EXAMINING THE RELATIONSHIPS BETWEEN NEONATAL PAIN-RELATED FACIAL ACTIONS AND CORTICAL ACTIVITY

OANA BUCSEA

A THESIS SUBMITTED TO THE FACULTY OF GRADUATE STUDIES IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF ARTS

GRADUATE PROGRAM IN PSYCHOLOGY

YORK UNIVERSITY

TORONTO, ONTARIO

AUGUST 2020

Abstract

The current study examined the relationships between preterm and full-term neonates' pain-related facial actions and cortical activity following a heel lance. Participants consisted of 41 late preterm and 37 full-term neonates, ranging in age from 0-14 days. Pain-related facial actions were micro-coded on a second-by-second basis using three constellations of facial actions to assess which is most optimal for capturing the full range of neonates' pain-related facial expressions. Results indicated that a cluster of three pain-related facial actions (brow bulge, eye squeeze, and naso-labial furrow), coded on a second-by-second basis, captured the distribution in neonates' pain-related facial expressions. Using this facial cluster, differences in pain-related cortical activity across the whole scalp using Global Field Power (GFP) were assessed between lower and higher pain-related facial activity. Underlying cortical activity in preterm and full-term neonates were found to vary with levels of pain-related facial activity. Implications for future research practices are discussed.

Acknowledgments

First, I would like to thank my supervisor and mentor, Dr. Rebecca Pillai Riddell, whose guidance for the past two years has made this project and all my other achievements possible. Thank you for all the opportunities you have afforded me and for continually inspiring me to be a better researcher.

I would also like to thank my committee (Drs. Louis Schmidt, Steven Wang, Mary Desrocher, and Jennifer Steeves) for their valuable contributions and input on this project. My deepest gratitude to the entire Fitzgerald-Fabrizi Lab at University College London for sharing their data with us and working with us to bridge the gap between behavioural and brain pain research. A special shout-out to Dr. Laura Jones for her patience as she embarked on the impossible journey of teaching me neuroscience from 5709 km away.

Next, I'd like to thank my CD area cohort, faculty, and administrative staff for their help with any problems I've had these past years, no matter how big or small. Also, I am grateful to all my OUCH Lab mates (Jordana Waxman, Monica O'Neill, Hannah Gennis, Miranda DiLorenzo, Shaylea Badovinac, and Ilana Shiff) for all their encouragement, pep talks, and wisdom they have shared with me throughout these years.

I am grateful to have received generous funding throughout my two M.A. years from the Social Sciences and Humanities Research Council (SSHRC), the Ontario Graduate Scholarship (OGS) program, the Meighen Wright Foundation, and the Pain in Child Health (PICH) program.

Finally, the biggest thank you to my family and friends for putting up with all the late nights, deadlines, and scholarship anxiety, and for loving and supporting me unconditionally through it all.

TABLE OF CONTENTS

List of Tables

List of Figures

Introduction

Approximately 7% of newborns are hospitalized after birth for medical reasons (Harrison et al., 2018) and are exposed to an average of 7-17 painful procedures daily (Cruz et al., 2016). Research has linked neonatal pain exposure with multiple adverse effects relating to pain reactivity (Taddio et al., 2009), neurodevelopment (Chau et al., 2019; Duerden et al., 2018; Ranger et al., 2015; Schneider et al., 2018), as well as cognitive, behavioural, and emotional outcomes (Duerden et al., 2018; Schneider et al., 2018; Ranger et al., 2015; Vinall et al., 2013). However, newborns cannot report on their pain experience. Thus, they are wholly dependent on proxy methods of pain assessment whereby an adult makes judgments. This underscores the importance of utilizing validated methods of proxy infant pain assessment during painful medical procedures, not only to optimally manage the pain-related distress that infants experience during these procedures, but also to mitigate the long-term adverse impacts on their development.

Pain-Related Facial Activity

Behavioural indicators of pain-related distress are commonly employed in clinical settings as a proxy for neonates' pain levels due to their ease of implementation. The most commonly used behaviours are facial actions, body movements, and cry. Of all pain behaviours, facial actions have repeatedly emerged as the most valid indicator of infant pain (DiLorenzo et al., 2018; Holsti et al., 2005; Stevens et al., 2007). These studies are consistent with evolutionary perspectives delineating the significance of facial actions in distressed preverbal infants as a means of communicating distress and eliciting a response from caregivers (Williams, 2002). Although facial actions are considered a valid indictor of newborns' pain-related distress by health care providers and caregivers, past research has yielded mixed findings regarding their associations with underlying pain-related cortical processes.

Pain-Related Cortical Activity

Using electroencephalography (EEG) measures, previous studies examining pain indicators in neonates have established the existence of a cortical response specific to painful stimulation i.e. the noxious event related potential occurring about 500 milliseconds after the application of a painful stimuli ([nERP]; Fitzgerald, 2015; Slater et al., 2010a; Verriotis et al., 2015). Green and colleagues (2019) have determined that neonates' ability to display distinct facial expressions to painful versus innocuous stimulation, which emerges at approximately 33 weeks gestational age, is related to brain maturation processes and thus, following this developmental milestone, a consistent relationship should emerge between these two pain-related responses. However, past studies examining the links between neonates' pain-related cortical activity and facial actions have still generated inconsistent results. While some studies have demonstrated a significant association between changes in pain-related facial expressions and the magnitude of cortical activity (Jones et al., 2017; Slater et al., 2008), others have failed to find any significant relationships (Hartley et al., 2015; Verriotis et al., 2015). Furthermore, discrepancies between pain-related facial actions and cortical activity have been uncovered in specific circumstances, such as during high stress contexts (Jones et al. 2017) and post-sucrose administration (Slater et al., 2010b).

The Current Study

In light of these mixed past findings, the goal of the current study was to examine a more fine-grained and comprehensive approach to both the measurement of pain-related facial actions and cortical activity in order to gain a better understanding of how these two pain-related responses are related. Both full-term and preterm neonates' pain responses to heel lance were examined. Specifically, this project sought to address two limitations in past literature. First,

neonates have been shown to demonstrate more subtle pain-related facial actions compared to older infants (Craig et al., 1993). Although pain-related facial actions are evaluated in the context of clinical pain assessment tools, these tools often seek to maximize clinical utility and ease of implementation and thus rely on more gross coding schemes measured over a longer time epoch (e.g. one score for each facial action based on the 30 seconds of post-painful stimulus). Therefore, the current study coded facial actions on a second-by-second basis in a more proximal time frame (10 seconds post heel-lance) in order to obtain a more fine-grained measure of pain-related behaviours. To our knowledge, no research using cortical measures of pain has utilized such a comprehensive measure of facial activity. Second, a limitation of EEG studies to date that have examined neonates' pain-related brain-behaviour relationships has been the reliance on a singular noxious evoked potential from only one location on the scalp, most often at the crown of the scalp (i.e. at the vertex Cz or CPz electrodes) (Green et al., 2019; Hartley et al., 2015; Jones et al., 2017; Slater et al., 2010b). However, this approach does not account for the widespread network of coordinated pain- and attention- related brain regions activated following a painful stimulus, called the dynamic pain connectome (Kucyi & Davis, 2015). Although research on the dynamic pain connectome has been primarily conducted in adults, Goksan and colleagues (2015) have shown that 18 of the 20 brain regions involved in pain-related processes in adults were also activated in neonates, suggesting an extensive network of pain-related cortical activity which must be better understood. However, no studies to date have examined how a comprehensive analysis of widespread pain-related cortical activity across the whole scalp relates to changes in facial activity in neonates.

The present project includes two sequenced studies that seek to address the aforementioned gaps in literature by selecting an optimal measure of pain-related facial actions

and then using this as a basis of comparison with a more comprehensive analysis utilizing pain-related cortical activity across the whole scalp. The goal of study 1 was to examine second-by-second patterns of pain-related facial expressions to a clinically required heel lance in order to determine the optimal measure of facial actions that best captures the full range of neonates' pain-related distress for research purposes. Utilizing the results from Study 1, both the samples of full-term and preterm neonates were optimally grouped into high pain-related facial activity and low pain-related facial activity. The goal of study 2 was to then examine the associations between the groups of facial activity found in study 1 and pain-related cortical activity using Global Field Power (GFP), a measurement of activity across the whole EEG array on the scalp. Overall, it was hypothesized that underlying pain-related cortical activity would differ between the high pain-related facial activity and low pain-related facial activity groups, in both full-terms and preterm neonates.

Method

Study Sample

The current study draws upon an archival sample of 78 neonates (41 late preterm and 37 full-term neonates; Table 1), ranging from 0-14 days postnatal age recruited from the postnatal ward, special care, or intensive care wards at the Elizabeth Garett Anderson Obstetric Wing, University College London Hospital (UCLH) in London, England in the period of June 2010 to May 2018. Infants that had grade 4 hypoxic ischaemic encephalopathy, periventricular haemorrhage, > grade 2 intraventricular haemorrhage, trisomy 21, intrauterine growth restriction, or were prescribed opioids at the time of the study were not eligible to participate.

Table 1

Participant Demographics

	Preterm (N = 41)	Full Term (N = 37)
GA[a] (weeks)	35.24 (1.03)	38.91 (1.18)
PNA[b] (days)	6.10 (3.91)	5.03 (2.87)
Females	24 (58.54)	12 (32.43)
Apgar [c]	9.20 (0.99)	9.27 (1.19)
Birth weight (grams)	2111.37 (374.53)	3066.41 (610.63)
Skin-breaking procedures [d]	17.83 (9.08)	11.91 (7.82)

Note. Counts (%) provided for sex ratios. *M(SD)* provided for all other variables.

[a]GA refers to gestational age – number of weeks from the first day of the mother's last menstrual cycle to the birth.

[b]PNA refers to postnatal age – number of days since birth.

[c] Five minutes Apgar scores.

[d] Total number of skin-breaking procedures neonates were exposed to before participating in the study.

Measures

Pain-Related Facial Actions (Study 1 and Study 2)

Coding. Video footage of neonates' pain-related facial actions was coded using the 7-item version of the Neonatal Facial Coding System (NFCS) (brow bulge, eye squeeze, nasolabial furrow, open lips, vertical stretch mouth, horizontal stretch mouth, and taut tongue; See Appendix A) (Grunau & Craig, 1987; Craig et al., 1993). NFCS has demonstrated good psychometric properties, such as reliability (Stevens et al., 2007), convergent validity (Lilley et al., 1997), and construct validity (Taddio et al., 1997). Three separate NFCS variants of the most common iterations (i.e. most common combinations of the above seven facial actions) were coded using a comprehensive micro-coding (i.e. second-by-second) method, as per the original validation of the scale (Grunau & Craig, 1987). First, a 3-item NFCS score was computed, specifically consisting of eye squeeze, vertical stretch mouth, and horizontal stretch mouth based on work with older full-term infants (DiLorenzo et al., 2018; NFCS-3). Second, another 3-item cluster of facial items was computed, consistent with the facial actions included in the well-

validated Premature Infant Pain Profile (brow bulge, eye squeeze, and naso-labial furrow; NFCS-P-3; usually coded coarsely over a 30-second period) (Stevens et al., 2014). Finally, a 7-item version was used with all facial actions coded (NFCS-7) (Ahola Kohut & Pillai Riddell, 2009).

Facial actions were coded for two distinct epochs: (1) 10 seconds immediately pre-needle and (2) the first 10 seconds immediately post-needle depicting infants' pain reactivity. Facial actions were coded per second as either 0 (not present) or 1 (present) and then summed for each 10 second epoch. Therefore, the maximum score for each facial action per epoch was 10, and then when summed over the different facial actions would be either 70 (seven facial actions) or 30 (three facial actions). Higher scores on NFCS are indicative of more pain-related facial actions and presumably greater pain-related distress. Video coders were blinded to the study hypothesis. Thirty percent of the data was coded for inter-rater reliability. Ongoing reliability throughout the coding process was also examined to prevent coder drift. Intraclass correlations (ICCs) ranged from 0.90 to 1.

Missing Data Management. Coding NFCS in a hospital setting in the context of acute painful procedures is challenging as infants' facial actions are often obstructed due to infant movement or medical equipment (i.e., endotracheal tube obstructing the mouth, tape obscuring nasolabial furrows). To prevent the systematic bias inherent in excluding infants who moved during the procedure or those who required more intensive medical care, missing data were managed using three previously established procedures (e.g. Pillai Riddell et al., 2007; Ahola Kohut & Pillai Riddell, 2009), which allowed coders to make conservative judgements about missing facial actions. First, if half of the infant's face could be seen on video and all facial actions were able to be coded from the visible half of the face, then the items would be coded based on the assumption of facial symmetry. This method was used with 15% of participants. Second, if facial

actions were actually obstructed, a blinded coder reviewed the video and determined the cause of missing data (e.g., infant turned face away from camera). Then, the coder examined whether two other commonly employed pain-related distress behaviours (e.g., cry, body movements) (Taddio et al., 1995) remained constant while the infants' face was obstructed. The assumption was made that if cry and body movements remained constant, it is highly probable that facial actions also remained constant during that time, and the missing value was exchanged for the closest preceding value available. In order to use this constancy method, three conditions had to be satisfied: a) facial activity had to be available and codable for at least 60% of the 10 second epoch; b) cry and body movements had to be available and remain constant throughout the 10 second epoch; and c) the coder did not have any other reason to believe facial activity did not remain constant when obstructed. Across the seven facial actions, 5% of data was imputed using this constancy rule. Third, if the first criteria of the constancy rule was not met (e.g., due to a facial action being available for less than 60% of the 10 second epoch), but the other pain-related distress behaviours (e.g., cry and body movements) remained constant, data were prorated if at least 60% of the overall coding across all the actions was available. For instance, if nasolabial furrow was obstructed by medical tape and could not be coded at all during the 10 second epoch, but the other six actions were coded (meaning 60/70 = 86% of data were available), then the total score out of 60 would be prorated to reflect the expected sum out of a total score of 70. Consensus had to be reached among at least two coders that the constancy rule could be applied before scores were prorated. This procedure was used with 5% of participants with 10 to 33 % missing data. Finally, if less than 60% of data was available and proration was not feasible, the participant was excluded from the sample for this current analysis. Overall, only one participant had a missing NFCS-P-3 score and was thus excluded from the analyses.

Electroencephalogram (EEG) (Study 2)

Recording. EEG responses were recorded from 18 disposable electrodes (Ag/AgCl cup electrodes) time-locked to the heel lance. Electrodes were placed on the scalp according to a modified international 10/20 electrode placement system (See Appendix B), covering the primary visual (O1, O2), primary auditory (T7, T8), association (F7, F3, F4, Fz, F8, P7, P8, TP9, TP10) and somatosensory (C3, Cz, C4, CP3, CPz, CP4) cortices. A reference electrode was placed at Fz and the ground electrode at FC1/2, depending on the positioning of the infant during the procedure (Appendix B). Electrode impedance was minimized by rubbing the scalp with a prepping gel (NuPrep, Weaver & Co), then applying the electrodes using a conductive paste (10/20 Weaver & Co). A soft bonnet was placed on the scalp to secure the electrodes. The Neuroscan SynAmps2 EEG/EP recording system was used to record activity from DC to ≥500 Hz. Signals were digitised using a sampling rate of 2kHz and resolution of 24 bit. All EEG data was examined by a trained neurophysiologist and no EEG abnormalities were observed. A description of how the topographic analyses were operationalized is contained in the analysis section.

Pre-processing. Cortical data were pre-processed in EEGLAB and MATLAB. The raw data was epoched to one second following the noxious stimulus and second-order bidirectional Butterworth bandpass (1-30 Hz) and notch (48-52 Hz) filters were applied. Electrode noise was removed using independent component analysis in EEGLAB. If the entirety of the noise could not be removed using this method, or if electrodes had missing recordings, spherical interpolation was used in EEGLAB for a maximum of four channels per trial (average = 0.4 electrodes, range 0-4). Finally, data was re-referenced to the common average. This EEG pre-processing was conducted by coders blinded to the study hypothesis.

Microstates. Once microstates were identified separately within each group, they were subsequently compared across groups using microstate analysis to examine whether there were significant differences in the onset, offset, duration, total state field power (area under the curve; AUC), and peak state field power (global field power; GFP) between neonates with varying patterns of pain-related facial actions. See below for more details.

Procedure

Informed written parent consent was obtained before the study. The painful event was always a clinically required heel lance performed by a trained research nurse. Standard hospital practice was followed during all heel lances. The heel was cleaned with sterile water, after which the nurse placed the lancet on the infants' skin for 30 seconds without releasing it to obtain baseline data free of other stimulation. The release of the heel lance was time-locked to the ongoing EEG recording using an accelerometer mounted onto the lancet. After the lancet was released, the nurse squeezed the infants' foot for another 30 seconds to ensure that the post-lancet data recorded is again free of other stimulation. Parents were allowed to comfort their infants as desired, which is consistent with hospital protocol encouraging parent-led pain management techniques during painful procedures. More information regarding this study's methodology is described in Jones and colleagues (2018).

Data Analysis Plan

Study 1

Preliminary data screening showed that the three clusters of pain-related distress facial actions (NFCS-7, NFCS-3, and NFCS-P-3) were not normally distributed; therefore, non-parametric tests were initially conducted. The non-parametric results were reported only if they differed from their parametric counterparts; otherwise, the parametric statistics were reported.

Frequency distributions, repeated measures ANOVA, and t-tests were employed to examine differences in patterns of pain-related facial activity in preterm and full-term neonates.

Study 2

Topographic cortical analyses were conducted separately for late preterm (33-36 weeks gestational age) and full term (37-40 weeks gestational age) neonates due to previous research uncovering distinct pain-related cortical activity across these groups (Willers Moore, 2020). These analyses were conducted using Ragu, a multivariate statistical approach allowing for the analysis of multi-channel event-related potential (ERP) data using randomization statistics (Habermann et al., 2018). When neural networks are activated post stimulation, they produce meta-stable states within the brain that produce measurable changes in scalp potential. Throughout the processing of a stimulus, various cortical areas get engaged, reflecting different brain states, depending on the source of underlying neuronal ensembles firing. In contrast to past studies' singular measurement of the noxious ERP elicited at the vertex of the scalp at a latency of approximately 500ms, Ragu permits the study of simultaneous pain-related activity across the whole scalp in order to better understand the temporal and spatial relationships of these pain related brain states. Underlying brain states produce measurable patterns of changes in scalp potential which can be measured via microstate analysis in Ragu (Habermann et al., 2018).

Ragu allows for the comparison of within-group and between-group differences using non-parametric permutation statistics (n = 1000 randomizations, alpha level 0.05). First, using a topographic consistency test (TCT), the topography of scalp potentials was examined within groups in order to detect periods of significant neural activation post stimulation. Random fluctuations in cortical activity will not be consistent across participants at a given latency and thus will not produce a significant event. Microstate analysis was then employed to determine

the microstates underlying the periods of significant activation in each group. In understanding the link between Study 1 and Study 2, it is critical to note that Ragu requires a categorical approach. Thus Study 1, aside from providing valuable comparative data, also justified our groupings of high and low facial responding to heel lance within the full-term and preterm groups.

Results

Study 1: Analysis of Pain-Related Facial Actions

As described, 3 iterations of pain-related facial activity scores were computed (NFCS-7, NFCS-3, NFCS-P-3) separately for preterm and full-term infants. The distribution of scores in each group was examined to better understand the constellation of pain-related facial actions that best captures the variability in neonates' pain-related distress levels. In both the preterm and full-term group, there was low occurrence of any pain-related facial activity during baseline, with 70.5% of all infants displaying no pain-related facial activity. Of the remaining 29.5%, the majority displayed only open lips, an item previously criticized for its lack of pain specificity (DiLorenzo et al., 2018; Stevens et al., 1996), with only 3.8% of the sample showing expressions other than open lips during baseline (e.g., brow bulge, eye squeeze). In light of this lack of variability, baseline pain-related distress scores were not included in subsequent analyses. Moreover, there were no sex differences on any of the three NFCS variants in either preterm or full-term neonates, so no further sex analyses were conducted. Thus, descriptive and comparative analyses proceeded in three clusters. First, an analysis of the individual facial actions was conducted, then total scores on the three NFCS variants were examined, and finally within-group variability and distributions of pain-related facial activity were explored.

Analysis of Discrete Facial Actions

Mean scores for each of the seven facial actions are displayed in Table 2, separately for preterm and full-term neonates. Descriptive analyses of the occurrences of each of the seven facial actions (percentage of infants who showed any occurrence during the 10-second post lance epoch and the percentage of infants who showed maximal occurrence during the 10-second post lance epoch) were then conducted (Table 3).

Table 2

Mean Scores for the Seven Individual Pain-Related Facial Actions

	Preterm *M*(*SD*); Range	Full-Term *M*(*SD*); Range
Brow bulge	5.20(4.32); 0-10	3.73(4.21); 0-10
Eye squeeze	5.35(4.20); 0-10	4.00(4.33); 0-10
Nasolabial furrow	4.48(4.29); 0-10	3.42(4.22); 0-10
Open lips	5.40(4.57); 0-10	6.46(4.39); 0-10
Horizontal stretch mouth	4.44(4.09); 0-10	3.05(3.87); 0-10
Vertical stretch mouth	1.15(2.32); 0-9	0.89(1.97); 0-7
Taut tongue	0.66(2.08); 0-9	0.24(0.80); 0-4

Table 3

Percentages of Pain-Related Facial Actions Expressed in Preterm and Full-Term Neonates

	Preterm		Full-Term	
	% showing any occurrence (scores of 1-10)	% showing maximal response (score of 10)	% showing any occurrence (scores of 1-10)	% showing maximal response (score of 10)
Brow bulge	65	22.5	51	19
Eye squeeze	72.5	22.5	51	22
Nasolabial furrow	60	20	47	17

Open lips	65	35	73	43
Horizontal stretch mouth	63	17	46	8
Vertical stretch mouth	27	0	24	0
Taut tongue	10	0	11	0

Brow bulge, eye squeeze, nasolabial furrow, open lips, and horizontal stretch mouth were most common across neonates of both groups with between 46% and 73% infants showing any occurrence. Examining maximal occurrence (i.e., scores of 10/10 on any individual facial action) also showed similar patterns across the two groups. Between 17 - 35% of preterms and 8 - 43% of full-terms showed a maximal response across the aforementioned five actions. Both groups had no maximal response occurrences on vertical stretch mouth and taut tongue.

Analysis of NFCS Variants Total Scores

Mean scores on the three NFCS variants are displayed below in Table 4.

Table 4

Total Scores on the Three NFCS Variants

	Preterm *M(SD)*; Range	Full-Term *M(SD)*; Range
NFCS-7	26.45(22.54); 0-65	21.90(20.55); 0-57
NFCS-3	11.08(9.54); 0-29	7.95(9.38); 0-27
NFCS-P-3	15.23(12.48); 0-30	11.29(12.45); 0-30

Within-subjects ANOVAs demonstrated significant differences between the three NFCS variants in both preterm, ($F(2, 78) = 34.90$, $p = .00$), and full-term, ($F(2, 72) = 17.21$, $p = .00$), neonates. Post-hoc analyses were then conducted to explore the origins of significant differences in both groups. As parametric and non-parametric tests yielded different results, the non-

parametric Wilcoxon signed-rank test results are reported in Table 5. Post-hoc results indicated that NFCS-P-3 clusters, for both preterms and full-terms, were significantly higher (i.e. captured higher behavioural activity related to the heel lance) than both NFCS-7 and NFCS-3 scores.

Table 5

Within-Subject Differences Between the NFCS Variants in Preterm and Full-Term Neonates

	Preterm		Full-Term	
	Z	*p*	*Z*	*p*
NFCS-3 vs. NFCS-P-3	-4.43	.00	-3.93	.00
NFCS-7* vs. NFCS-P-3	-4.41	.00	-2.65	.01
NFCS-3 vs. NFCS-7*	-1.90	.06	-3.67	.00

Note. NFCS-7* total scores were linearly scaled to 0-30 for comparison (M_{preterm} = 11.62; $M_{\text{full-term}}$ = 9.38).

Within-Group Variability of Pain-Related Facial Activity

An examination of the within-group variability of NFCS-7, NFCS-3, and NFCS-P-3 total scores was conducted for both preterm and full-term neonates. Both preterm and full-term groups showed a similar pattern of total scores, with the majority of participants clustering at the extreme ends of the distribution and a minority (approximately 10%) falling in between these two clusters. For the sake of parsimony, only the NFCS-P-3 total score distributions are shown (rest of figures in Appendix C). Dot plots illustrating the distribution of NFCS-P-3 total scores in preterm (Figure 1) and full-term (Figure 2) neonates are provided, with each dot representing a neonate (i.e., two circles above a score of four means that two neonates obtained total NFCS-P-3 scores of four).

Figure 1

Distribution of NFCS-P-3 Total Scores (Range 0-30) in Preterm Neonates

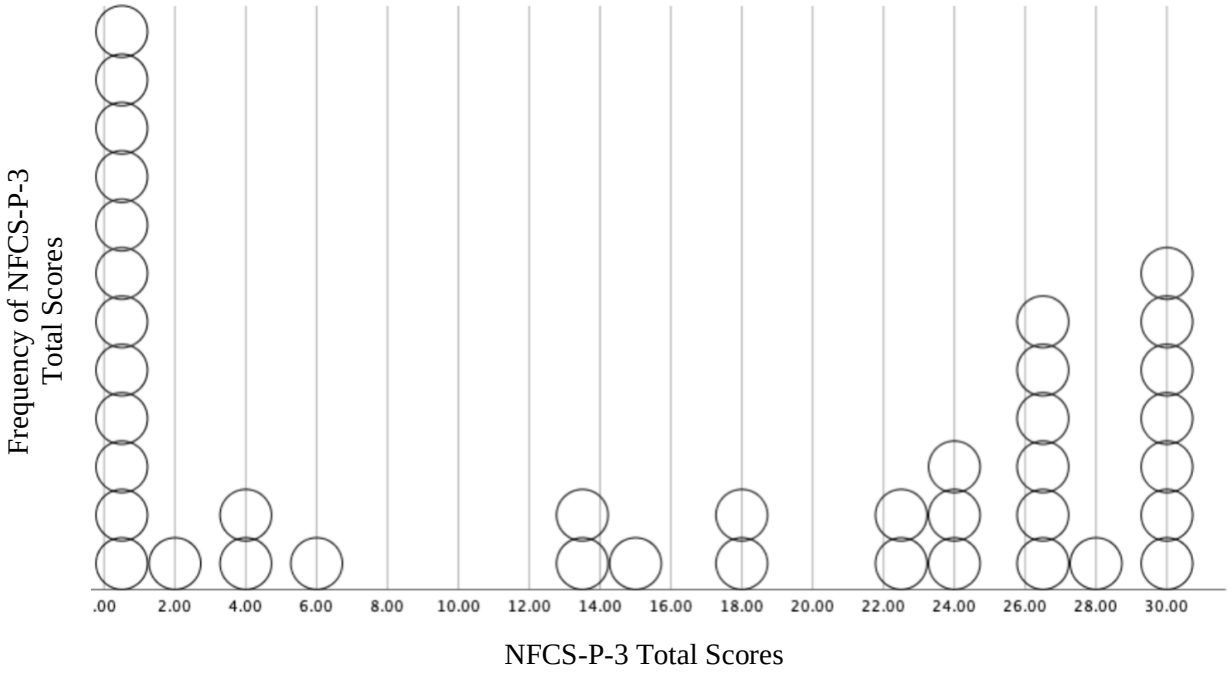
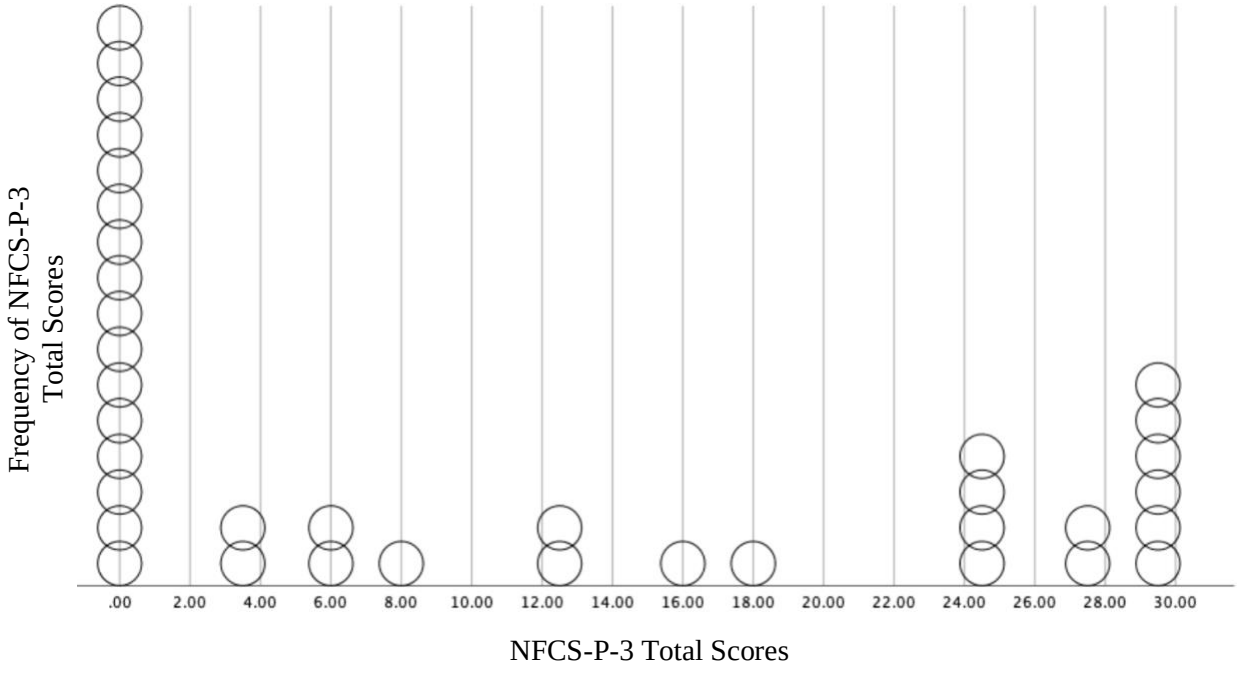


Figure 2

Distribution of NFCS-P-3 Total Scores (Range 0-30) in Full-Term Neonates

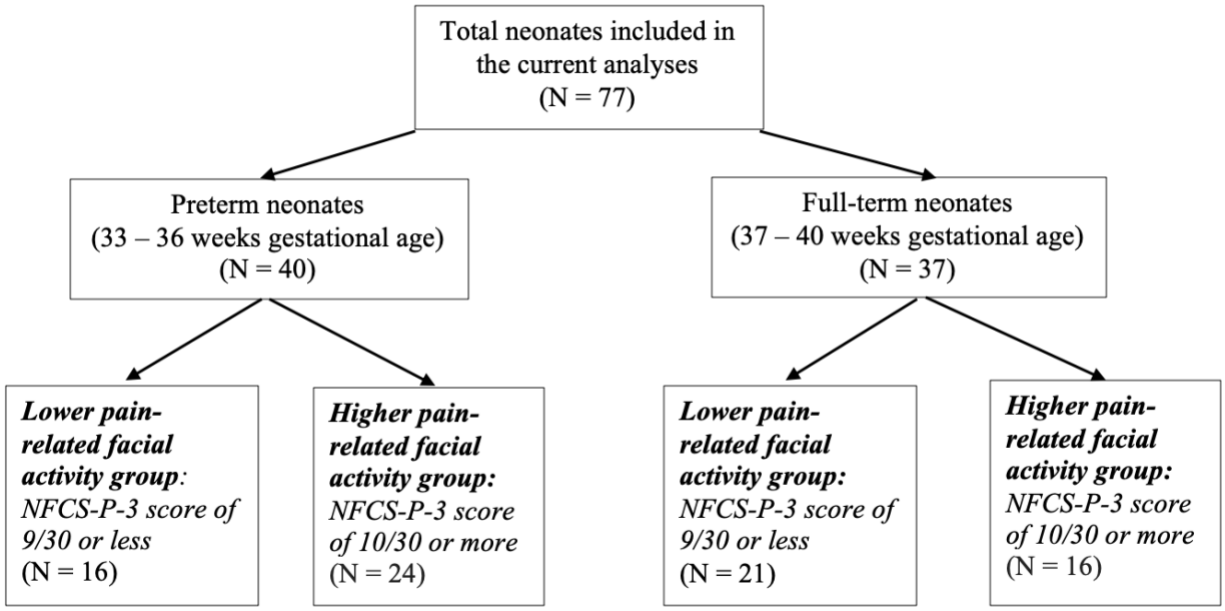


Selecting a Measure of Pain-Related Facial Activity for Study 2

Overall, the first study examined three variants of pain-related facial actions in preterm and full-term neonates post heel lance. Based on the synthesis of study 1 results, the NFCS-P-3 cluster of facial actions (brow bulge, eye squeeze, and nasolabial furrow) coded on a second-by-second basis was selected to move forward with the comparisons utilizing Ragu. The NFCS-P-3 was deemed most optimal for capturing the widest range of preterm and full-term neonates' pain-related distress due to the variability it captured particularly with infants demonstrating higher pain-related facial activity along with reflecting a natural dichotomy, with only minor exceptions. Therefore, this NFCS variant was used in subsequent analyses to examine the relationships between pain-related facial activity and cortical processes in preterm and full-term neonates. Ragu requires a categorical grouping and past infant research on the minimal clinically significant pain scores falls around 2/10 (Taddio et al., 2017), generally coinciding with the naturally occurring gap at approximately 9/30 demonstrated in NFCS-P-3 scores. Thus, based on current analyses and informed by past work in the literature, both samples of neonates with scores equal to 9/30 or less were categorized as the lower pain-related facial activity group (lower pain group), and those with scores equal to or above 10/30 were deemed the higher pain-related facial activity (higher pain group) group (Figure 3).

Figure 3

Breakdown of Preterm and Full-Term Groups Compared in Study 2



Study 2: Patterns of Pain-Related Cortical Activity Underlying Pain-Related Facial Actions

Differences in demographics between the lower and higher pain groups compared in study 2 were examined. In both the preterm and full-term samples, the lower and higher pain groups did not significantly differ in terms of gestational ages (GA), postnatal ages (PNA), and sex ratios (Table 6).

Table 6

Demographic Information of Lower and Higher Pain Groups Compared in Study 2

	Preterm (*N* = 40)				Full-Term (*N* = 37)			
	Lower pain (*N* = 16)	Higher pain (*N* = 24)	*t*(38)/$\chi 2$(1)	*p*	Lower pain (*N* = 21)	Higher Pain (*N* = 16)	*t*(35)/$\chi 2$(1)	*p*
GA (weeks)	35.31 (1.10)	35.24 (1.00)	0.22	0.83	38.99 (1.20)	38.80 (1.18)	0.48	0.63
PNA (days)	6.31 (3.95)	5.96 (4.04)	0.27	0.79	5.52 (2.99)	4.38 (2.65)	1.21	0.23
No (%) females	10 (62.5)	13 (54.2)	0.27	0.60	7 (33.3)	5 (31.25)	0.18	0.89

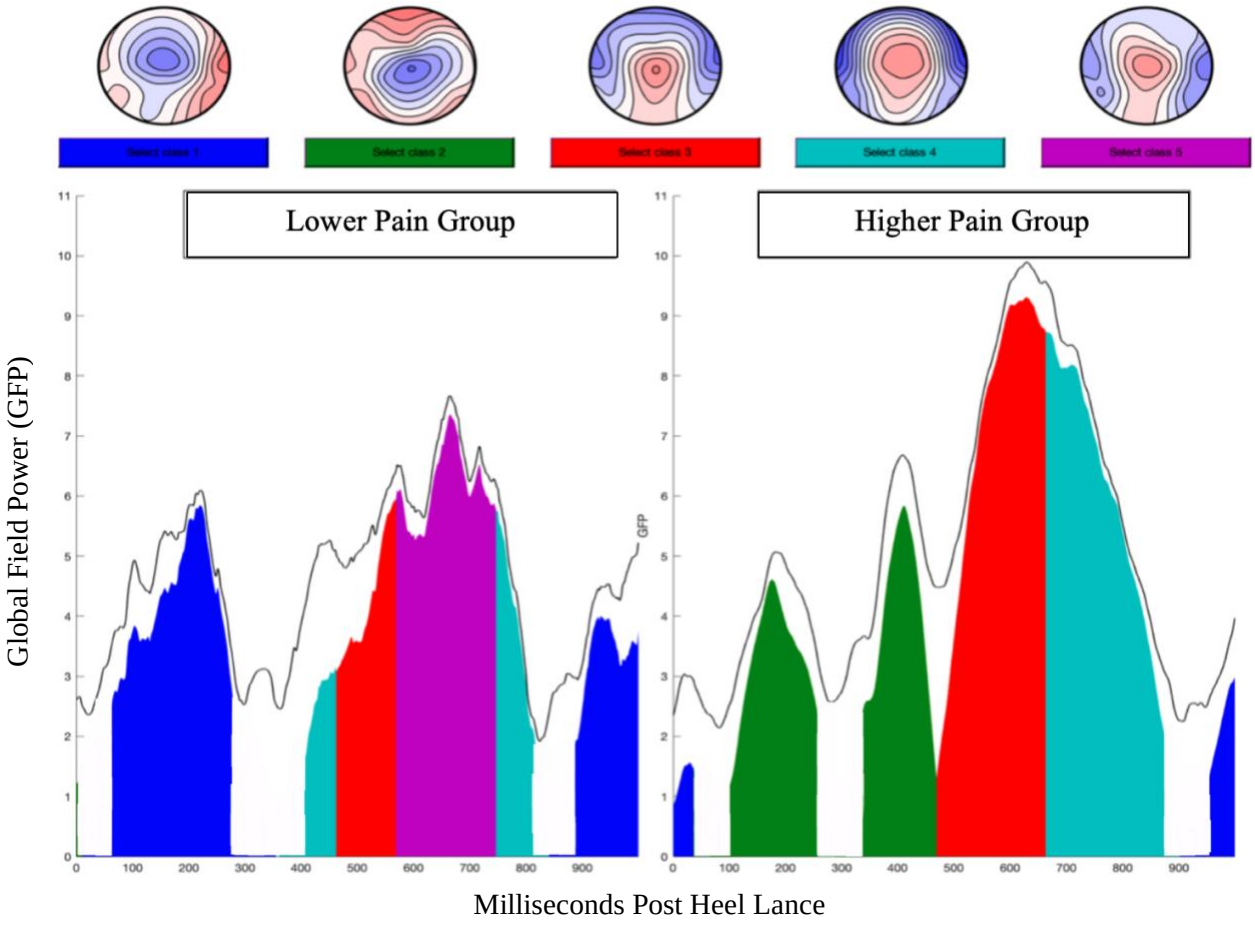
Note. T-test values provided for gestational age (GA) and postnatal age (PNA); χ^2 statistics provided for sex ratios.

As aforementioned, past research on pain-related cortical activity has focused solely on the nERP emerging at approximately 500 ms at electrode Cz and/or CPz. However, recent research has revealed a sequence of pain-related cortical events that emerges before 500ms (Willers Moore, 2020). Therefore, the relationships between pain-related facial actions and cortical activity was inspected across the whole one second epoch, starting with the shorter latency events. Over the first second post-lance, preterms displayed a sequence of pain related cortical activity related to five microstates with a positive or negative peak at the vertex, which predicted approximately 84% of the variance in the data (Figure 4). Eighty-one percent of the data in full-terms was predicted by five microstates which varied in topography, with three displaying a positive or negative peak at the vertex (Figure 5). Analyses are reported by group and timing since heel lance.

Preterm Neonates

Figure 4

Microstate Analysis Between the Lower and Higher Pain Preterm Groups



The black line represents the magnitude of cortical activity across the whole electrode array (Global Field Power; GFP). Gaps in the microstate sequence represent periods in which no significant event occurred in that respective group, as per the topographic consistency test (TCT) results; therefore, those periods were not inspected further.

Differences in early pain-related cortical activity between lower and higher pain groups. Immediately following the painful stimulus, preterms who exhibited lower pain scores engaged distinct microstates compared to those with higher pain scores, meaning distinct neural sources were activated between these two groups in this early somatosensory processing stage of

the heel lance. As can be seen in Figure 4, preterms in the lower pain group showed engagement of a central negative and parietal temporal positive microstate (navy) (duration = 346ms and AUC = 1129.2 ms x uV) until about 358ms. Although this state was also briefly activated immediately following the heel lance for approximately 40ms in the higher pain group (duration = 37.5ms and AUC = 49.5ms x uV), its duration (p = 0.022) and total state field power (p = 0.015) were significantly lower in this group, and it only predicted approximately 51% of variance in GFP in this group. Interestingly, this group instead engaged a different microstate, negative over the vertex and positive in the frontal regions (green), lasting until approximately 470ms post painful stimulus (Figure 4). This microstate was engaged twice, peaking at approximately 200ms and 400ms post heel lance.

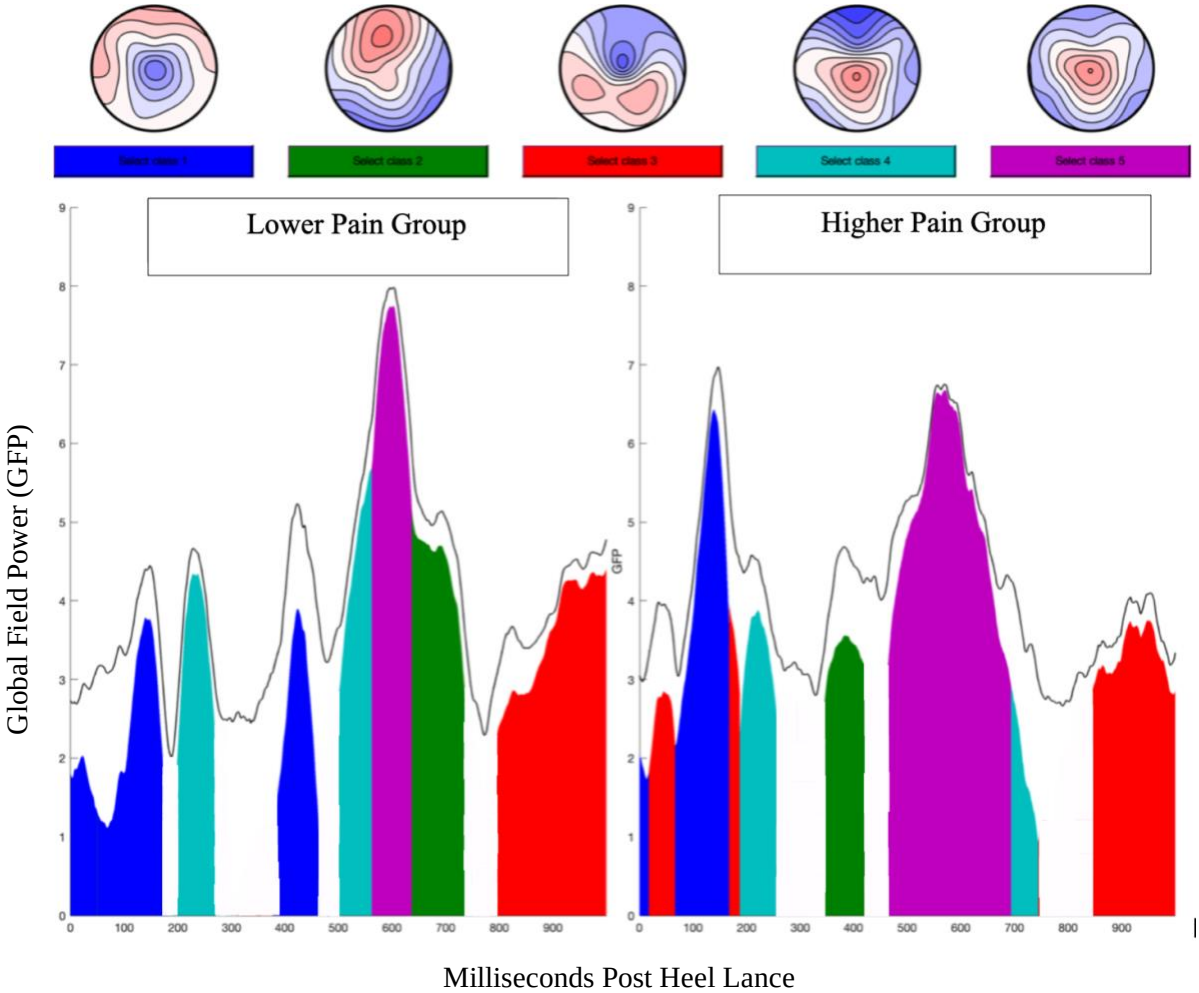
Differences in later pain-related cortical activity between lower and higher pain groups. Following the early pain-related activity in the initial 400ms post stimulus, the lower pain group engaged three microstates (turquoise, red, pink) while the higher pain group engaged two microstates (red, turquoise), each with slightly different topographies but showing a central positive peak and negative activation over the temporal and frontal areas. A notable difference in this stage of processing was that the lower pain group engaged a microstate that the higher pain group did not, with a positive peak at the vertex and bilateral negative potentials in the temporal areas which emerged at approximately 570ms post noxious stimulus and lasted 178ms (Figure 4). Furthermore, one of the microstates engaged at approximately 356ms in the lower pain group was re-engaged at around 750ms, coinciding with its activation in the higher pain behaviours group. Finally, the longest latency event (from 850/900ms onwards; navy) engaged the same microstate in both groups with no differences in power, onset, or duration (Figure 4).

To summarize, the earliest pain-related cortical activity (in the first 350/400ms post heel-lance) was different across the two preterm groups, indicating that distinct neural networks were engaged between them. In addition, much of their later pain-related activity (post 400ms) was similar, with one exception. The lower pain preterm group demonstrated an additional state that was not activated in the higher pain group.

Full-Term Neonates

Figure 5

Microstate Analysis Between the Lower and Higher Pain Full-Term Groups



The black line represents the magnitude of cortical activity across the whole electrode array (Global Field Power; GFP). Gaps in the microstate sequence represent periods in which no significant event occurred in that respective group, as per the topographic consistency test (TCT) results; therefore, those periods were not inspected further.

Differences in early pain-related cortical activity between lower and higher pain groups. Early pain-related cortical activity in both full-term groups consisted of two microstates with a negative peak (navy), followed by a positive peak (turquoise), at the vertex in the first 300ms post stimulus. The first microstate (navy; characterized by a negative peak at the vertex and positive activation over the frontal and temporal regions) was engaged in both groups at a similar latency, but it demonstrated increased peak state field power in the higher pain group (GFP = 4.86 uV) compared to the lower pain group (GFP = 3.32 uV) (p = 0.036). During this early processing stage, the higher pain group also engaged a third microstate (red) at two separate latencies (approximately 17ms and 170ms post stimulus); however, this state was only briefly engaged for 69ms, had a low total state field power (AUC = 196.8ms x uV), and only explained approximately 60-70% of the variance in GFP, suggesting that this microstate does not adequately capture the pain-related activity occurring at this latency in this group (Figure 5).

Differences in later pain-related cortical activity between lower and higher pain groups. Interestingly, the lower pain group showed re-engagement of an earlier microstate (navy) characterized by a negative peak at the vertex at 425ms, which was not also re-engaged in the higher pain group. Following this, three microstates (green, pink, turquoise) were engaged in both groups, but in a separate order. Two of these microstates were characterized by a central positive peak (turquoise, pink), while the third had a more frontal positive peak (green). No differences in power, duration, or onset were observed between the two groups for these three

states (Figure 5). Once again, there were no differences between the two groups in the longest latency microstate (red) that emerged at about 745/765ms which was characterized by a central/frontal negative with a posterior/temporal positive amplitude (Figure 5).

Overall, the two full-term groups engaged similar early pain-related cortical activity in the first 300ms following the heel lance; however, the higher pain group showed greater activation in one of these early brain states. Later pain-related activity was predominantly consistent across the two groups, with the same brain states being engaged but in a different sequence. However, the lower pain group engaged an additional microstate during this period, which was also engaged during earlier stages of processing.

Discussion

Study 1: Patterns of Pain-Related Facial Actions

The goal of study 1 was to examine the constellation of facial actions in preterm and full-term neonates that most accurately captures the variability in pain-related distress, and thus is optimal for research use. No sex differences were found in either preterm or full-term neonates. Overall, study 1 demonstrated that NFCS-P-3, a coding system based on brow bulge, eye squeeze, and nasolabial furrow, and micro-coded on a second-by-second basis, was the only one that captured the full range (total scores ranging from 0/30 – 30/30) of pain-related facial activity in both preterm and full-term neonates (i.e., the NFCS-7 and NFCS-3 ranges of total scores did not encompass the maximal score of 30/30 in any infant, even though 17.5% of preterm and 13.5% of full-term neonates showed a maximal response of 30/30 on the NFCS-P-3). The NFCS-P-3 cluster of facial actions was based in the cluster utilized by the well-established Premature Infant Pain Profile (PIPP) (Stevens et al., 1996; Stevens et al., 2014).

The neonates in the current sample showed patterns of facial actions characterized by frequent brow bulge, eye squeeze, naso-labial furrow, open lips, and horizontal stretch mouth, and infrequent displays of vertical stretch mouth and taut tongue. Interestingly, regardless of gestational age, preterms and full-terms in the current sample showed pain-related facial actions similar to each other, but different from older healthy term-born infants (DiLorenzo et al., 2018). A major discrepancy had to do with the occurrence of vertical stretch mouth. Although the occurrence of this action carried significant information about infants' pain levels in older full-term infant samples (DiLorenzo et al., 2018), it appears to be less common in newborns. Past research has stated that more effort is involved when engaging in vertical stretch mouth as opposed to other facial actions, such as horizontal stretch mouth (i.e., whereas only soft tissue is involved in horizontal stretch mouth, tension in both the soft tissue and mandibula are required for vertical stretch mouth) (Johnston et al., 1993). Due to it being a more effortful action, older infants may be better able to mount this facial action compared to newborns, particularly with a more vigorous cry. Taut tongue was also infrequently observed in newborns in past research (Stevens et al., 1996). Therefore, NFCS variants that relied on vertical stretch mouth and taut tongue appear to not be optimal for research purposes as their rare occurrences led to a restricted range of pain-related facial activity scores on the NFCS-7 and NFCS-3 variants. This study bolsters evidence for the unique developmental stage of pain-related facial expressions of neonates (within the first two weeks of life) and a more detailed coding approach which is integral information for deciding on the optimal facial activity coding methods for infant pain researchers.

Study 2: Relationships Between Pain-Related Facial Actions and Cortical Activity

The goal of study 2 was to examine the associations between patterns of pain-related facial actions and cortical activity in the one second epoch following a painful stimulus. Overall, as hypothesized, changes in pain-related facial activity were linked to differences in underlying cortical activity. However, the present study revealed more nuanced relationships between these two pain-related responses. Specifically, although one magnitude difference was observed at approximately 150ms post heel lance in full-term neonates, the majority of cortical differences between lower and higher pain groups were not related to magnitude, but instead to the activation of distinct patterns of neural networks when processing the painful stimulus. Thus, it was not the overall magnitude of cortical activation driving the difference in the two facial activity groups, but rather how the activation was distributed.

Building on past literature, the present study uncovered cortical differences relating to pain-related facial activity which would have been missed by EEG analyses focusing solely on evaluating the nERP at 500ms. First, significant differences in pain-related cortical activity were exhibited between the lower and higher pain groups during the early stages of sensory processing, specifically in the initial 300 - 400ms post stimulus. Whitehead and colleagues (2019) demonstrated a hierarchical organization in the sequence of neonates' sensory-evoked potentials following tactile stimulation, suggesting that shorter latency potentials are associated with lower levels of sensory processing (i.e., processing of basic stimulus features) which subsequently increase in complexity. Furthermore, past research has concluded that pain-related brain states engaged at approximately 800ms post stimulus are related to higher level processes responsible for integrating sensory information with contextual factors (Jones et al., 2020). However, in the present study, there were no differences between the lower and higher pain

groups in pain-related cortical activity at 800ms post stimulus. Taken together, these findings suggest that differences in pain-related facial expressions are linked to distinctions in the initial sensory processes responsible for processing basic stimulus features, before more complex processes, such as the integration of sensory and contextual information and stimulus recognition, unfold.

Second, although past studies linking pain-related cortical activity and behaviours have measured the magnitude of the nERP at approximately 500ms, the present study demonstrated that, in these later stages of sensory processing, differences in pain-related facial actions are related to variations in the brain states engaged during this stage of cortical processing, not their magnitude. Past research using microstate EEG analyses to study pain-related cortical activity has suggested that differences in the patterns of engaged pain-related brain states could be indicative of perceptual differences regarding the painful stimulus (Willers Moore, 2020). Therefore, a more nuanced relationship between pain-related facial actions and cortical activity may exist, such that those displaying higher pain-related facial expressions may not just be perceiving the stimulus more intensely at a cortical level, but instead are perceiving it differently than those expressing lower pain-related facial activity. Overall, this finding could account for the previous mixed results regarding pain-related behavioural-cortical relationships, as past studies have solely relied on linking the magnitude of cortical activity with the intensity of behavioural responses (Green et al., 2019; Hartley et al., 2015; Jones et al., 2017; Slater et al., 2010b).

Limitations & Future Directions

The current findings have to be interpreted in light of some potential limitations. First, small sample sizes precluded the examination of pain-related cortical differences across neonates

who displayed clinically significant pain scores of varying intensities. Although a clinically relevant gap in facial activity at 9/30 (coinciding with the minimal clinically significant pain scores from past research) was used to dichotomize the lower versus higher pain groups, a second gap in scores at 20/30 was uncovered for the first time in literature due to the nuanced coding scheme employed in the present study, reflective of varying intensities of clinically significant pain scores (i.e., moderate versus severe pain scores). However, as can be observed in Figures 1 and 2, only five preterm and four full-term neonates fell between 9/30 – 20/30, precluding the separation of this group from those scoring above 20/30. Future research should aim to examine pain-related cortical differences not just between newborns exhibiting clinically significant pain behaviour scores versus those that do not, but also across those demonstrating moderate versus severe or maximal scores to better understand how various facial expressions are linked to variations in underlying cortical processes. Second, sex ratios were unequal among the groups being compared in study 2, particularly the full-term neonates who were predominantly male (composed of approximately only 30% females). Sex-matching was not conducted in the present study as it would have led to a loss of power due to small sample sizes. While this study did not find sex-related differences in pain-related facial activity, past research has uncovered sex-based differences in pain-related cortical activity at the crown of the scalp (Verriotis et al., 2018). Therefore, particularly in full-term neonates, the current data might primarily reflect males' pain-related cortical processing. Future research is needed to disentangle the effects of infant sex on pain-related cortical activity across the whole scalp, not just at the crown of the scalp. Finally, an important component of research with preterm neonates is the steep trajectory of development causing intraindividual variability in the sample (e.g., skull thickening), which the current study did not control for. Future research should seek to

disentangle the impacts of these developmental differences on preterm versus full-term neonates' pain-related responses.

Conclusions & Research Implications

In summary, the present study examined the associations between pain-related facial actions and cortical activity in the initial second following a clinically-required heel lance. By employing more finely grained and comprehensive measurements of both facial actions and cortical activity, results suggested that differences in pain-related facial activity are linked to distinctions in underlying cortical processes, a topic which has been disputed due to mixed findings yielded by previous studies. As importantly, it provides an explanatory mechanism for the mixed findings in previous work. Future research examining these relationships should incorporate the methodological considerations discussed in the present study, namely the importance of employing developmentally-sensitive, facial coding schemes in research that optimize the variability in pain-related behavioural distress scores along with comprehensive cortical measures that capture the full range of pain-related cortical activity across the scalp. Employing these more comprehensive measurements of cortical and behavioural pain responses in research will be integral for furthering our understanding of brain-behaviour relationships in hospitalized infants frequently exposed to painful medical procedures, ultimately laying the foundation for the development of evidence-based pain assessment tools in infancy.

References

Chau, C. M. Y., Ranger, M., Bichin, M., Park, M. T. M., Amaral, R. S. C., Chakravarty, M., Poskitt, K., Synnes, A. R., Miller, S. P., & Grunau, R. E. (2019). Hippocampus, amygdala, and thalamus volumes in very preterm children at 8 years: neonatal pain and genetic variation. *Frontiers in Behavioral Neuroscience, 13*, article 51.

Craig, K. D., Whitfield, M. F., Grunau, R. V., Linton, J., & Hadjistavropoulos, H. D. (1993). Pain in the preterm neonate: behavioural and physiological indices. *Pain*, *52*(3), 287-299.

Cruz, M. D., Fernandes, A. M., & Oliviera, C. R. (2016). Epidemiology of painful procedures performed in neonates: A systematic review of observational studies. *European Journal of Pain, 20*, 489-498.

DiLorenzo, M. G., Pillai Riddell, R., Flora, D. B., & Craig, K. D. (2018). Infant clinical pain assessment: Core behavioural cues. *The Journal of Pain*, *19*, 1024-1032.

Duerden, E. G., Grunau, R. E., Guo, T., Foong, J., Pearson, A., Au-Young, S., Lavoie, R., Chakravarty, M., Chau, V., Synnes, A., & Miller, S. P. (2018). Early procedural pain is associated with regionally-specific alterations in thalamic development in preterm neonates. *The Journal of Neuroscience*, *38*(4), 878-886.

Fitzgerald, M. (2015). What do we really know about newborn infant pain? *Experimental Physiology, 100*, 1451-1457.

Goksan, S., Hartley, C., Emery, F., Cockrill, N., Poorun, R., Moultrie, F., Rogers, R., Campbell, J., Sanders, M., Adams, E., Clare, S., Jenkinson, M., Tracey, I., & Slater, R. (2015). fMRI reveals neural activity overlap between adult and infant pain. *Elife, 4*, e06356.

Green, G., Hartley, C., Hoskin, A., Duff, E., Shriver, A., Wilkinson, D., Adams, E., Rogers, R., Moultrie, F., & Slater, R. (2019). Behavioural pain discrimination of noxious stimuli in infants is dependent on brain maturation. *Pain, 160,* 493-500.

Grunau, R. V., & Craig, K. D. (1987). Pain expression in neonates: Facial action and cry. *Pain, 28,* 395-410.

Habermann, M., Weusmann, D., Stein, M., & Koenig, T. (2018). A student's guide to randomization statistics for multichannel event-related potentials using Ragu. *Frontiers in Neuroscience, 12,* Article 355.

Harrison, W. N., Wasserman, J. R., & Goodman, D. C. (2018). Regional variations in neonatal intensive care admissions and the relationship to bed supply. *The Journal of Pediatrics, 192,* 73-79.

Hartley, C., Goksan, S., Poorun, R., Brotherhood, K., Mellado, G. S., Moultrie, F., Rogers, R., Adams, E., & Slater, R. (2015). The relationship between nociceptive brain activity, spinal reflex withdrawal and behaviour in newborn infants. *Scientific reports, 5,* 12519.

Holsti, L., Grunau, R. E., Oberlander, T. F., Whitfield, M. F., & Weinberg, J. (2005). Body movements: An important factor in discriminating pain from stress in preterm infants. *Clinical Journal of Pain, 21,* 491-498.

Johnston, C. C., Stevens, B., Craig, K. D., & Grunau, R. V. (1993). Developmental changes in pain expression in premature, full-term, two-and four-month-old infants. *Pain, 52*(2), 201-208.

Jones, L., Laudiano-Dray, M. P., Whitehead, K., Meek, J., Fitzgerald, M., Fabrizi, L., & Riddell, R. P. (2020). *The impact of parental contact upon cortical nociceptive activity in human neonates*. Figshare. Preprint. https://doi.org/10.6084/m9.figshare.11830860.v1

Jones, L., Fabrizi, L., Laudiano-Dray, M., Whitehead, K., Meek, J., Verriotis, M., & Fitzgerald, M. (2017). Nociceptive cortical activity is dissociated from nociceptive behaviour in newborn human infants under stress. *Current Biology*, *27*, 3846-3851.

Jones, L., Laudiano-Dray, M., Whitehead, K., Verriotis, M., Meek, J., Fitzgerald, M. & Fabrizi, L., (2018). EEG, behavioural and physiological recordings following a painful procedure in human neonates. *Scientific Data*, 5:180248.

Kohut, S. A., & Riddell, R. P. (2009). Does the neonatal facial coding system differentiate between infants experiencing pain-related and non-pain-related distress?. *The Journal of Pain*, *10*(2), 214-220.

Kucyi, A., & Davis, K. D. (2015). The dynamic pain connectome. *Trends in neurosciences*, *38*(2), 86-95.

Lilley, C. M., Craig, K. D., & Grunau, R. E. (1997). The expression of pain in infants and toddlers: Developmental changes in facial action. *Pain*, *72*, 161-170.

Pillai Riddell, R., Stevens, B. J., Cohen, L. L., Flora, D. B., & Greenberg, S. (2007). Predicting maternal and behavioral measures of infant pain: The relative contribution of maternal factors. *Pain*, *133*, 138-149.

Ranger, M., Zwicker, J. G., Chau, C. M., Park, M. T., Chakravarthy, M. M., Poskitt, K., Miller, S. P., Bjornson, B. H., Tam, E., Chau, V., Synnes, A. R., & Grunau, R. E. (2015). Neonatal pain and infection relate to smaller cerebellum at school age. *The Journal of Pediatrics*, *167*, 292-298.

Schneider, J., Duerden, E. G., Guo, T., Ng, K., Hagmann, P., Bickle Graz, M., Grunau, R. E., Chakravarty, M. M., Huppi, P. S., Truttman, A. C., & Miller, S. P. (2018). Procedural pain

and oral glucose in preterm neonates: brain development and sex-specific effects. *Pain, 159*, 515-525.

Slater, R., Cantarella, A., Franck, L., Meek, J., & Fitzgerald, M. (2008). How well do clinical pain assessment tools reflect pain in infants? *PLoS Medicine, 5*, e129.

Slater, R., Worley, A., Fabrizi, L., Roberts, S., Meek. J., Boyd, S., & Fitzgerald, M. (2010a). Evoked potentials generated by noxious stimulation in the human infant brain. *European Journal of Pain, 14*, 321-326.

Slater, R., Cornelissen, L., Fabrizi, L., Patten, D., Yoxen, J., Worley, A., Boyd, S., Meek, J., & Fitzgerald, M. (2010b). Oral sucrose as an analgesic drug for procedural pain in newborn infants: A randomized controlled trial. *The Lancet, 376*, 1225-1232.

Stevens, B., Johnston, C., Petryshen, P., & Taddio, A. (1996). Premature infant pain profile: development and initial validation. *The Clinical Journal of Pain, 12*(1), 13-22.

Stevens, B. J., Pillai Riddell, R., Oberlander, T. E., & Gibbins, S. (2007). Assessment of pin in neonates and infants. In: *Pain in neonates and infants*. Edinburgh: Elsevier Unlimited.

Stevens, B. J., Gibbins, S., Yamada, J., Dionne, K., Lee, G., Johnston, C., & Taddio, A. (2014). The premature infant pain profile-revised (PIPP-R): Initial validation and feasibility. *The Clinical Journal of Pain, 30,* 238-243.

Taddio, A., Nulman, I., Koren, B. S., Stevens, B., & Koren, G. (1995). A revised measure of acute pain in infants. *Journal of Pain and Symptom Management, 10*, 456-463.

Taddio, A., Riddell, R. P., Ipp, M., Moss, S., Baker, S., Tolkin, J., Dave, M., Feerasta, S., Govan, P., Fletcher, E., Wong, H., McNair, C., Mithal, P., & Stephens, D. (2017). A Longitudinal Randomized Trial of the Effect of Consistent Pain Management for Infant Vaccinations on Future Vaccination Distress. *The Journal of Pain, 18*(9), 1060-1066.

Taddio, A., Shah, V., Atenafu, E., & Katz, J. (2009). Influence of repeated painful procedures and sucrose analgesia on the development of hyperalgesia in newborn infants. *Pain, 144,* 43-48.

Taddio, A., Stevens, B., Craig, K., Rastogi, P., Ben-David, S., Shennan, A., Mulligan, P., & Koren, G. (1997). Efficacy and safety of lidocaine-prilocaine cream for pain during circumcision. *New England Journal of Medicine, 336,* 1197-1201.

Verriotis, M., Jones, L., Whitehead, K., Laudiano-Dray, M., Panayotidis, I., Patel, H., Meek, J., Fabrizi, L., & Fitzgerald, M. (2018). The distribution of pain activity across the human neonatal brain is sex dependent. *NeuroImage, 178,* 69-77.

Verriotis, M., Fabrizi, L., Lee, A., Ledwidge, S., Meek, J., & Fitzgerald, M. (2015). Cortical activity evoked by inoculation needle prick in infants up to one-year old. *Pain, 156,* 222-230.

Vinall, J., Miller, S. P., Synnes, A. R., & Grunau, R. E. (2013). Parent behaviors moderate the relationship between neonatal pain and internalizing behaviors at 18 months corrected age in children born very prematurely. *Pain, 154,* 1831-1839.

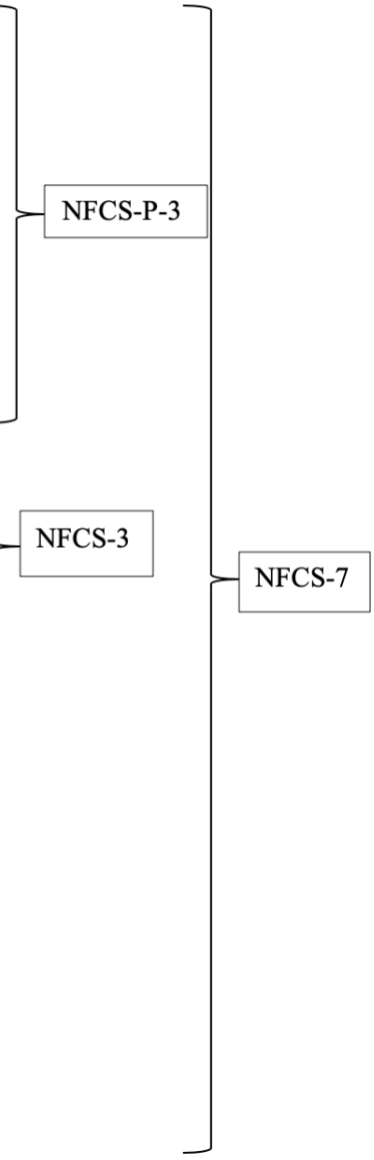
Willers Moore, Jucha (2020): Development of nociceptive brain states in preterm neonates. figshare. Thesis. https://doi.org/10.6084/m9.figshare.12613448.v1

Williams, A.C. (2002). Facial expressions of pain: An evolutionary account. *Behavioural and Brain Sciences, 25,* 439-455.

Appendix A

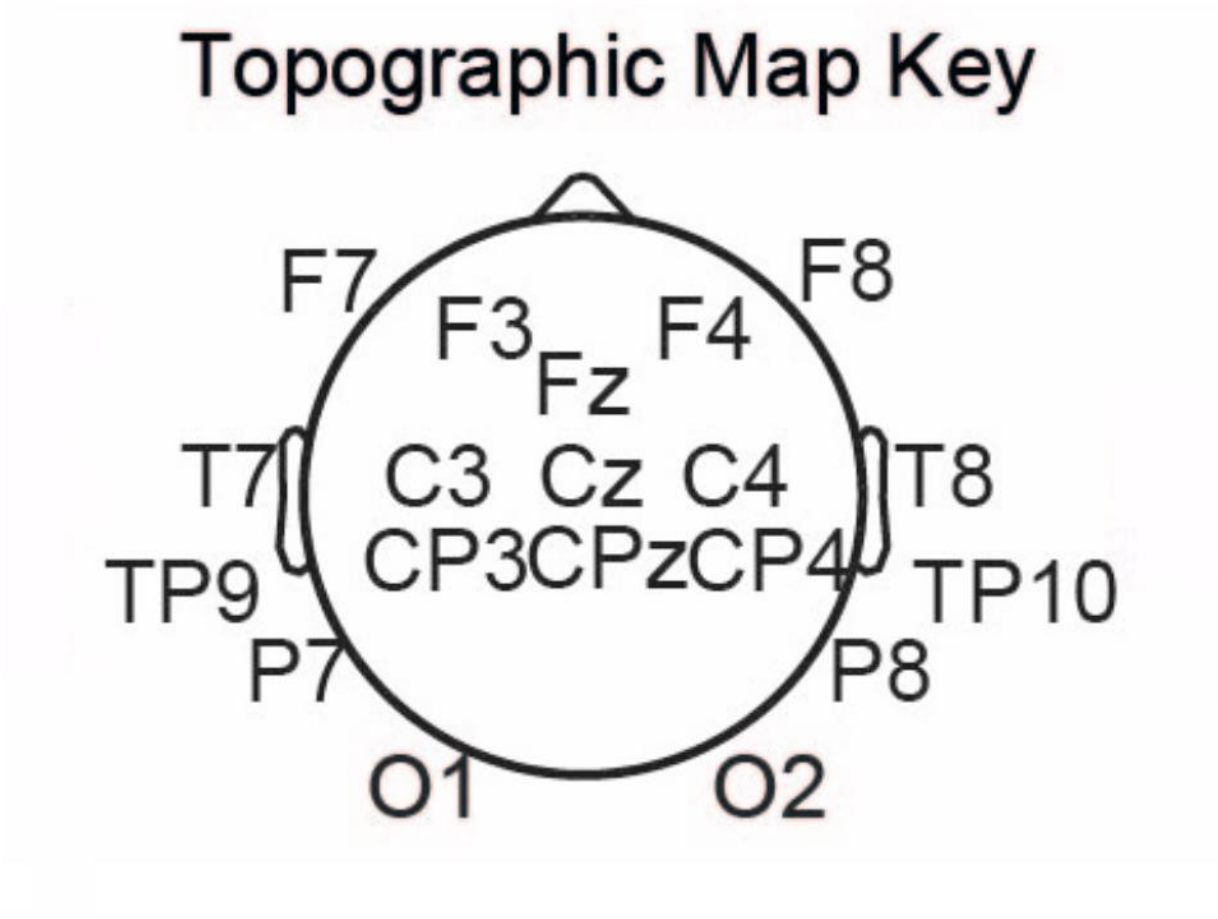
Breakdown of Pain-Related Facial Actions for the Three NFCS Variants

Facial Action	Description
Brow Bulge (BB)	Bulging, creasing and vertical furrows above and between brows occurring as a result of the lowering and drawing together of the eyebrows.
Nasolabial Furrow (NLF)	Primarily manifested by the pulling upwards and furrow deepening of the nasolabial furrow (a line or wrinkle that begins adjacent to the nostril wings and runs down and outward beyond the lip corners).
Eye Squeeze (ES)	Identified by the squeezing or bulging of the eyelids. Bulging of the fatty pads about the infant's eyes is pronounced.
Vertical Stretch Mouth (VSM)	Characterized by a tautness at the lip corners (vertical) coupled with a pronounced downward pull of the jaw. Often stretch mouth is seen when an already wide-open mouth is opened a fraction further by an extra pull at the jaw.
Horizontal Stretch Mouth (HSM)	Appears as a distinct horizontal pull at the corners of the mouth.
Open Lips (OL)	Any separation of the lips.
Taut Tongue (TT)	Characterized by a raised, cupped tongue with sharp tensed edges. The first occurrence of taut tongue is usually easy to see, often occurring with a wide-open mouth. After this first occurrence, the mouth may close slightly. Taut tongue is still scoreable on the basis of the still-visible tongue edges.



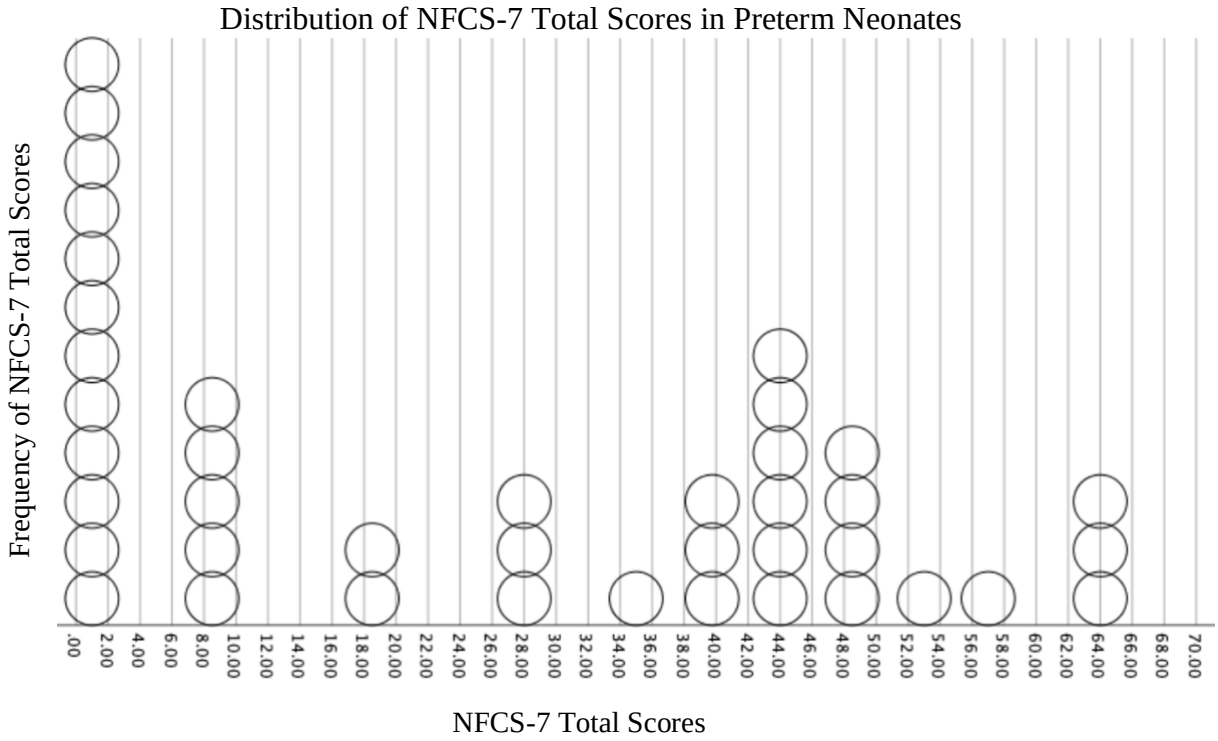
Appendix B

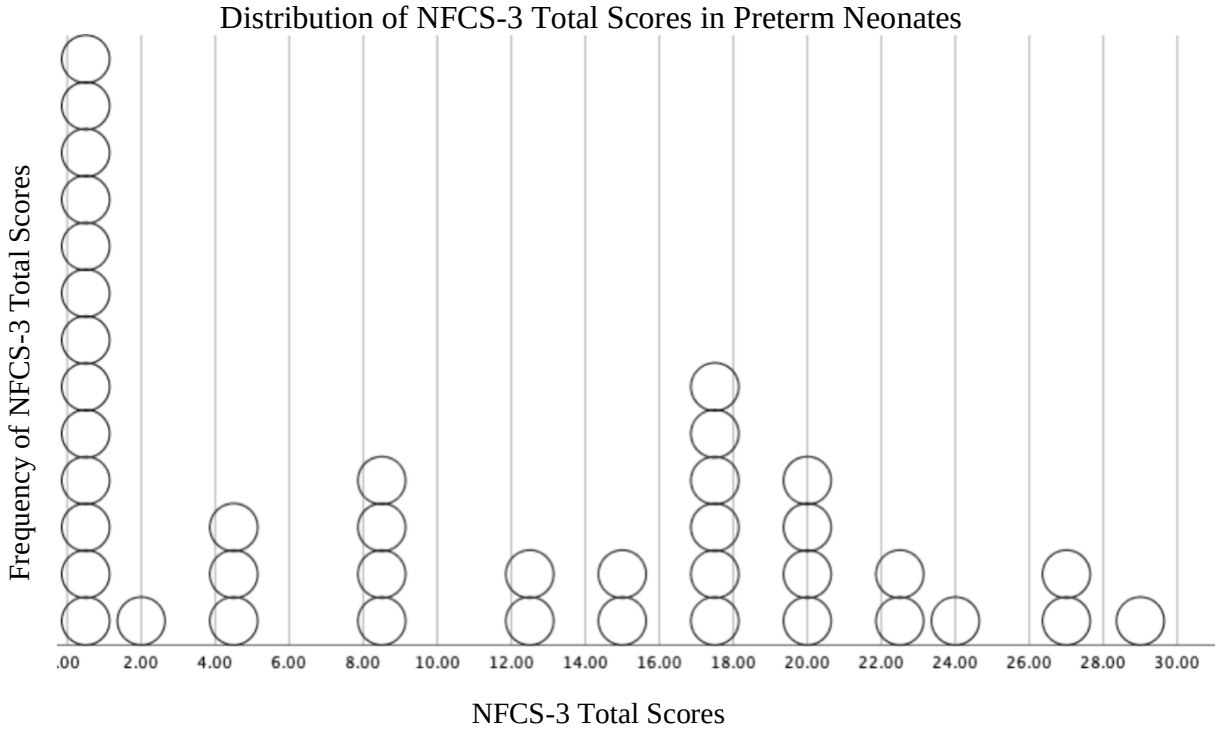
Map of the 18 Electrodes Scalp Placement



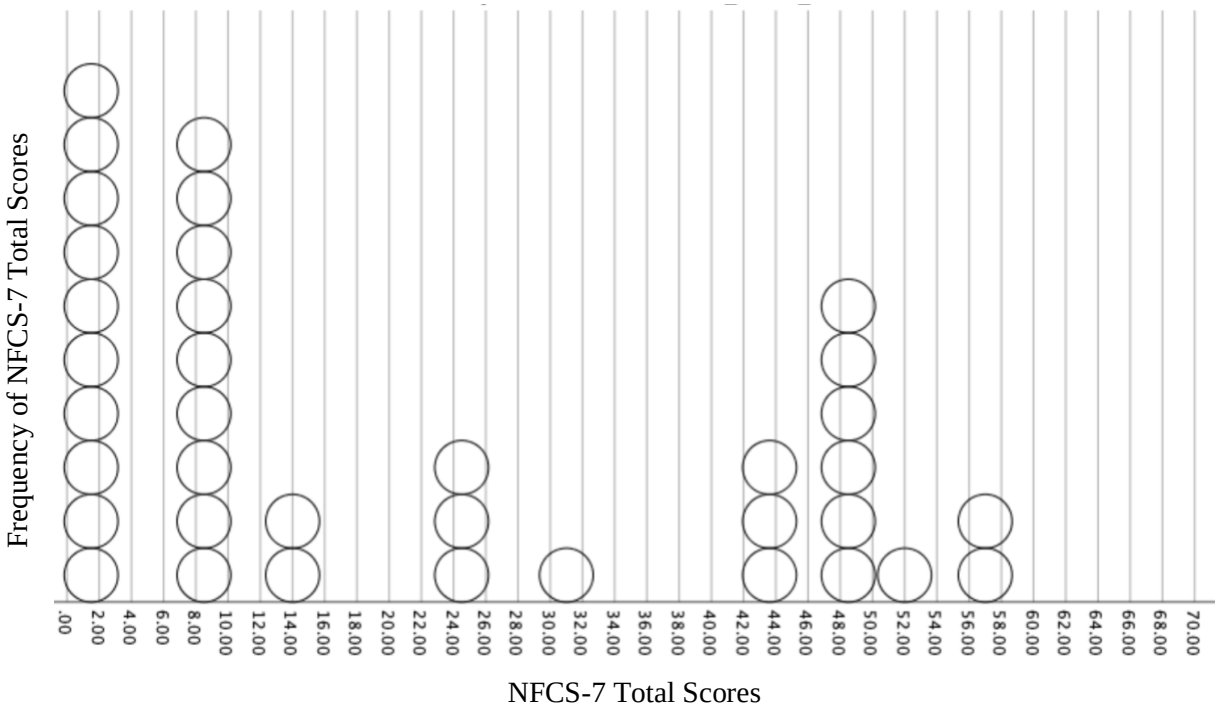
Appendix C

Distributions of NFCS-7 and NFCS-3 Total Scores





Distribution of NFCS-7 Total Scores in Full-Term Neonates



Distribution of NFCS-3 Total Scores in Full-Term Neonates