

# Early Procedural Pain Is Associated with Regionally-Specific Alterations in Thalamic Development in Preterm Neonates

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Very preterm human neonates are exposed to numerous invasive procedures as part of life-saving care. Evidence suggests that repetitive neonatal procedural pain precedes long-term alterations in brain development. However, to date the link between pain and brain development has limited temporal and anatomic specificity. We hypothesized that early exposure to painful stimuli during a period of rapid brain development, before pain modulatory systems reach maturity, will predict pronounced changes in thalamic development, and thereby cognitive and motor function. In a prospective cohort study, 155 very preterm neonates (82 males, 73 females) born 24–32 weeks' gestation underwent two MRIs at median postmenstrual ages 32 and 40 weeks that included structural, metabolic, and diffusion imaging. Detailed day-by-day clinical data were collected. Cognitive and motor abilities were assessed at 3 years, corrected age. The association of early (skin breaks, birth–Scan 1) and late pain (skin breaks, Scans 1–2) with thalamic volumes and *N*-acetylaspartate (NAA)/choline (Cho), and fractional anisotropy of white-matter pathways was assessed. Early pain was associated with slower thalamic macrostructural growth, most pronounced in extremely premature neonates. Deformation-based morphometry analyses confirmed early pain-related volume losses were localized to somatosensory regions. In extremely preterm neonates early pain was associated with decreased thalamic NAA/Cho and microstructural alterations in thalamocortical pathways. Thalamic growth was in turn related to cognitive and motor outcomes. We observed regionally-specific alterations in the lateral thalamus and thalamocortical pathways in extremely preterm neonates exposed to more procedural pain. Findings suggest a sensitive period leading to lasting alterations in somatosensory-system development.

**Key words:** critical period; DTI; MRI; MRS; pain; preterm

## Significance Statement

Early exposure to repetitive procedural pain in very preterm neonates may disrupt the development of regions involved in somatosensory processing, leading to poor functional outcomes. We demonstrate that early pain is associated with thalamic volume loss in the territory of the somatosensory thalamus and is accompanied by disruptions thalamic metabolic growth and thalamocortical pathway maturation, particularly in extremely preterm neonates. Thalamic growth was associated with cognitive and motor outcome at 3 years corrected age. Findings provide evidence for a developmentally sensitive period whereby subcortical structures in young neonates may be most vulnerable to procedural pain. Furthermore, results suggest that the thalamus may play a key role underlying the association between neonatal pain and poor neurodevelopmental outcomes in these high-risk neonates.

## Introduction

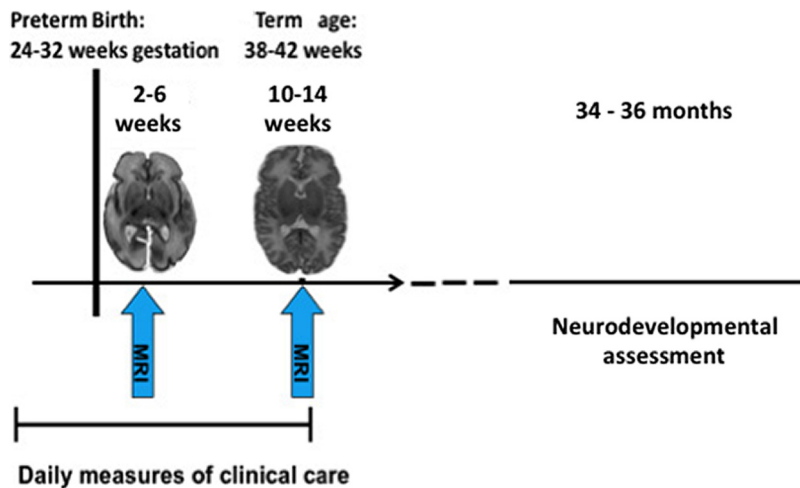
Infancy is characterized by critical periods of experience-induced plasticity when the brain is adaptable to novel sensory informa-

tion. Little research has focused on sensitive windows for nociceptive and somatosensory systems despite evidence suggesting neonatal tissue damage may have long-lasting effects on pain

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**Figure 1.** Timeline of study procedures.

processing systems (R. V. Grunau et al., 1994a) and functional outcomes (R. E. Grunau et al., 2009; Brummelte et al., 2015). The thalamus is a relay for nociceptive pathways and developing thalamocortical projections may be disrupted by early pain.

Preterm-born neonates often undergo hundreds of invasive procedures during intensive care (Carbajal et al., 2008). Extremely preterm born neonates ( $\leq 28$  weeks' gestation) may be most susceptible to pain exposure, occurring at a time of rapid and vulnerable brain development (Back and Miller, 2014). Nociceptive systems become functional at 24–28 weeks' gestation with the development of thalamocortical connections (Andrews and Fitzgerald, 1994); however, brainstem-mediated endogenous pain modulation only develops closer to term-equivalent age (Fitzgerald, 2005). Thus, exposure to pain at earlier gestational ages may have differential effects on the developing brain.

Neonatal pain contributed to poorer cognitive and motor outcome at 18 months corrected age (CA; R. E. Grunau et al., 2009), and predicted IQ at school age (Vinall et al., 2014) in preterms. Pain in preterms precedes specific alterations in the frontal cortices and somatosensory system (Ranger et al., 2013) and somatosensory processing (R. V. Grunau et al., 1994a; Hermann et al., 2006; Goffaux et al., 2008; Walker et al., 2009). Neonatal pain may have lasting consequences on subcortical gray and white-matter development subserving somatosensory, cognitive, and motor processes.

Our earlier work suggests that neonatal intensive care unit (NICU)-related invasive procedures are associated with widespread alterations in thalamic metabolic development and white-matter microstructural pathways in very preterm born neonates (Brummelte et al., 2012). The long-term sequelae of early exposure to noxious stimuli in animals suggests that the first weeks of life may be a period of enhanced vulnerability, with susceptibility increasing with earlier exposure during development (Anand et al., 1999; Ren et al., 2004; Beggs et al., 2012). In the present study, our central hypothesis was that early skin breaks administered to

extremely preterm neonates would be associated with regionally-specific alterations in thalamic and thalamocortical fiber pathway maturation. We used three complementary neuroimaging measures to examine congruence of findings across thalamic development: structure, metabolism, and microstructure in a group of extremely (24–28 weeks' gestation) and very (29–32 weeks' gestation) preterm born neonates who underwent MRI early in-life and at term-equivalent age.

To address our primary aim, models testing the association of early (birth–Scan 1) and late (Scans 1–2) invasive procedures accounted for repeated measures and were applied to the neuroimaging data. Given previous research suggesting an association among invasive procedures, brain development and poorer

functional outcomes in preterm neonates, our secondary aim was to assess the association between thalamic development and two assessments of neurodevelopmental outcome: cognitive and motor function. To address our second aim, cognitive and motor abilities were assessed using the Bayley Scales of Infant and Toddler Development, third edition (Bayley-III) at 3 years CA in relation to thalamic growth.

## Materials and Methods

**Participants.** One hundred fifty-five neonates (82 males and 73 females) admitted to the NICU at BC Women's Hospital, Canada, were enrolled in the study between 2006 and 2013. Birth at 24–32 weeks' gestation was the sole eligibility criterion. Neonates were excluded based on the following criteria: congenital malformations/syndromes, antenatal infections, or large ( $>2$  cm) parenchymal hemorrhagic infarctions. Neonates with two scans and detailed daily clinical data were included in this study. The Clinical Research Ethics Board at the University of British Columbia and Children's and Women's Health approved the study. Written informed consent was obtained from parents or legal caregivers.

**Magnetic resonance imaging.** Neonates were scanned on a Siemens 1.5T Avanto MRI (VB 13A software) inside an MR-conditional incubator (Lammers Medical Technology) using a single-channel neonatal head coil (Advanced Imaging Research; Fig. 1). Neonates underwent their first MRI scan when clinically stable for transport (median postmenstrual age 32 weeks; IQR = 30.7–33.0) and again at term-equivalent age (median age 40 weeks; IQR = 38.7–42). Anatomical imaging included the acquisition of T1-weighted [repetition time (TR) = 36; echo time (TE) = 9.2; field-of-view (FOV) =  $192 \times 88$  mm; voxel size:  $1.04 \times 1.00 \times 1.04$  mm; no gap] and T2-weighted images (TR = 4610; TE = 107; FOV =  $20 \times 402$  mm; voxel size =  $0.4 \times 0.4 \times 4.48$  mm; gap = 0.2 mm). The magnetic resonance spectroscopic imaging (MRSI) data were acquired using multivoxel chemical shift imaging (TR: 1500 ms; TE: 144; averaging 4). Diffusion tensor imaging (DTI) data were acquired using a multirepetition, single-shot echoplanar sequence [TR = 4900, TE = 104; motion-probing gradient in 12 diffusion-encoding directions with 3 averages of 2 diffusion weightings of 600 and 700  $s/mm^2$  ( $b$  value) and a non-diffusion-weighted image; FOV = 160 mm; 1.3 mm in-plane resolution; slice thickness = 3 mm].

Anatomical images were reviewed for the presence and severity of white matter injury (WMI). WMI was defined as foci exhibiting T1 hyperintensity without significant T2 hypointensity. WMI volumes were calculated through a manual segmentation protocol (Guo et al., 2017).

**Volumetric measurements of the thalamus.** Volumetric measurements of the thalamus were performed on the early-in-life and term-equivalent age scans using the T1-weighted images. A semiautomatic method previously described by Guo et al. (2015) was applied to the T1-weighted images ac-

for database development; Dr. Kenneth Poskitt for scoring the MRI scans acquired in the neonates; Dr. Rollin Brant for review and comments on the statistical analyses; and Christopher Hammill for technical support for the deformation based morphometry analyses.

The authors declare no competing financial interests.

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quired at the two time points. In brief, one of the authors (A.P.) manually segmented the left and right thalami on a selection of randomly chosen early and term T1-weighted images using the Display software package ([en.wikibooks.org/wiki/MINC/VisualTools/Display](http://en.wikibooks.org/wiki/MINC/VisualTools/Display)). T1-weighted images were visualized and the thalamus was segmented in simultaneous coronal, sagittal, and axial views of the brain. Manual thalamic segmentations were used as templates for subsequent automatic thalamic segmentation of all T1-weighted images by using the Multiple Automatically Generated Templates (MAGeT) Brain (Chakravarty et al., 2013) pipeline. Using the pipeline, the manual segmentations of the thalamus were propagated to the T1-weighted images of the entire cohort permitting the volume calculation of the left and right thalami for each participant. The left and right values were averaged for each participant's thalamic volumetric measurements and were used in subsequent statistical models.

**Deformation-based morphometry analysis.** Regionally-specific alterations in thalamic volume were estimated using deformation-based morphometry (DBM; Ashburner et al., 1998). First, templates for the early-in-life and term-equivalent age scans were created using the T1-weighted images. Templates were created by developing an average of all T1-weighted images collected at the two time points. T1-weighted images were nonlinearly coregistered using Advanced Normalization Tools (Avants et al., 2008, 2011). Each individual T1-weighted image was aligned to the early-in-life or term-equivalent age templates depending on the age of the neonates at scan using affine transformations. A nonlinear registration algorithm was then used for precise alignment of the native T1-weighted images to the template images. The final registrations create the Jacobian determinants of the deformation fields describing the shape differences in expansion and contraction at each voxel in the template MRI. Log-transformed Jacobian determinants were used in the final statistical analyses as they are more representative of the normal distribution.

**MRSI.** The MRSI data were analyzed using a region-of-interest approach by one of the authors (V.C.). A volume-of-interest (VOI; 50 × 50 × 10 mm thick) was selected at the level of the basal nuclei. Within the VOI, the peak-area spectra were analyzed in the thalamus using Siemens' MRS processing software. Voxels with adequate signal-to-noise ratio and that were fully included in the VOI were used for statistical analyses as described previously (Chau et al., 2009; Brummelte et al., 2012). The mean *N*-acetylaspartate (NAA)/choline (Cho) and lactate/Cho ratios for each ROI were calculated bilaterally; these methods have high intra-rater reliability (Brummelte et al., 2012).

**DTI analysis.** Diffusion-weighted images were processed using the FMRIB software library (<http://www.fmrib.ox.ac.uk/fsl/>; Jenkinson et al., 2012). Data were corrected for eddy-current effects. Each participants' diffusion-weighted volumes were registered to a single non-diffusion-weighted volume using affine transformations (Jenkinson and Smith, 2001; Jenkinson et al., 2002). The estimated diffusion tensor data were masked using the Brain Extraction Tool (Jenkinson et al., 2002) to include only brain tissue. A diffusion tensor model was fit to the data at each voxel and to calculate voxelwise fractional anisotropy (FA) values. To better determine the spatial location of alterations in FA values in the neonatal brain, volumes were processed using the Tract-based spatial statistics (TBSS) pipeline (Smith et al., 2006). FA images were nonlinearly aligned (Avants et al., 2008, 2011) to age-appropriate templates as previously described (Duerden et al., 2015) to calculate voxelwise statistics. Last, the Johns Hopkins University (JHU) atlas was nonlinearly registered to the age-based templates and the FA values from the corpus callosum, posterior white matter (posterior corona radiata, posterior thalamic radiation), internal capsule (anterior, posterior limbs, corticospinal tract), and cingulum (cingulum, fornix) from the left and right sides were averaged leaving a total of four ROIs. The FA data were extracted and used for statistical modeling.

**Clinical data collection.** Clinical data were systematically collected from the infants' NICU charts by a neonatal nurse and a pediatric neurologist. As a measure of pain each individual invasive procedure involving skin breaks was prospectively documented by nursing staff, including but not limited to heel lances, intubations, intravenous or central line insertion, and intramuscular injection (described by R. E. Grunau et al., 2009;

Brummelte et al., 2012). Each attempt at an invasive procedure was counted daily. Cumulative doses of midazolam, morphine, and dexamethasone in milligrams were calculated (intravenous dose plus converted oral dose) as the average daily dose adjusted for daily body weight. Other clinical variables included: gestational age (GA) at birth, biological sex, days of life (DOL) at scan, postmenstrual age (PMA) at scan, morphine dose, midazolam dose, dexamethasone dose, and days of mechanical ventilation.

**Neurodevelopmental outcomes at 3 years.** Toddlers and their families returned for neurodevelopmental follow-up at the clinic at BC Women's Hospital at 3 years of age corrected for prematurity (CA). Neurodevelopmental abilities were assessed using the Bayley-III, which yields cognitive and motor composite scores (mean = 100; SD = 15).

**Statistical analysis.** The Statistical Package for the Social Sciences (v24, SPSS) was used for the volumetric, microstructural and brain metabolite statistical analyses. The DBM analyses were performed using the R statistical software package.

To investigate the association of early and late invasive procedures on thalamic macrostructural and metabolic growth, as well as the microstructural development of thalamocortical pathways, the thalamic volumes, NAA/Cho, and FA values from the early in life and term-equivalent age scans were modeled in three separate analyses [(1) volumes, (2) NAA/Cho, (3) FA] using generalized estimating equations to account for repeated measures. Early invasive procedures included the total number of skin breaks recorded from birth until the time of the first scan, whereas the late invasive procedures included the total number of skin breaks from the time immediately after the first scan until the second scan. Invasive procedures were entered into the analysis as categorical or continuous variables. For categorical variables, the median number of skin-breaking procedures from birth to Scan 1 (low early skin breaks: <49; high early skin breaks: >50) and from Scan 1 to Scan 2 (low late skin breaks: <31; high early skin breaks: >32) were used to divide the cohort. Variables known to influence brain development were entered as covariates and included: DOL at MRI, GA at birth (weeks), biological sex, total morphine, midazolam and dexamethasone doses (mg), days of intubation, and total cerebral volumes (volumetric analyses only). The variables of DOL at MRI and GA at birth were used to account for the PMA at scan in the regression analyses. We conducted an exploratory analysis in which we visualized the standardized residuals of the model and created scatterplots of the residuals to assess their distribution. If the final models assessing macrostructural growth were significant and the residuals were homoscedastic, then models were repeated to examine the effects of early and late skin breaks on thalamic metabolic maturation in the extremely preterm neonates compared with neonates born very preterm. If the final model was significant in the extremely preterm population, then the models were repeated to examine the association of early and late skin breaks with thalamic metabolic development and microstructural development of thalamocortical pathways in neonates born ≥29 weeks' gestation, adjusting for factors related to prematurity and illness severity. As we had one a priori central hypothesis regarding the association of early skin breaks in the extremely preterm neonate with thalamic maturation, the  $\alpha$  level for statistical tests for the volumetric and metabolic data were set to 0.05.

Neurodevelopmental outcome scores (cognitive and motor) were assessed in relation to the weekly growth of the thalamus (thalamic volume Scan 2 – thalamic volume Scan 1/PMA at Scan 2 – PMA at Scan 1), adjusting for GA at birth and biological sex. For our secondary aim regarding thalamic development and neurodevelopmental outcome, we had two hypotheses in relation to cognitive and motor outcomes and the  $\alpha$  level was set at 0.025.

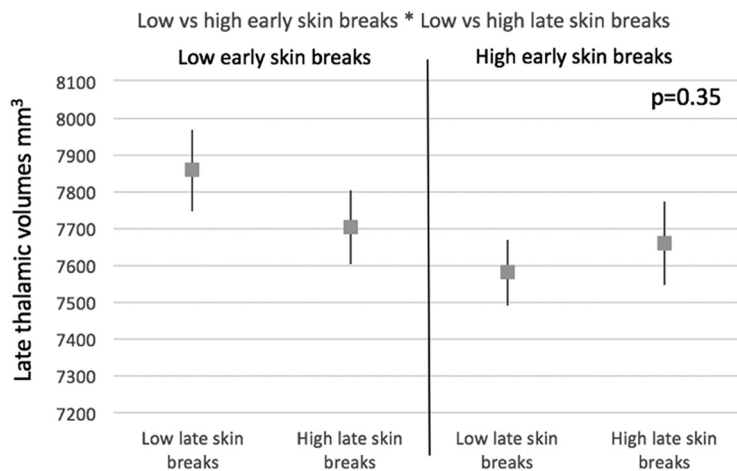
For the DBM analyses, the association of early and late skin breaks were assessed in relation to the log-transformed Jacobians developed from the first and second scan data using two separate linear regression models (early skin breaks and Scan 1, late skin breaks and Scan 2), adjusting for the ages at scan, using the RMINC toolkit with the R statistical software package (<http://www.R-project.org/>). *t* Tests at each voxel assessed the significance of the regression models. As the primary area of interest was the thalamus, a thalamic mask was applied to the data. Masked data were corrected for multiple comparisons using the false

**Table 1. Characteristics for the full cohort (N = 155) and comparisons of demographic information for neonates born before or after 28 weeks**

Characteristic	Total N = 155	Birth GA 24–28 weeks n = 94	Birth GA 29–32 weeks n = 61
Birth GA	27.6 (25.9–29.9)	26.3 (25.4–27.3)	30.1 (29.6–31.4)
Median weeks (IQR)			
Male, no. (%)	82 (53)	49 (52)	33 (54)
PMA MRI 1, median (IQR)	32.3 (30.7–33.7)	32 (30–35)	32.3 (31.4–33.43)
PMA MRI 2, median (IQR)	40.3 (39–42)	40.3 (39–42)	40.3 (39.3–43)
DOL MRI 1, median (IQR)	21 (12–46)	38 (20–60)	12 (8–17)
DOL MRI 2, median (IQR)	90.5 (70–101)	97 (89–105)	69 (60–84)
Days of mechanical ventilation, median (IQR)	4 (1–27)	16.5 (0–121)	1 (0–2)
Fentanyl, median dose <sup>a</sup> (IQR); no. exposed	0.007 (0.005–0.022); 34	0.008 (0.005–0.02); 31	0.003 (0.003–0.006); 3
Morphine, median dose <sup>a</sup> (IQR); no. exposed	1.8 (0.18–7.9); 96	2.85 (0.4–13.7); 74	0.15 (0.13–1.5); 22
Midazolam, median dose <sup>a</sup> (IQR); no. exposed	6.8 (4.3–12.7); 29	7.1 (4.1–12.8); 28	0 (0); 1
Dexamethasone, median dose <sup>a</sup> (IQR); no. exposed	1.2 (0.75–2.4); 28	1.2 (0.75–2.4); 28	0 (0); 0
Total skin breaks, median (IQR)	101 (65–168)	142 (97–208)	58 (46–74)
Early skin breaks, median (IQR)	49 (30–185)	84 (42–158)	34 (23–45)
Late skin breaks, median (IQR)	31 (18–57)	44 (25–66)	24 (13–31)

Probability values provide results using *t* tests for continuous measures and  $\chi^2$  tests for categorical measures comparing neonates born extremely (24–28 weeks' gestation) and very preterm (29–32 weeks' gestation).

<sup>a</sup>Cumulative dose in milligrams adjusted for daily body weight.



**Figure 2.** Late-skin-breaking procedures were not significantly associated with variations in thalamic volumetric development at term equivalent age and were not dependent on neonates' prior exposure to early skin-breaking procedures ( $p = 0.35$ ). Estimated marginal means of thalamic volumes adjusting for male sex, GA at birth, DOL at scan, days of intubation, morphine dose, midazolam dose, dexamethasone dose, and total cerebral volume. Error bars are the SE of the estimated marginal means.

discovery rate (Genovese et al., 2002). A *p* value of 0.05 was considered statistically significant.

For the DTI analysis, as we had four ROIs in the white matter, we corrected for the total number of comparisons and the  $\alpha$  level was set at 0.013.

## Results

### Clinical characteristics and diagnostic MRI findings

One hundred fifty-five preterm neonates participated in the study [53% boys; median birth gestational age (GA): 27.6 weeks; interquartile range (IQR): 25.9–29.9; Table 1]. Of these neonates, 94 (61%) were born extremely preterm (24–28 weeks' gestation; 52% boys; median birth GA: 26.3 weeks; IQR: 25.4–27.3) and 61 (39%) were born very preterm (29–32 weeks' gestation; 54% boys; median birth GA: 30.1 weeks; IQR: 29.6–31.4). As expected, extremely preterm born neonates had a longer NICU stay before the first scan, were on a ventilator for more days, had greater total doses of morphine, midazolam, and dexamethasone compared with neonates born 29–32 weeks' gestation. Extremely preterm born neonates did not exhibit significantly greater volume of WMI ( $\beta = 463.6, p = 0.16$ ) measured by manual segmen-

tation compared with their counterparts born at 29–32 weeks' gestation, adjusting for birth GA, DOL at scan, and total cerebral volume. The WMI volume did not significantly differ between neonates that received a high or low number of early invasive procedures ( $\beta = 40.4, p = 0.7$ ), adjusting for the same demographic and clinical care factors noted above.

### Early skin breaks are associated with slower thalamic macrostructural growth

Higher early skin-breaking procedures (>median 50 skin breaks), when used as a categorical variable, demonstrated slower thalamic growth across both scans ( $\beta = -194.6, p = 0.04$ ), adjusting for DOL at MRI, GA at birth (weeks), biological sex, total morphine, midazolam, and dexamethasone doses (mg), days of intubation, and total cerebral volumes. In comparison, higher later acquired skin-breaking procedures (>32 skin breaks)

did not predict thalamic volume compared with those that received a low number of skin-breaking procedures in the late neonatal period ( $\beta = -3.5, p = 0.96$ ). Furthermore, late skin-breaking procedures were not associated with differences in thalamic volume at term regardless of whether neonates had received low or high numbers of early skin-breaking procedures (Fig. 2; Table 2).

The model was repeated examining the association of early and late skin breaks with thalamic growth during the neonatal period in groups of neonates born before or after 28 weeks' gestation. Extremely preterm born neonates exposed early to higher numbers of skin-breaking procedures showed a significant decrease in thalamic growth ( $p = 0.04$ ; Table 3; Fig. 3a), but thalamic volume was not associated with later pain ( $p = 0.9$ ), adjusting for sex, birth GA, DOL at scan, total morphine dose, midazolam dose, dexamethasone dose, days of mechanical ventilation, and total cerebral volume. In contrast with extremely preterm born neonates, thalamic growth in very preterm born neonates (29–32 weeks' gestation) was not associated with early

**Table 2. Results of a generalized estimating equation for late thalamic volumes (Scan 2) describing the interaction between early × late skin-breaking procedures**

	$\beta$ values	$p$ values
Male sex	100.7	0.27
Birth GA	73.2	0.06
DOL at MRI	12.2	<0.001 <sup>a</sup>
Morphine dose	2.9	0.37
Midazolam dose	2.0	0.85
Dexamethasone dose	−5.9	0.93
Days of intubation	−5.0	0.16
Early pain × late pain		0.35
Low early skin breaks * low late skin breaks	197.6	0.29
Low early skin breaks * high late skin breaks	43.5	0.78
High early skin breaks * low late skin breaks	−78.9	0.55
High early skin breaks * high late skin breaks <sup>b</sup>	—	—
Total cerebral volume	0.01	<0.001 <sup>a</sup>

Estimated coefficients and  $p$  values for the interaction of early (low/high) by late (low/high) skin-breaking procedures.

<sup>a</sup>Statistically significant,  $p < 0.05$ .

<sup>b</sup>Comparison group.

**Table 3. Results of linear regression models for thalamic volumes relative to early skin breaks**

	24–28 weeks' gestation		29–32 weeks' gestation	
	$\beta$ values	$p$ values	$\beta$ values	$p$ values
Male sex	7.6	0.93	−12.6	0.89
Birth GA	96.4	0.2	137.4	0.02 <sup>a</sup>
DOL at scan	23.3	0.02 <sup>a</sup>	10.3	0.037 <sup>a</sup>
Morphine dose	1.7	0.33	104.66	0.002 <sup>a</sup>
Midazolam dose	−4.2	0.47	−806.79	0.029 <sup>a</sup>
Dexamethasone dose	−63.3	0.21	—	—
Days of mechanical ventilation	−3.13	0.37	−100.07	0.0001 <sup>a</sup>
High early skin breaks	−211.81	0.04 <sup>a</sup>	−20.45	0.85
High late skin breaks	9.44	0.93	133.46	0.21
TCV	0.01	0.0001 <sup>a</sup>	0.02	0.0001 <sup>a</sup>

Estimated coefficients and  $p$  values for early skin-breaking procedures in relation to thalamic volumes in extremely (24–28 weeks' gestation) and very preterm (29–32 weeks' gestation) neonates. TCV, Total cerebral volume.

<sup>a</sup>Statistically significant,  $p < 0.05$ .

( $\beta = -20.45$ ,  $p = 0.85$ ) or late skin breaks ( $\beta = 133.46$ ,  $p = 0.2$ ), controlling for the above clinical factors.

### Early skin breaks are associated with reduced volume in the lateral thalamus

To determine the spatial localization of the volumetric changes in the thalamus the T1-weighted images were subjected to DBM analyses to better understand relative change in volume on a voxelwise level. Early skin-breaking procedures were entered into a linear model as continuous variables against the log-transformed Jacobian determinants describing the changes in shape relative to the average models for the early-in-life and term-equivalent age scans, adjusting for PMA at scan.

Early skin-breaking procedures were found to be significantly associated with a reduction in volume in the territory of the lateral thalamus ( $t_{(152)} = -5.9$ ,  $p < 0.001$ , corrected) in the early-life scans, which contains the somatosensory relay nuclei. Those neonates receiving >100 skin breaks in the early neonatal period exhibited a 4–6% relative decrease in volume in the lateral thalamus (Fig. 4).

### Early skin breaks and reduced thalamic metabolites (NAA/Cho)

Early and late skin breaks in the neonatal period were assessed as categorical variables in relation to NAA values extracted from the

thalamus relative to the reference metabolite Cho in neonates born extremely and very preterm in two separate models. In agreement with the volumetric findings, in the neonates born extremely preterm early skin breaks were associated with slower metabolic growth ( $p < 0.0001$ ; Table 4; Fig. 3b), adjusting for sex, birth GA, DOL at scan, total morphine dose, midazolam dose, dexamethasone dose; and days of mechanical ventilation. Later pain was not associated with NAA/Cho values ( $p = 0.5$ ) in extremely preterm born neonates.

### Early skin-breaking procedures are associated with white matter microstructure

Early and late skin-breaking procedures were first assessed as continuous variables in relation to FA values obtained from four white matter ROIs including the corpus callosum, posterior white matter, the internal capsule, and cingulum in the JHU neonatal atlas, adjusting for clinical variables in extremely preterm born neonates.

In the extremely preterm born cohort, early skin-breaking procedures were associated with FA in the corpus callosum ( $p < 0.0001$ ; Table 4; Fig. 3), posterior white matter comprised of the posterior corona radiata and posterior thalamic radiations ( $p = 0.005$ ), and the cingulum and fornix ( $p = 0.01$ ). Late skin-breaking procedures were not found to be significantly associated with FA values in any of the assessed white-matter fiber pathways (corpus callosum:  $\beta = 0.00001$ ,  $p = 0.9$ ; posterior white matter:  $\beta = 0$ ,  $p = 0.4$ ; cingulum and fornix:  $\beta = 0.00001$ ,  $p = 0.9$ ).

### Neurodevelopmental outcome at 3 years

The majority of the children ( $n = 122$ , 79% of survivors) returned for assessments at 33–36 months corrected age (median age: 34.7 months; IQR: 33.7–36.1). The median scores for cognitive (100; IQR: 95–110) and motor (103; IQR: 94–110) outcomes were in the normal range.

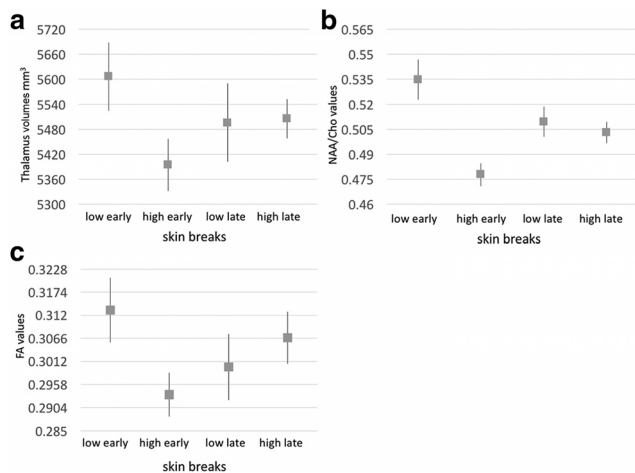
### Relationship between neurodevelopmental outcome at 3 years and macrostructural thalamic growth

Bayley-III outcome scores were assessed in two separate linear regression models as dependent variables in relation to the predictor variable weekly thalamic macrostructural growth (volume Scan 2 – volume Scan 1/age Scan 2 – age Scan 1), adjusting for birth GA and sex. In linear regression models, cognitive ( $\beta = 0.04$ ,  $p = 0.003$ ) and motor scores ( $\beta = 0.06$ ,  $p = 0.0001$ ) were predicted by thalamic volumetric growth adjusting for sex and GA at birth.

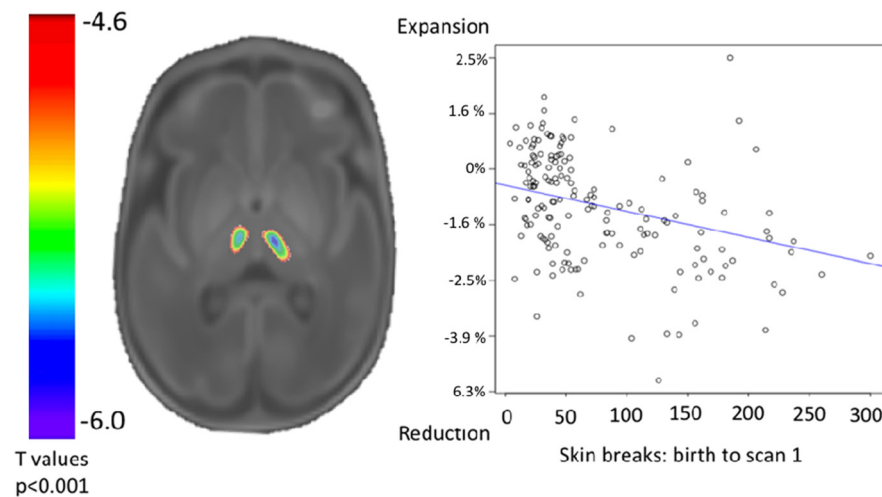
### Discussion

Exposure to painful stimuli early in the neonatal period is independently associated with adverse alterations in thalamic macrostructural and metabolic growth. Alterations in macrostructural, metabolic, and microstructural growth were most pronounced in neonates born <28 weeks' gestation. Extremely preterm born neonates may represent a vulnerable group whereby painful life-saving procedures may have long-term adverse effects on somatosensory system development.

Early but not later pain was associated with thalamic macrostructural, metabolic, and microstructural alterations as well as thalamocortical pathways and commissural and association white matter fibers. Our findings indicate some regional specificity in the thalamus for exposure to invasive procedures, compared with the hippocampus that showed no association with procedural pain after accounting for benzodiazepine exposure in this cohort (Duerden et al., 2016). Crucially, changes were most



**Figure 3.** Extremely preterm neonates exposed to high early skin breaks demonstrate slower (a) macrostructural ( $\beta = -211.81, p = 0.04$ ) and (b) metabolic thalamic growth ( $\beta = -0.06, p < 0.0001$ ) and (c) microstructural development of posterior white matter ( $\beta = -0.02, p = 0.02$ ) across early and term-equivalent age scans. Late skin breaks were not associated with delays in macrostructural ( $\beta = 9.4, p = 0.9$ ), metabolic ( $\beta = -0.006, p = 0.5$ ), and microstructural development ( $\beta = 0.007, p = 0.5$ ). Boxes represent the estimated marginal means of high/low early/late skin breaks fixed at the values for sex, birth GA, DOL at scan, total morphine dose, midazolam dose, dexamethasone dose, days of mechanical ventilation, and total cerebral volume (volume analysis only) in three separate models. Error bars are the SE of the estimated marginal means.



**Figure 4.** Volume loss in the lateral thalamus is associated with a greater number of early skin-breaking procedures ( $t_{(152)} = -5.9, p < 0.001$ , corrected). Left, DBM *t*-statistic map overlaid on a template MRI developed from the T1-weighted images collected at the early-in life time point (median PMA: 32 weeks). *t* Values reflect regional reductions in volume associated with the number of early skin-breaking procedures (birth–Scan 1). Right, Scatterplot of the relative increase and decrease values associated with early skin breaking procedures (birth–Scan1).

evident in neonates born extremely preterm. Animal studies reported age-sensitive periods in relation to the exposure of painful stimuli early in development (Anand et al., 1999; Ruda et al., 2000; Knaepen et al., 2013). Behavioral signs of enhanced sensitivity to painful stimuli at the site of injury are evident long-term in adult rats (Ren et al., 2004); however, early injuries are also accompanied by widespread alterations in basal thresholds at pain-naïve sites reflected in hyposensitivity to somatosensory and noxious stimuli. Altered sensitivity to painful stimuli will not occur in neonatal rat pups if inflammation or tissue damage is applied after the first weeks of life suggesting that young neonates are more vulnerable to the adverse effect of pain. Milder injuries are associated with more general hyposensitivity to painful stimuli later in adulthood (Ren et al., 2004; Sternberg et al., 2005; Laprairie

and Murphy, 2009). The anatomic localization of the most prominent changes within the lateral thalamus is consistent with these experimental observations.

Early pain may act through several mechanisms resulting in hypersensitivity or hyposensitivity to noxious stimuli (Ren et al., 2004). Of note is that early life injury will result in developmentally-regulated effects on nociceptive pathways, not occurring at older ages in response to the same injury (Fitzgerald and Walker, 2009). Neonatal injury will result in hyperinnervation in the periphery (Reynolds and Fitzgerald, 1995; Moss et al., 2005) and will increase the receptive field sizes of dorsal horn neurons (Torsney and Fitzgerald, 2003). Also originating from the dorsal horn are neurons expressing the neurokinin 1 (NK1) receptor that project to pain modulatory systems in the brainstem. Dorsal horn neurons expressing the NK1 receptor terminate in brainstem pain modulatory systems and early pain may lead to increased opioid tone resulting in a decrease in pain sensitivity. Moreover, brainstem-mediated pain modulation exhibits age-sensitive periods (Fitzgerald and Koltzenburg, 1986; Hathway et al., 2009), where painful stimulation is facilitatory during the first weeks of postnatal development that becomes inhibitory later on in development. Additional mechanisms include the activation of the hypothalamic-pituitary-adrenal (HPA) axis causing the release of cortisol and sensitizing dorsal horn neurons in the spinal cord. Sensitization of dorsal horn neurons may be an underlying cause of central sensitization resulting in alterations in the neuronal function of nociceptive pathways and thalamus. The HPA axis does not mature until late childhood (McCormick and Mathews, 2010; Foilb et al., 2011). Yet, pain in the preterm neonate is associated with an altered stress response through childhood (R. E. Grunau et al., 2013). Additionally, the activation of immune system modulators will release inflammatory molecules that sensitize fibers in the periphery (Schwaller and Fitzgerald, 2014; Zouikr et al., 2016), consistent with our finding that inflammatory factors were implicated in altered cortisol levels at school-age in very preterm children (R. E. Grunau et al., 2013).

During the development of the mammalian brain, afferent thalamocortical projections and their refinement into a topographic organization occurs through an activity-dependent manner (Shatz, 1990; Goodman and Shatz, 1993). During this process, axons from the thalamus contend for representation in the cortex. Should activity in this pathway be disrupted, the thalamocortical projections will be abnormally distributed. In the somatosensory system, projections from the ventral posterior nucleus of the thalamus to the primary somatosensory cortex are regulated by NMDA-dependent mechanisms (Crair and Malenka, 1995). Ad-

**Table 4. Results of linear regression models for thalamic NAA/Cho and FA values from white-matter fibre pathways relative to early skin breaks in neonates born extremely preterm**

	NAA/Cho values				FA values			
	Thalamus		Corpus callosum		Posterior white matter		Cingulum + fornix	
	$\beta$ values	$p$ values	$\beta$ values	$p$ values	$\beta$ values	$p$ values	$\beta$ values	$p$ values
Male sex	−0.012	0.262	−0.038	0.001 <sup>b</sup>	−0.041	<0.0001 <sup>b</sup>	−0.025	0.002
Birth GA	0.021	<0.0001 <sup>a</sup>	0	0.95	−0.005	0.392	−0.006	0.188
DOL at scan	0.003	<0.0001 <sup>a</sup>	0.001	<0.0001 <sup>b</sup>	0.001	<0.0001 <sup>b</sup>	0	0.202
Morphine dose	0	0.414	0	0.17 <sup>b</sup>	−0.0001	0.678	0	0.008
Midazolam dose	−0.001	0.408	−0.0001	0.89	0.0001	0.889	−0.001	0.299
Dexamethasone dose	−0.007	0.121	0.015	0.89	0.01	0.02	0.01	0.015
Days of mechanical ventilation	0	0.389	−0.001	0.006 <sup>b</sup>	−0.001	0.002 <sup>b</sup>	0.011	0.12
High early skin breaks	−0.057	<0.0001 <sup>a</sup>	0	<0.0001 <sup>b</sup>	0	0.005 <sup>b</sup>	0	0.01 <sup>b</sup>
High late skin breaks	−0.006	0.546	0.00001	0.937	0	0.408	0.00001	0.876

Estimated coefficients and  $p$  values for early skin-breaking procedures in relation to NAA/Cho (left) values and FA (right) values in extremely (24–28 weeks' gestation) preterm neonates.

<sup>a</sup>Statistically significant,  $p < 0.05$ .

<sup>b</sup>Statistically significant,  $p < 0.013e$ .

ditionally the NMDA-dependent mechanisms are only active early on in development and with age the ability to modify thalamocortical pathways fades (Crair and Malenka, 1995). Thus, these data indicate that activity in thalamocortical pathways is crucial for typical brain development. Early pain may therefore disrupt thalamocortical pathway development and underlie adverse cognitive and motor outcomes. Previous research has demonstrated regionally-specific alterations in white-matter fiber pathway maturation, including corpus callosum volume (Malavolti et al., 2017) and DTI measures of white matter microstructural development (Krishnan et al., 2007; Boardman et al., 2010; Chau et al., 2013; Duerden et al., 2015), and an association with adverse cognitive and motor outcome in the very preterm born population. In conjunction with the current findings, alterations in both thalamic maturation and thalamocortical projections appear to be key predictors of neurodevelopmental outcome in preterm born infants. Our findings also extend these observations made at 18 months of to 2 years age with neurodevelopmental outcomes assessments at 3 years of age.

Few studies have examined the association between alterations in gray matter and neonatal pain during the neonatal period (Smith et al., 2011; Brummelte et al., 2012). However, one study examined functional and structural cortical changes in a group of neonates exposed to NICU-related stress (Smith et al., 2011). Neonatal stress was associated with decreased brain width particularly impacting frontal and parietal lobes in preterms when assessed at term-equivalent age with structural MRI (Smith et al., 2011). In adults, painful dystonia was associated with regionally-specific alterations in lateral thalamic nuclei reflected in 5–7% decrease in volume (Südmeyer et al., 2012).

Long term follow-up studies of preterm-born children exposed to pain have examined pain reactivity and the association between exposure to noxious stimuli and brain morphometry. We found that extremely preterm low-birthweight neonates were reported as less reactive to painful stimuli based on parent report (R. V. Grunau et al., 1994b). Extremely preterm born children had a reduced ability to modulate pain compared with preterm children born at later gestational ages (Goffaux et al., 2008). Early exposure to tissue damage in the neonatal period may result in long-term structural plasticity in somatosensory and/or pain modulatory systems. Early skin breaks during the development of nociceptive and somatosensory systems may represent a mechanism by which preterm-born adults are predisposed to develop complex chronic pain conditions (Buskila et al., 2003; Kling-

mann et al., 2008; Low and Schweinhardt, 2012). Together with our findings of altered neonatal thalamic development, these data suggest that early pain is associated with long-term alterations in somatosensory processing and somatosensory system development.

A challenge for clinical studies examining the association between neonatal pain and brain development is the difficulty of distinguishing between direct effects of exposure to painful stimuli and the confounding clinical variables. Previous neuroimaging findings with the preterm population have identified associations among clinical care factors and regionally-specific alterations in brain development (Tam et al., 2011; Duerden et al., 2016; Zwicker et al., 2016). Our findings suggest an association between early invasive procedures and altered development of the thalamus and thalamocortical pathways particularly in extremely preterm neonates. In our analyses, we adjusted for demographic and clinical factors, including the age of the participants at the time of birth and scan reflecting a lengthier stay in the NICU, which may have influenced the development of the thalamus. After adjusting for these factors, the association of early pain and thalamic development was maintained. Clinical care factors can have significant impact on neonatal brain development, for instance preterm neonates exposed antenatally to steroids demonstrated a 10% decrease in volume of the cerebellum (Tam et al., 2011). Our results do not provide a causal link between early pain and brain development, however, this has been demonstrated in a controlled neonatal animal experiment (Anand et al., 2007). Rather our clinical findings suggest an association between pain and brain development that is consistent with experimental work and that should be replicated in future cohorts of preterm born neonates. Strengths of the current study included multimodal neuroimaging assessments at two time points, detailed day-by-day neonatal clinical data collection, and neurodevelopmental assessments in a rare cohort of extremely preterm and very preterm born neonates studied from birth until the third year of life.

Neonates treated in intensive care undergo life-saving invasive procedures. Those who participated in the study were all exposed to early and late invasive procedures making it challenging to examine thalamic growth in subsets of neonates who were not exposed to any early pain relative to neonates having received a high number of early invasive procedures. Therefore, defining a critical period when the thalamus may be most vulnerable to invasive procedures during development would be imprecise based on the results of this study; instead our findings suggest a window of enhanced vulnerability.

Our findings suggest a pathway by which early exposure to pain contributes to the neurodevelopmental consequence of extremely preterm birth. Future follow-up studies of preterm infants are needed to determine how the consequences of early pain can be mitigated by appropriate analgesia.

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