infants with and without colic. *Child Dev.* 2000; 71:862–877. [PubMed: 11016553]
40. Price DA, Close GC, Fielding BA. Age of appearance of circadian rhythm in salivary cortisol values in infancy. *Arch Dis Child.* 1983; 58:454–456. [PubMed: 6859940]

FIGURE 1.

Flow diagram of recruitment and inclusion. *The provincial immunization schedule changed during the study period; only infants receiving 3 injections were included.

FIGURE 2.

Facial activity (mean, se) on the Neonatal Facial Coding System (NFCS) across phases of immunization in infants born extremely low gestational age (ELGA 24 to 28 wk), very low gestational age (VLGA 29 to 32 wk) and full-term (38 to 41 wk), adjusted for age at test day.

FIGURE 3.

Heart rate (mean, se) across phases of immunization in 30 seconds epochs [Baseline (Base), 1st and 3rd Injections, Recovery (Rec)] comparing infants born extremely low gestational age (ELGA 24 to 28 wk), very low gestational age (VLGA 29 to 32 wk) and full-term (38 to 41 wk), adjusted for age at test day.

FIGURE 4.

Cortisol (mean, se) across phases of immunization (Basal and 20 min and 30 min after the injections began) in extremely low gestational age (ELGA 24 to 28 wk), very low gestational age (VLGA 29 to 32 wk) and full-term (38 to 41 wk), adjusted for age at test day.

FIGURE 5.

Cortisol (mean, se) in boys, across phases of immunization (Basal and 20 min and 30 min after the injections began) in extremely low gestational age (ELGA 24 to 28 wk), very low gestational age (VLGA 29 to 32 wk) and full-term (38 to 41 wk) boys, adjusted for age at test day.

TABLE 1Infant Characteristics (Mean $\pm$ SD)

	ELGA ($\leq$ 28 wk GA) n=29	VLGA (29–32 wk) n=39	Full-term (38–41 wk) n=32	P<
Gestational age (wk)	26.7 (1.7)	31.3 (1.1)	40.0 (1.2)	0.0001
Birthweight (grams)	943.9 (277.0)	1608.5 (334.8)	3524.0 (345.0)	0.0001
Corrected age at vaccination (mo)	3.6 (1.2)	4.5 (0.7)	4.5 (0.9)	0.0001
SNAP score (Day 1)	13.6 (11.5)	7.2 (9.0)	n/a	0.01
Number of skin-breaking procedures from birth to term	128.7 (66.6)	51.6 (36.1)	n/a	0.0001
Number of days on intravenous morphine	7.0 (11.6)	0.89 (1.7)	n/a	0.002
Mechanical ventilation (d)	20.0 (21.4)	1.8 (3.7)	n/a	0.0001