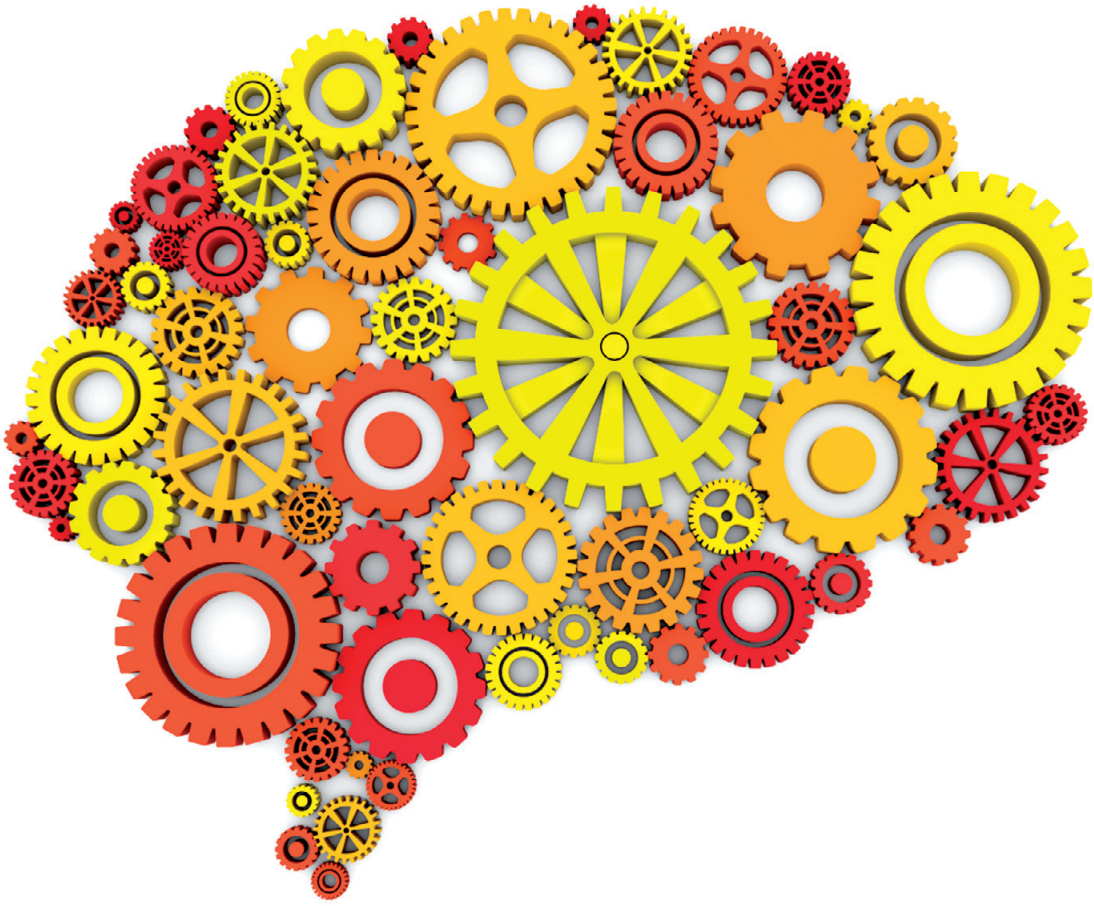

Projections of Pain

Neonatal pain in children, what remains in the brain after the wheels of time

Gerbrich E. van den Bosch

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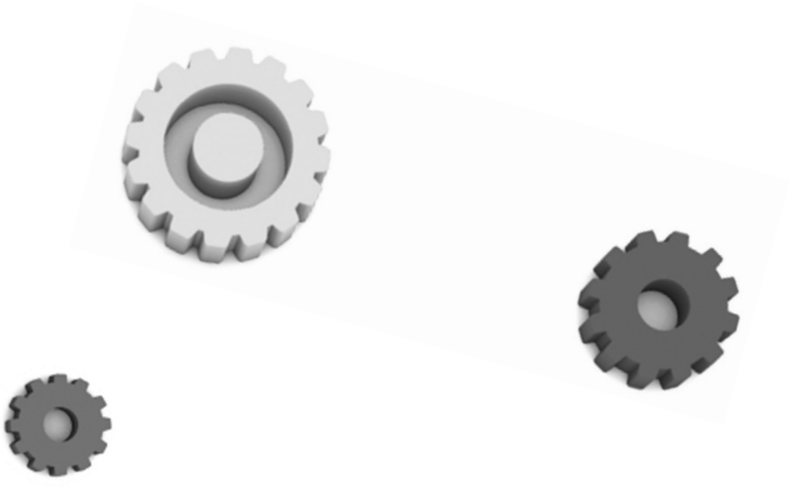

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Projections of Pain

Neonatal pain, what remains in the brain after the wheels of time

Projecties van pijn

Neonatale pijn, de gevolgen voor de hersenen op de lange termijn

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties.

De openbare verdediging zal plaatsvinden op
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Gerbrich Engelien van den Bosch
geboren te Leeuwarden

ERASMUS UNIVERSITEIT ROTTERDAM

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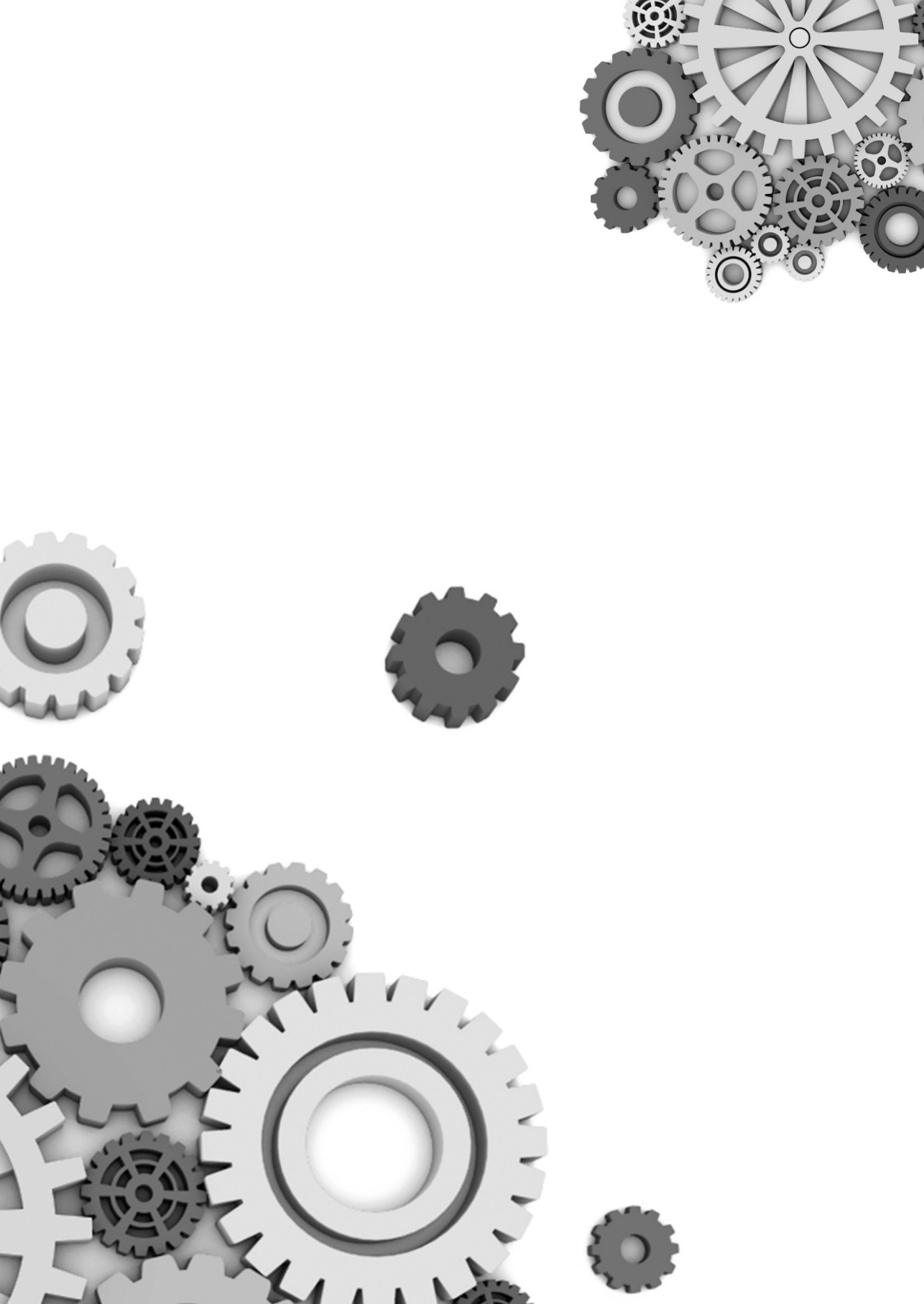

Chapter 1

General Introduction

FOREWORD

The International Association for the Study of Pain (IASP) has defined 'pain' as '*An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*' with the note that *'Pain is always subjective. Each individual learns the application of the word through experiences related to injury in early life'*.[1] Unfortunately some individuals experience pain from injuries in very early life, such as preterm born children, who will need to undergo painful procedures, and children who require major surgery shortly after birth. These children will therefore receive painkillers or anaesthetics in a life stage in which the brain is rapidly developing. There is ongoing concern about the potential negative effects of both early exposure to pain, analgesia with opioids and exposure to anaesthetics in infancy.[2-6]

HUMAN DATA

Worldwide, 9.6 per cent of the newborns are born before 37 weeks of gestation.[7] Prematurely born babies admitted to Neonatal Intensive Care Unit (NICU) will inevitably undergo painful procedures, with an estimated number of 10-14 per day, often without adequate pain treatment.[8-10] A follow-up study at our department among preterm born mechanically ventilated children found that morphine administration was significantly negatively correlated with one IQ subtest at the age of 5 years.[11] Interestingly, a positive correlation with respect to executive functioning was found in the same cohort at age 8 or 9 years.[12] Other studies among extremely preterm born children found a relation between exposure to neonatal pain and poorer corticospinal tract development[13] and reduced white matter fractional anisotropy and subcortical gray matter at term-equivalent age.[14] Former preterm born children showed differences in functional cortical brain activity,[15] and altered brain activation during pain at a later age.[16]

Moreover, approximately 5000 newborns are born with congenital anomalies each year in the Netherlands.[17,18] Many require immediate surgical correction resulting in exposure to the combination of pain-inducing tissue damage, anaesthesia and analgesic therapy. Exposure to anaesthetics in infancy is associated with an increased rate of learning disabilities, higher incidence of developmental and behavioral disorders and lower scores on academic achievement tests.[19-21] Other studies, however, found no differences in cognitive and educational outcome at age 12 after surgery and related exposure to anaesthetics.[22] With regard to pain sensitivity, surgery in the first months of life induced hyperalgesia to subsequent surgery, especially if the tissue damage was in the same area,[23] and stronger pain responses in infancy.[24]

EXPERIMENTAL FINDINGS

Pain

Early pain exposure has been associated with cell death in rat brains.[25] Moreover, neonatal inflammatory pain resulted in decreased baseline nociceptive sensitivity at adult age, and enhanced hyperalgesia after a subsequent inflammatory insult.[26,27] However, in animal models, the pain is often induced by chronic inflammation rather than by repeated painful procedures, which is more comparable to the human situation with procedural pain. A previous study from our group therefore exposed animals to repeated skin-breaking procedures and found that those pain stimuli induced acute hypersensitivity but did not affect basal nociceptive thresholds later in life.[28]

Opioids

Early opioid exposure in rodents was found associated with degeneration of red neurons in the brain[29] as well as apoptosis in brain regions associated with sensory and emotional memory functioning,[30] impaired cued fear extinction,[31] and impaired adult cognitive functioning.[32] While these negative effects occurred in the absence of pain, neuroprotective effects of opioid exposure in combination with pain experience are also observed, such as less neurological damage after preemptive morphine administration,[25] and significantly attenuated hypoalgesia and faster recovery after subsequent inflammatory pain.[33] Interestingly, preemptive morphine also ameliorated some of the negative long-term effects with respect to pain behavior resulting from exposure to neonatal inflammatory pain.[34]

Anaesthesia

Previous studies in rodents have reported neuronal cell death after blockade of N-methyl-D-aspartate (NMDA) glutamate receptors,[35,36] memory deficits and a decrease in neural stem cells after anaesthesia with the gamma-amino butyric acid (GABA) receptor agonist isoflurane.[37] Other rodent studies reported immediate neuroapoptosis, learning deficits, abnormal social behaviour,[38] and memory deficits[39] in adulthood after sevoflurane exposure early in life. Furthermore, a combination of widespread neuroapoptosis, deficits in hippocampal synaptic functioning and cognitive problems was observed after administration of a commonly used combination of midazolam, nitrous oxide and isoflurane.[40] Moreover neuronal cell death and apoptotic activity were significantly increased after exposure to desflurane, isoflurane, or sevoflurane.[41] Non-human primates developed apoptosis of neurons and oligodendrocytes after foetal and neonatal exposure to propofol, ketamine and isoflurane.[42-46]

Potential underlying mechanisms

The development of pain pathways extends into the neonatal period. Structural and functional fine-tuning of the nociceptive system and spinal circuit has been shown to be activity-dependent and could therefore be affected by noxious stimuli during the neonatal period.[28,47] With respect to opioids and anaesthetics, GABA, NMDA, and opioid receptors have a direct role in human neuronal development,[48] and this justifies the fear of negative effects of both types of drugs in humans. One of the theories for the underlying mechanisms of neurotoxicity holds neuronal inactivity induced by the drug responsible, since excess cells are removed by apoptosis and neuronal survival is based on activity.[48] Activity of the GABA receptor induces neuronal inactivity in line with this hypothesis. A hypothesis with regard to the NMDA receptor has it that the anaesthetic-induced NMDA blockade produces an acute upregulation of the NMDA receptor and that excitotoxic neurotoxicity occurs when administration of a NMDA receptor blocker is stopped.[48] Whether pain, opioids and anaesthetics induce negative alterations with respect to pain sensitivity, brain functioning and brain morphology in humans as well is an important but largely unstudied topic.

OVERALL RESEARCH QUESTION

Can we find projections of pain, exposure to opioids and anaesthetics later in life? Or do negative effects not remain in the brain after the wheels of time have run their course?

GENERAL OBJECTIVES

The studies presented in this thesis address the following research questions:

Part I - FMRI and pain studies: methods and feasibility

- Is quantitative sensory testing feasible in children and which tests are to be preferred?
- Is it beneficial to employ individualized stimuli in pain studies using fMRI or do standardized stimuli induce the same brain activation patterns?
- Are fMRI studies without sedation feasible in children?
- Are there developmental differences in functional connectivity associated with working memory in healthy children?

Part II - Long-term consequences of early pain and opioid exposure

- Do early pain experiences, exposure to opioids and/or exposure to anaesthetics during neonatal life induce alterations in thermal detection and pain perception, brain functioning during pain, brain morphology, neuropsychological functioning or the incidence of chronic pain later in life?
- Does a child with a sensory neuropathy have disturbed detection- and pain threshold and alterations in brain activation during pain?

GENERAL DESIGN

To answer the research questions of part II, we evaluated five models in which exposure to pain, opioids and anaesthetics were studied at different points along the continuum from no pain to intense pain and from no opioid exposure to very high opioid exposure.

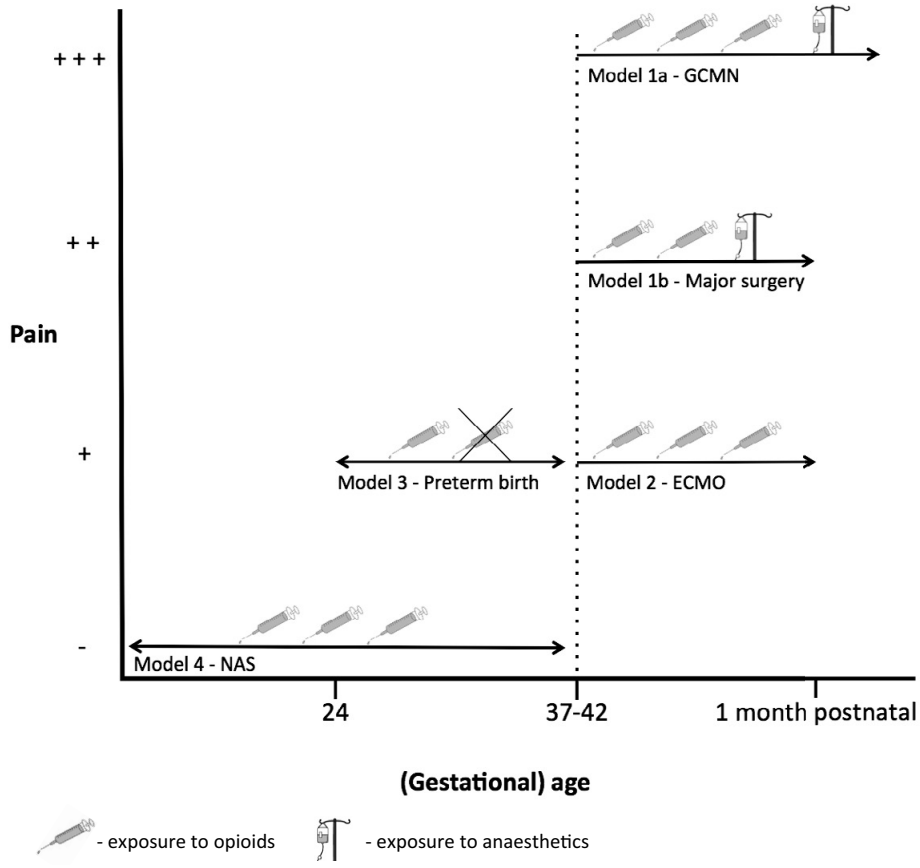

Figure 1 - Study models

Models 1a and 1b in the figure also involve exposure to anaesthetics. The cohorts included in the different studies were specifically chosen and also encompassed two groups of children who at neonatal age had participated in two randomized controlled trials performed in our department – with the advantage that all the neonatal characteristics were available (model 1b and model 3).[49-51] The figure presents the different models studied (*GCMN-giant congenital melanocytic naevus, ECMO-extracorporeal membrane oxygenation, NAS-neonatal abstinence syndrome*).

OUTLINE OF THIS THESIS

This thesis is in two parts. **Part I** focuses on the methodology for pain studies and fMRI studies in children. We evaluated whether standardized pain stimuli gave the same results of brain activation compared to the golden standard of individualized stimuli in **chapter 2**. A standardized protocol for quantitative sensory testing, including reference values for children, is presented in **chapter 3**. **Chapter 4** shows that children enjoyed participation in a pain related fMRI study and were not scared in general. In **chapter 5** we present a paradigm for the measurement of brain connectivity during working memory and an overview of changes in working memory during development.

Part II evaluates the long-term effects of early exposure to pain, opioids and anaesthesia by describing five models. The consequences of the combination of pain induced by tissue damage, opioid exposure and general anaesthesia are described in **chapters 6 and 7** (models 1a and 1b). The effects of prolonged continuous opioid and sedative exposure in the absence of severe pain are discussed in **chapter 8** (model 2). **Chapter 9** (model 3) deals with the long-terms effects of procedural pain in combination with low doses of opioids in former preterm born children. In **chapter 10** we present the long-term effects of the last model regarding exposure to high doses op opioid related substances such as heroin and methadone in the absence of pain. **Chapter 11** presents a case study of a child with hereditary sensory and autonomic neuropathy.

In **chapter 12** the main findings of this thesis are discussed in a broader perspective. Moreover, suggestions for future studies are presented as well as a summary of our findings in **chapter 13**.

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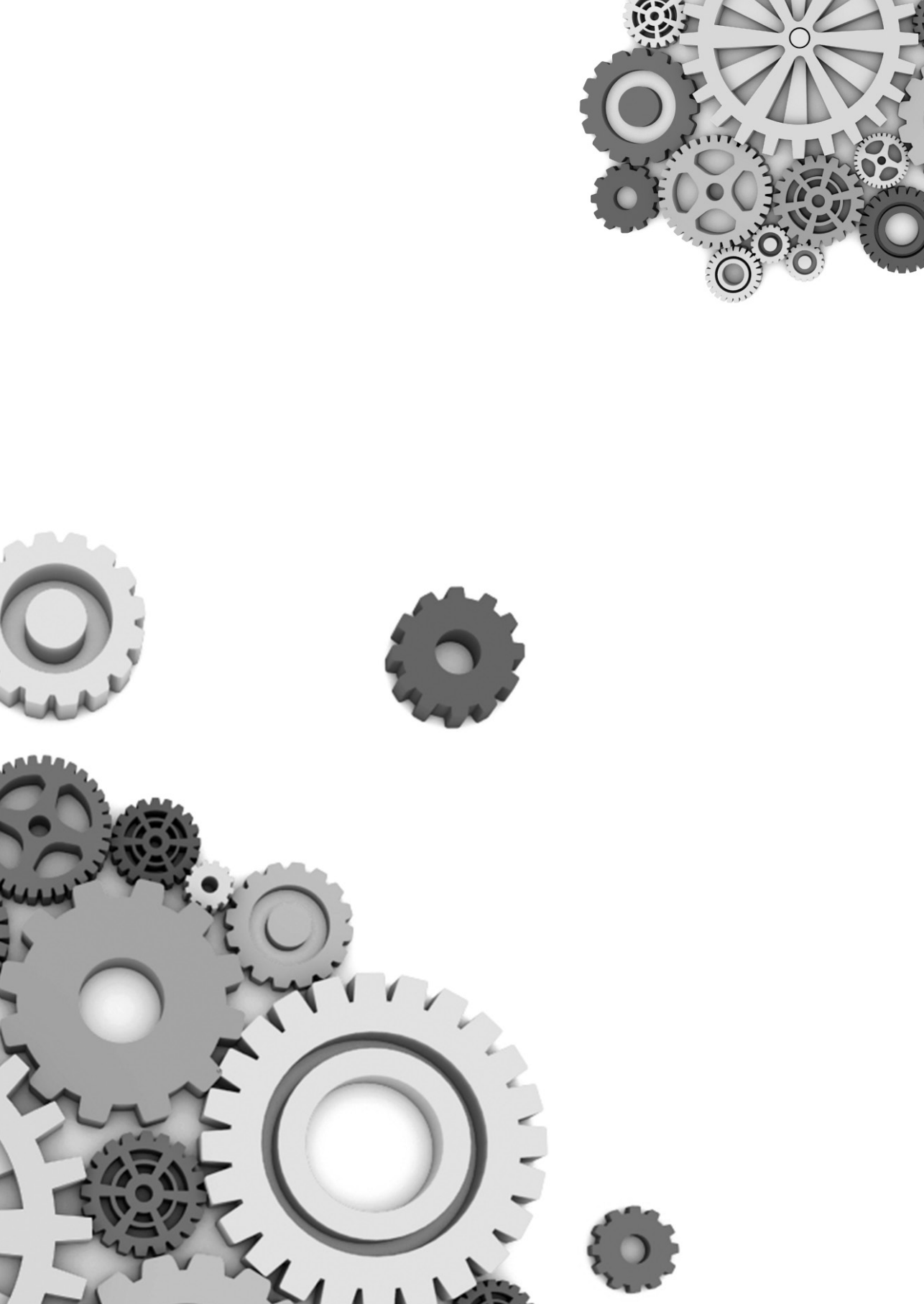
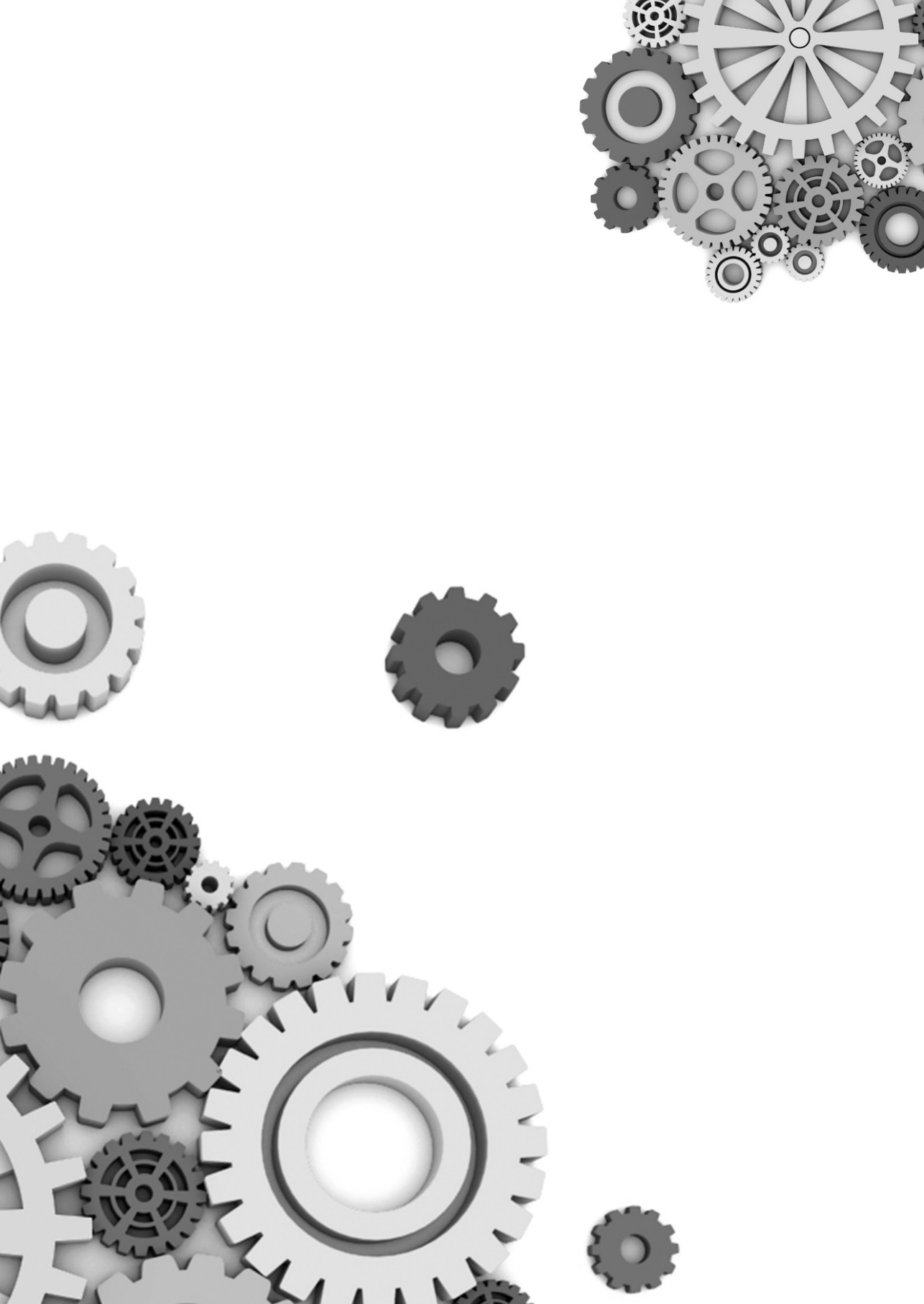


Part 1

FMRI and pain studies

Methods and feasibility



Chapter 2

Standard and individually determined thermal pain stimuli induce similar brain activations

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Dick Tibboel, Jeroen W.B. Peters, Jos N. van der Geest

* contributed equally

European Journal of Pain 2013; Oct 17(9):1307-15

ABSTRACT

Background Several functional magnetic resonance imaging (fMRI) studies use thermal pain stimuli to determine brain activation patterns during pain. Studies use either a standard temperature condition for all participants or an individualized temperature condition based on the individually determined pain threshold of the participant. The aim of the present study was to compare both conditions in the same participants.

Methods Eighteen healthy participants (21 - 29 years) underwent four fMRI runs, in each of which they received three types of thermal stimuli: neutral (32°C), warm (37°C) and painfully hot. In two runs the painfully hot stimulus was set at a standard temperature of 46°C; in the other two runs the temperature was set at the subject's individual pain threshold (46 - 48°C). FMRI (blood oxygen level dependent) was performed on a 1.5T MR scanner (GE Signa). Pre-processing and statistical analyses were performed using Statistical Parametric Mapping (SPM8) software.

Results While the stimulation temperatures were lower in the standard temperature condition, both conditions activated the same brain regions. When comparing the conditions directly to each other, we did not find significantly different grey matter activation patterns.

Conclusions The similar activation patterns between the two conditions suggest that it is not necessary to use individualized stimuli per se. The temperature of 46°C appeared to be an adequate temperature for standardized stimulation to observe significant brain activations related to thermal pain.

INTRODUCTION

Pain processing in the human brain is thought to involve several brain regions, including the insula, thalamus, primary and secondary somatosensory cortices (S1 and S2), anterior cingulate cortex (ACC) and the motor areas.[1-3] These regions have been identified using functional magnetic resonance imaging (fMRI) or positron emission tomography (PET). However, different studies have found different patterns of brain activation during pain. For instance, Bucher and colleagues[4] describe activation in the insula and thalamus, while Hoffman and colleagues[5] describe additional activation in the S1, S2, and ACC. Activation in the frontoparietal cortex has also been reported.[6] These differences may be related to the different types of stimulation used to induce pain. Most studies used thermal stimulation to induce pain, however, laser-light, electricity, and mechanical pressure have also been applied. Moreover, differences occur even when using a similar technique, which might be related to variations in stimulation methods.

Here we focus on brain activations related to thermal pain induced by heat stimuli for which also different activation patterns have been reported. Davis et al.[7] showed that painful heat stimuli activated the thalamus, insula and S2, while Disbrow et al.[8] did not find these cortical brain activations in response to painful heat stimuli. This heterogeneity between reported activation patterns related to thermal pain might arise from various methodological differences in, for instance, MR machines, scanning parameters (i.e. 1.5 Tesla versus 3.0 Tesla), the various dermatomes that are stimulated (i.e. foot, face, thenar eminence of the hand, dorsal eminence of the hand), differences in duration of pain stimulation, and differences in the types of analyses that are performed (i.e., voxel-based versus region of interest analyses (ROI)). Peltz et al.[9] for instance, primarily focused on the insula, whereas Helmchen et al.[10] specifically looked at the cerebellum.

Another important factor that may have influenced the heterogeneity in pain-induced activation is the stimulus temperature that was used to induce pain. Some fMRI studies used a fixed, or standardized temperature for all subjects,[7,10-15] whereas others used a individualized stimulation temperature adjusted for every subject.[5,8,9,16-22] It can be argued that standardization could lead to differences in the pain experience between subjects, because the temperatures do not match individual pain thresholds. Therefore, adjusting the temperature to meet these individual thresholds might yield a more homogeneous pain experience across subjects, which in turn, could lead to more reliable activation patterns. However, the individualized condition has several disadvantages. For instance, it requires the determination of the individual pain threshold in each subject. This is often very difficult or unfeasible in young children and in individuals with problems expressing themselves. Interestingly, the question whether individual pain thresholds are necessary

to induce significant pain-related activation in the brain, has not been addressed in the literature.

Thus, the aim of the present study was to determine whether it is beneficial to employ individualized stimuli in pain studies using fMRI. Thereto, we compare pain-related activation patterns induced by standard stimuli to individually determined stimuli within the same subjects. We hypothesize that activation differences between the two stimulation conditions will be small.

MATERIALS AND METHODS

Participants

Twenty healthy subjects (10 females) between 19 and 33 years old were recruited for the study. Participants were students from the Erasmus University in Rotterdam. None of the participants used drugs related to pain suppression or had any contraindications for participation in an MRI study. The study was performed at the Erasmus MC in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board at the Erasmus MC. Informed consent was obtained from each subject prior to participation.

Materials

Thermal stimulation

Individual pain thresholds were determined and thermal stimuli were applied with the MRI-compatible, computer-controlled Thermal Sensory Analyzer (TSA type II, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel). The Peltier-based contact thermode (30 x 30 mm) was placed at the ball of the thumb of the right hand of the participant.

Numerical rating scale

Verbal numerical rating scales (NRS) were used to collect information about the intensity and the unpleasantness of the thermal stimuli. Participants were asked two questions in Dutch, 'How much pain did you experience?' and 'How unpleasant was the pain stimulus?' They were asked to provide a number between 0 (no pain at all / not unpleasant at all) and 10 (worst imaginable pain/extremely unpleasant).

Image acquisition

The MRI images were acquired using a 1.5T MRI scanner with an 8-channel head coil (Signa CV/I; GE Healthcare, Milwaukee WI, USA) located at the Department of Radiology

in the Erasmus MC, Rotterdam, the Netherlands. Cushions were used to comfortably support the participant's head in order to minimize head motion. Participants wore an MRI-compatible headphone to reduce the scanner noise and to enable communication.

For anatomical reference, a high-resolution three-deimensional inversion recovery (IR) fast-spoiled gradient echo (FSPGR) T1-weighted image was acquired (parameters: TR/TE/TI 9.9/2.0/400 ms; flip angle 20°; 320 x 224 matrix with a field-of-view of 240 x 240 mm^2; 86 slices; 1.6 mm slice thickness with no gap; ASSET factor 2; acquisition time 3 min and 10s). For the four functional scans single-shot gradient-echo echo-planar imaging (EPI) $T2^*$-weighted sequences in transverse orientation sensitive to blood oxygen level dependent (BOLD) contrast were used (parameters: TR/TE 3000/40 ms, flip angle 60°, 96 x 96 matrix with a field-of-view of 260 x 260 mm^2; 5 mm slice thickness with 1 mm gap, 22 slices and voxel sizes of 2.7 x 2.7 x 5 mm^3). The acquisition time for 136 volumes was 7 min and 3 seconds per run, including 15 seconds of initial dummy scans that were discarded.

Procedure

Examination of the individual pain thresholds

After the anatomical MRI scan was performed, the individual pain thresholds were measured using the TSA while the participants were lying in the MRI scanner with their eyes closed using the method of levels (MLE). No MRI acquisition was obtained during this period.

The thermal stimuli were presented in a series set by the computer. In each trial the baseline temperature of the thermode was 32°C. From this baseline the temperature increased at a rate of 2°C/s to the target temperature and returned back to baseline immediately. The target temperature of the first trial was 35°C, so the temperature step size between baseline and target stimulation was 3°C. Following the thermal stimulus the researcher asked whether the participant perceived the target temperature as painful or not. If the participant experienced no pain, the target temperature would be increased and if the participant did experience pain, the target temperature would be decreased. The temperature step size was halved every time the participant experienced pain. This was repeated until the step size was decreased to 0.5°C. The lowest temperature that was perceived as fairly painful (rounded up to half or whole degrees) was the pain threshold of that subject and was used as the individualized stimulation temperature in the MRI examinations.

After determination of the individual pain threshold outside the scanner, we tested the threshold temperature while the participant was still lying in the scanner to see if the

stimulation could be tolerated for 21 seconds, and whether it was painful enough. If needed, the temperature was adjusted and tested again until the participant tolerated the pain for 21 seconds and rated the stimulation temperature with an NRS of 6 or higher.

The maximum temperature for individualized stimulation was 48°C because the TSA cannot consistently maintain higher temperatures for a longer period of time. In case the individual pain threshold of a subject was higher than 48°C, the participant received a tonic stimulation for 21 seconds at 48°C and had to rate the pain intensity and pain unpleasantness using the numerical rating scales (NRS). If the subjects reported a score of 6 or lower, he or she was excluded from the study.

Functional MRI examination

After the individualized stimulation temperature was determined, the fMRI experiment was performed. During the four functional scans the participants were asked to keep their eyes closed. After each functional scan the participant was asked to rate the pain intensity and unpleasantness using the NRS.

Each functional scan consisted of a block design in which the participants received three types of thermal stimulation; four blocks of warm (37°C) and four blocks of painfully hot temperatures were alternated pseudo-randomly with nine baseline blocks of a neutral temperature of 32°C (Figure 1). Each scan started and ended with a baseline block. The warm and painfully hot stimulation blocks lasted 21 seconds each. In order to prevent anticipation to the stimulation, the baseline blocks lasted either short (24 seconds) or long (30 seconds).

In the standardized condition the painfully hot stimulation temperature was 46°C. In the individualized condition the stimulation temperature was set to the subject's individual pain threshold. Both conditions (standardized and individualized) were performed twice in alternation in four separate scans. Ten subjects started with the individualized con-

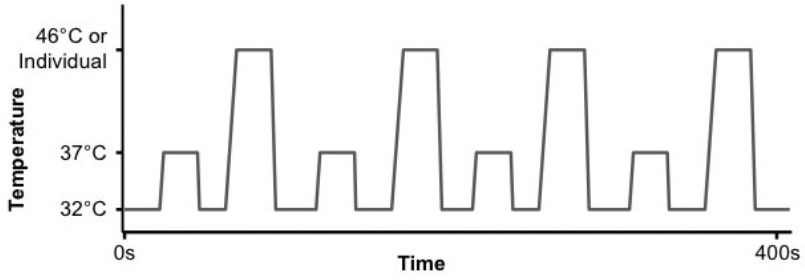

Figure 1 - Block design

dition, while the other ten started with the standardized condition. The subjects were blinded to the order of the conditions.

Statistical analysis

Stimulation temperatures and NRS scores

For each subject, the pain intensity and unpleasantness NRS scores were averaged over the two runs for each of the two conditions (standardized and individualized). A related-samples Wilcoxon signed-rank test was used to investigate whether these scores differed between the two conditions. For each subject, we also calculated the difference between the mean pain intensity scores, as well as the differences in the stimulation temperatures between the individualized and standardized condition. A Spearman rank correlation for these values was calculated (two-tailed). A Mann-Whitney test for two independent samples was performed to investigate whether the individual pain threshold temperatures differed between men and women.

Functional imaging analysis

All functional images were analyzed using the Statistical Parametric Mapping software (SPM8, Welcome Department of Cognitive Neurology, University College, London, UK). The anatomical scans were segmented into maps for white matter and grey matter. Normalization into Montreal Neurological Institute (MNI) space was performed with parameters obtained during segmentation. The normalized anatomical data had an isotropic resolution of 1 mm3. Functional scans were realigned, co-registered to the grey matter map, normalized with parameters obtained during segmentation and finally re-sliced into 2 mm^3 isotropic voxels. Subsequently, the images were spatially smoothed with a Gaussian kernel of 8 mm^3 FWHM (full width at half maximum).[23]

Single-subject statistical analysis was performed with the general linear model. The fMRI time-series were modeled as a series of event blocks convolved with a canonical hemodynamic response function. The event blocks were derived from the two levels of stimulation (warm and painfully hot) for each of the two conditions (standardized and individualized); movement parameters were included as regressors of no interest. The model was estimated with a high-pass filter with a cut-off period of 128 seconds.

Individual contrast maps were calculated for the contrast between painfully hot and warm blocks for each of the two conditions, which were used in the second level, random effects analyses. Firstly, whole brain group results for standardized hot stimulation versus warm stimulation and individualized hot stimulation versus warm stimulation were evaluated separately using a statistical threshold of $p < 0.001$ (uncorrected)

and a minimum cluster size of 20 voxels. Secondly, the comparison between the two stimulation conditions (standardized and individualized hot stimulation, corrected for warm stimulation to avoid potential confounding effects of stimulation per se) were contrasted using a paired t-test with a statistical threshold of $p < 0.001$ (uncorrected) and a minimum cluster size of 20 voxels. Additionally, we compared the two conditions using a family-wise error (FWE) correction for multiple testing ($p < 0.05$). Anatomical structures were defined with the Talairach Deamon Labels atlas of the WFU PickAtlas[24] in AAL (Anatomical Automatic Labeling).[25]

RESULTS

Study Population

From a total of 20 participants who completed scanning, one male participant was excluded due to morphological brain anomalies and one male was excluded due to a pain score of zero during scanning for the standardized condition, indicating that this subject did not experience pain. The 18 remaining participants (ten females) were between 21 and 29 years of age (22.9 ± 2.4 SD). Nine subjects started with the individualized condition and nine subjects started with the standardized condition. The mean individualized stimulus temperature was 47.56°C (± 0.64), and did not significantly differ between the male and female subjects ($p = 0.237$).

Pain intensity and unpleasantness scores

The mean NRS pain intensity score averaged over the two repetitions was lower in the standardized condition (4.3 ± 2.0) than in the individualized condition (7.2 ± 1.3; $p < 0.001$). Also, the mean NRS unpleasantness score was lower in the standardized condition (3.0 ± 2.5) than in the individualized condition (6.5 ± 1.7; $p < 0.001$). On average, the subjects perceived the standard stimulation temperature as mildly painful. The Spearman rank correlation of 0.574 between the differences in stimulation temperature and the differences in NRS pain intensity scores between the two conditions was significant ($p = 0.013$).

Figure 2 represents the pain intensity scores per subject during the standardized and individualized conditions. The stimulation temperatures as well as the pain intensity scores were always higher during the individualized condition, except for one subject for whom the individualized stimulation temperature was also 46°C (Figure 2).

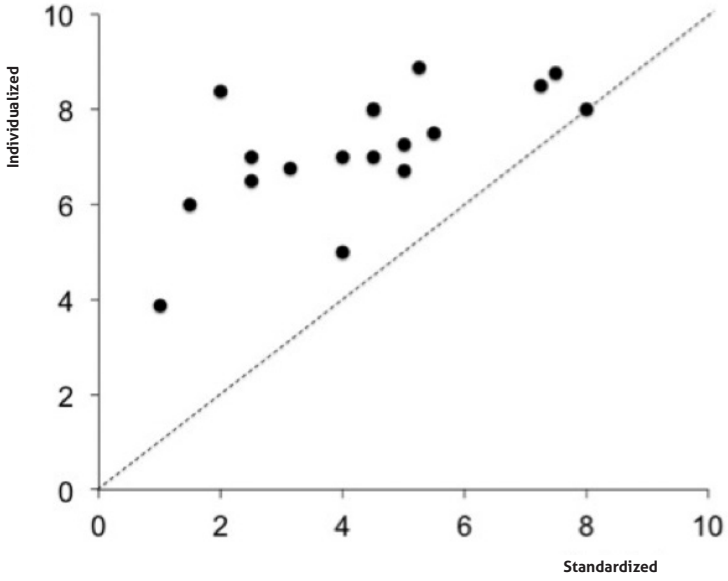

Figure 2 - Pain intensity scores
Each dot represents the NRS pain intensity score in the individualized condition versus the standardized condition for one subject, averaged over the two runs. Two subjects had exactly the same scores (point at 4.5, 8).

Imaging results

Activation during the standardized and individualized condition separately.
Standardized painfully hot stimulation corrected for warm stimulation induced activation in several brain areas, including areas in the frontal and parietal lobes in both hemispheres (Table 1, Figure 3).

The subtraction image of individualized painfully hot stimulation minus warm stimulation also induced activation in several brain areas including areas in the frontal and parietal lobe (Table 2, Figure 4). We found fewer clusters in the individualized in comparison with the standardized condition. However, the cluster sizes were larger in the former.

The direct comparison of the individualized condition to the standardized condition revealed one cluster (cluster size 181 voxels, T-value 5.05), which was, however, localized to white matter in the corpus callosum and ventricular region (MNI coordinates −2, −32, 13) (Figure 5). The direct comparison of the standardized condition to the individualized condition yielded no activations of clusters larger than 20 voxels. The direct comparisons with FWE correction yielded no significant differences between the two conditions.

Table 1 - Standardized hot stimulation

Clustersize (voxels)	T-value	MNI coordinates (mm)			Anatomical area	Side	No of voxels (*)
		X	Y	Z			
640	6.58	−60	−46	43	Parietal inferior lobe	L	329
					Supra Marginal	L	130
581	6.94	36	56	−11	Frontal mid. orbital lobe	R	363
					Frontal mid. lobe	R	125
					Frontal inf. orbital lobe	R	67
423	5.77	42	44	23	Frontal mid. lobe	R	390
					Frontal inferior tri	R	26
288	4.96	44	−54	57	Parietal inferior lobe	R	188
					Parietal superior lobe	R	51
					Angular	R	49
221	4.88	38	14	9	Insula	R	120
					Frontal inf. operculum	R	42
					Frontal inf. orbital lobe	R	22
185	6.59	−34	44	5	Frontal mid. lobe	L	111
					Frontal inferior tri	L	53
141	6.49	−42	4	−11	Insula	L	37
					Temporal superior lobe	L	33
					Temporal pole superior	L	25

Areas of activation (standardized hot > warm) with cluster size, T-values of the local maximum, Montreal Neurological Institute (MNI) coordinates, the anatomical areas within a cluster and the number of voxels within the cluster. All areas were thresholded at P < 0.001 (uncorrected) with a minimum cluster size of 20 voxels. (L: left hemisphere, R: right hemisphere). (*) The anatomically unassigned areas for each cluster are not listed in the table.

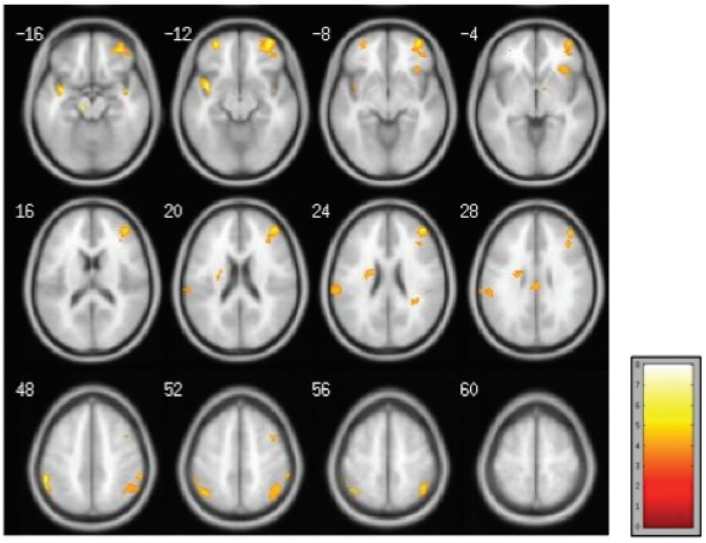

Figure 3 - Standardized hot stimulation
Twelve axial slices showing areas of activation during standardized painful hot stimulation versus warm stimulation. All areas were thresholded at P < 0.001 (uncorrected) with a minimum cluster size of 20 voxels.

Table 2 - Individualized hot stimulation

Clustersize (voxels)	T-value	MNI coordinates (mm)			Anatomical area	Side	No of voxels (*)
		X	Y	Z			
7918	8.64	−2	−36	13	Frontal mid. lobe	R	1190
					Insula	R	727
					Cingulum mid.	R	413
					Thalamus	L	351
					Cingulum mid.	L	278
					Frontal inf. operculum	R	248
					Cingulum anterior	L	236
					Thalamus	R	199
					Supp. motor area	L	186
					Rolandic operculum	R	172
					Caudate	L	157
					Frontal inferior tri	R	152
					Supp. motor area	R	144
					Caudate	R	133
					Temporal pole sup. lobe	R	132
					Cingulum anterior	R	130
					Frontal mid. orbital lobe	R	110
					Frontal sup. medial lobe	L	108
					Frontal inf. orbital lobe	R	94
					Putamen	R	76
					Pallidum	R	76
					Frontal sup. medial lobe	R	66
					Frontal superior lobe	R	64
					Cingulum posterior	L	54
1485	8.74	−36	10	7	Insula	L	534
					Putamen	L	309
					Temporal superior lobe	L	148
					Temporal pole sup. lobe	L	88
					Rolandic operculum	L	56
					Pallidum	L	55
					Amygdala	L	31
					Frontal inf. operculum	L	24
471	5.37	52	−46	51	Parietal inferior lobe	R	300
					Supra marginal	R	97
					Angular	R	55
450	4.99	−30	56	21	Frontal mid. lobe	L	412
					Frontal superior lobe	L	22
369	6.26	−32	−68	−35	Cerebellum (Crus 1)	L	240
					Cerebellum (Crus 2)	L	101

Areas of activation (individualized hot > warm) with cluster size, T-values of the local maximum, Montreal Neurological Institute (MNI) coordinates, the anatomical areas within a cluster and the number of voxels within the cluster. All areas were thresholded at P < 0.001 (uncorrected) with a minimum cluster size of 20 voxels. (L: left hemisphere, R: right hemisphere). (*) The anatomically unassigned areas for each cluster are not listed in the table.

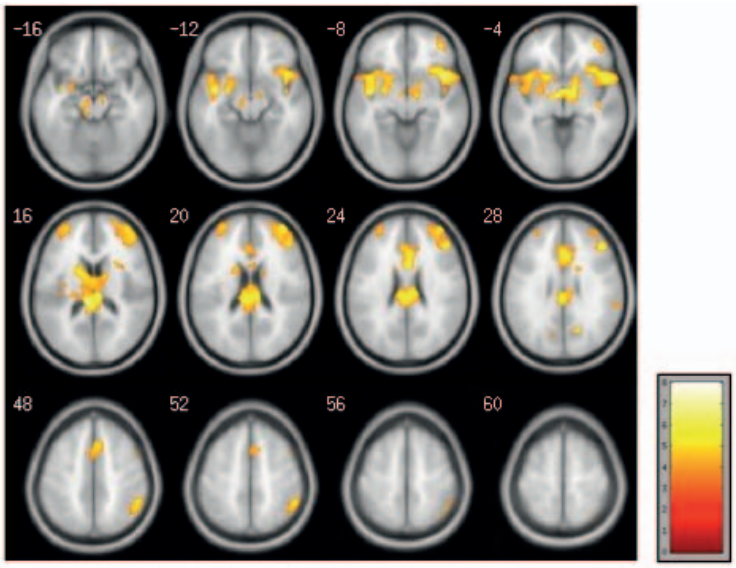

Figure 4 - Individualized hot stimulation
Twelve axial slices showing areas of activation during individualized painful hot stimulation versus warm stimulation. All areas were thresholded at P < 0.001 (uncorrected) with a minimum cluster size of 20 voxels.

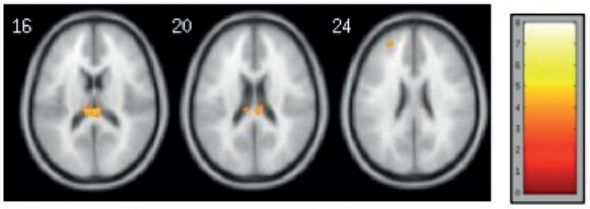

Figure 5 - Direct comparison
Three axial slices showing minimal areas of activation comparing individualized versus standardized hot stimulation (corrected for warm stimulation). All areas were thresholded at P < 0.001 (uncorrected) with a minimum cluster size of 20 voxels.

DISCUSSION

We compared two conditions of thermal stimulation in this neuroimaging study in healthy subjects. We demonstrated that both individualized and standardized hot thermal stimuli activate the same brain regions. While the stimulus temperatures and pain intensity scores were significantly higher in the individualized condition, we found significant activations in the insula, and in areas of the frontal, temporal and parietal lobes in both the individualized and standardized pain conditions. When directly comparing the individualized condition with the standardized condition, only one cluster consisting

within the white matter was significantly more activated during the individualized condition compared to the standardized condition. This finding was obtained using a rather liberal statistical threshold for differences ($p < 0.001$, uncorrected). These results suggest that both standardized and individualized stimulation temperatures are adequate stimuli to induce significant pain-related activation patterns in the brain.

Both individualized and standardized pain stimuli are commonly used in fMRI studies. To our knowledge no other studies have compared pain-related activation patterns induced by standard or individually determined pain stimuli within the same subjects.

The individualized condition has been considered the gold standard in pain studies. This is because the stimulation temperature is matched to the individual pain threshold, which may vary between participants. We found activation in the insula, thalamus, cerebellum and the frontal, temporal and parietal lobes in the individualized condition. Besides grey matter activation, we also found some white matter activation in the individualized condition. Although we included movement parameters as regressors of no interest, this is unlikely to capture all sudden movement, which may have occurred after stimulation onset, and affect the activation patterns. Previous studies using individualized stimulation temperatures have found activation patterns in similar brain regions as reported here, although it is difficult to directly compare results due to differences in experimental design and analyses methods (i.e., ROI analyses versus voxel-based). Most groups have observed activation in the insula,[5,9,18,19] which is regarded as the hallmark of effective pain stimulation in imaging studies.[2] In addition, activation in the anterior cingulate cortex (ACC) is also commonly observed with individualized stimulation.[9,17,18] The individualized condition has, however, several disadvantages. Since pain is very subjective in general, it could well be that different subjects may rate an equally experienced level of pain at a different level. Besides, a pain threshold temperature that is determined using brief exposure can be experienced quite differently when given as a 21 seconds long sustained stimulus. Moreover, subjects who have problems expressing themselves may also have problems providing a specific rating. For instance, young children and intellectually disabled subjects have difficulties providing reliable and accurate pain levels. Furthermore, determining individualized thresholds is more time-consuming as it requires assessment of the pain thresholds prior to the fMRI experiment.

Using a standardized temperature for all subjects circumvents these problems. In our study we found activation in the insula and the frontal, parietal and temporal lobes, similar to the individualized condition. Although it is again difficult to compare results due to differences in design and analysis methods, other studies using a standardized temperature reported activation patterns in the same brain regions.[7,11-14] For instance,

Becerra et al.[11] reported activation in the frontal gyrus, anterior and posterior cingulate gyrus, thalamus, motor cortex, S1, S2, SMA, insula, and cerebellum using 46°C as the stimulus temperature that was applied for 29 seconds.
One problem is how to choose a standardized stimulation temperature. In our study we opted for 46°C, based on the previous fMRI studies using a standardized stimulation temperature[7,11-15] and on reference values of thermal heat pain thresholds in healthy participants which were lower than 46°C.[26,27] When using the standardized condition, it is very important to collect pain intensity scores. Subjects who experience no pain during the fMRI scan can be excluded based on this score. We excluded only 1 subject out of 19 due to very low pain intensity scores, therefore we assume that 46°C is an adequate stimulation temperature.

The direct comparison of the individualized and standardized condition failed to show significant statistical differences in activation pattern. This seems to be incongruent with the differences in pain rating scores for the two conditions. When we analysed the two conditions separately, the activation patterns seem to be different. It has to be noted that subjects experienced pain in both conditions. Differences in activation patterns can therefore be quite subtle and it might well be that the BOLD signal is simply not sensitive enough to pick up the apparent differences in activation patterns between the two conditions. Newer scanners with higher field strengths might be able to overcome this issue in the future.

In summary, our study suggests that it is not necessary to use individualized thermal pain stimuli in imaging studies on pain processing. In most settings it might be even beneficial to use the standardized condition, for instance, in protocols involving young children or adults who have problems with expressing themselves; it is often difficult to determine accurate pain thresholds in these study populations. We have two recommendations for future studies that wish to implement a standardized stimulation condition in an imaging setting. Firstly, it is wise to adapt your standardized stimulation temperature to the reference values for the pain thresholds of your study population. These values might vary considerably.[26,27] Secondly, it is important to measure pain intensity and unpleasantness scores. In this way, subjects with too low pain intensity scores can be excluded, who otherwise could have contaminated the results.

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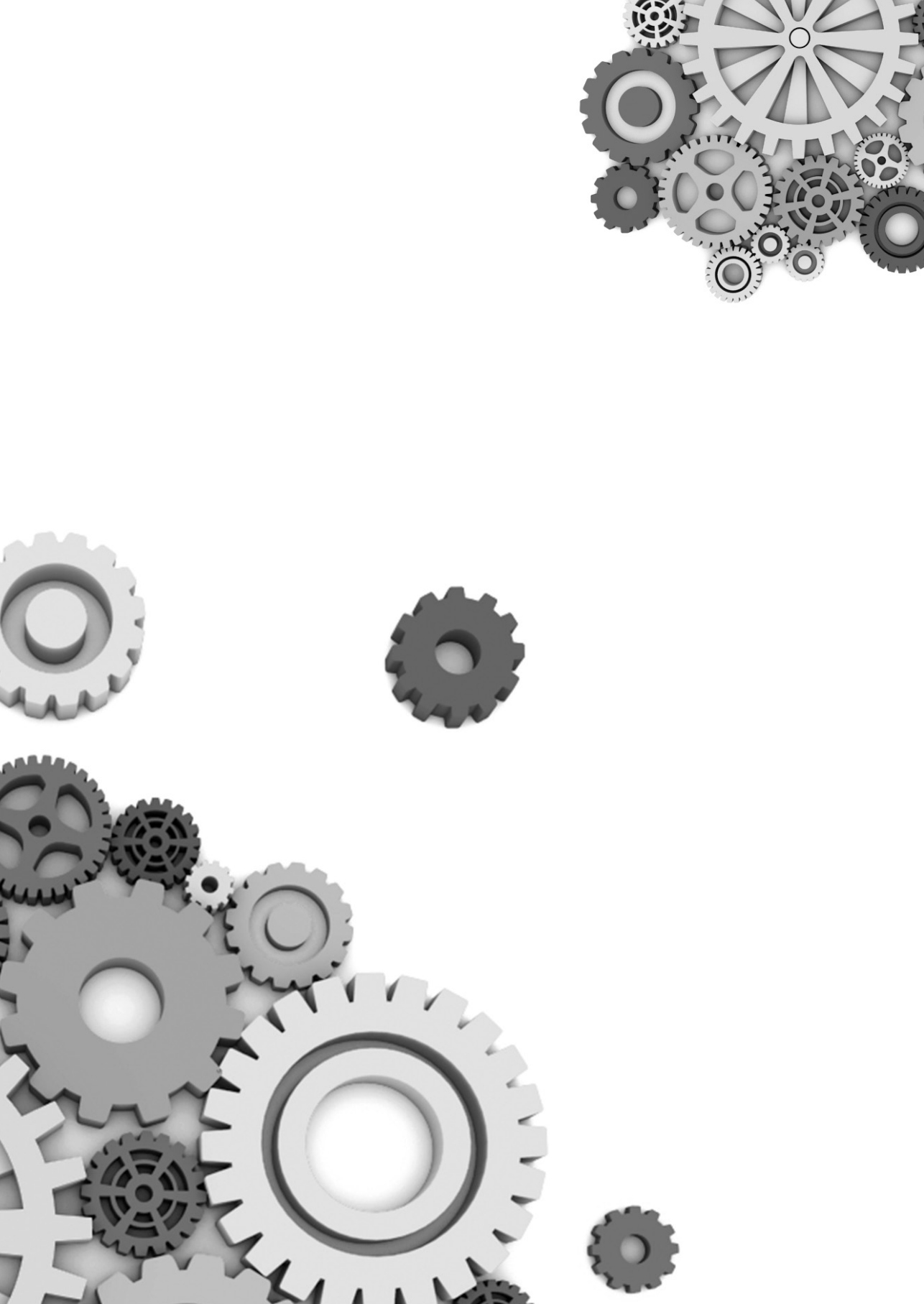

Chapter 3

Thermal Quantitative Sensory Testing in healthy Dutch children and adolescents

Standardized test paradigm and Dutch reference values

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Submitted for publication

ABSTRACT

Background Quantitative sensory testing (QST) is often used to measure children's and adults' detection- and pain thresholds in a quantitative manner. In children especially the Thermal Sensory Analyzer (TSA-II) is often applied to determine thermal detection and pain thresholds. As comparisons between studies are hampered by the different testing protocols used, we aimed to present a standard protocol and up-to-date reference values for thermal detection- and pain thresholds in children.

Methods Our standard testing protocol includes reaction time dependent and independent tests and takes about 14-18 minutes to complete. Reference values were obtained from a sample of 69 healthy term born children and adolescents with a median age of 11.2 years (range 8.2 to 17.9 years old). Twenty-eight males and 41 females were successfully tested and possible age and gender differences were studied.

Results This study provides Dutch reference values and presents a standard quantitative sensory testing protocol for children with an age from eight years onwards. This protocol appeared to be feasible since only two out of 71 participants were not able to reliably complete the protocol. We found some significant age and gender differences: females were statistically significantly more sensitive for both cold and heat pain compared to males, and the youngest children (8-9 years old) were less sensitive to detect a warm stimulus but more sensitive to heat pain in comparison to older participants.

Conclusions We present a feasible thermal quantitative sensory testing protocol for children and up-to-date reference values that are easy to interpret and may serve as normative values for future studies.

BACKGROUND

Quantitative Sensory Testing (QST) encompasses a group of assessments with the goal to systematically document the functioning of the sensory nervous system, and in particular, the nociceptive system. The advantage of QST in comparison with a classical neurological examination is its quantitative nature. Furthermore, depending on the type of stimuli, both large myelinated and small myelinated nerve fibers in combination with unmyelinated nerve fibers can be tested, because QST can involve thermal, pressure, vibration or electrical stimulation, among other things.[1] QST is widely used in adults to diagnose and monitor neuropathic and chronic pain disorders.[2] Therefore, the German research network on neuropathic pain (DFNS) developed a standard, comprehensive testing protocol for adults.[3]
The first use of QST in children with regards to the diagnosis and monitoring of pain syndromes was reported in 1987 for the diagnosis of diabetic complications.[4] Since then, many different devices to determine pain thresholds, pain intensity, and pain tolerance have been tested in children, for example the Cold Pressor Task,[5] the VibraMeter[6] and the Thermal Sensory Analyzer.[7] The German protocol has also been evaluated for the ability to diagnose chronic pain in children, and reference values for several different tests are available.[7] Those reference values showed that 6-8 year old children were in general less sensitive to detect a thermal or mechanical stimulus compared to older 9-12 year old children. On the other hand, the younger children were more sensitive to pain stimuli compared to the older children. Furthermore, girls appeared to be more sensitive to thermal detection and pain stimuli compared to boys.[7]

Besides the diagnosis of chronic and neuropathic pain, QST is used for basic mechanistic studies of pain as a neurobiological phenomenon in healthy volunteers, as well as in pharmacological studies evaluating the efficacy of analgesics.[2] QST is also an often-used technique for experimental pain research in children. Especially by using a thermal stimulation paradigm, detection- and pain thresholds can easily be determined in children. The assessment of thermal detection thresholds is feasible in children from the age of 5 years onwards.[8] The Thermal Sensory Analyzer (Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel), for example, is previously used to investigate the long-term effects of neonatal pain and analgesic treatments in children. Hermann and colleagues showed that former preterm (n=19) and term born (n=20) patients with a history of neonatal intensive care unit (NICU) admission were less sensitive for brief heat pain stimuli than controls (n=20).[9] In a larger study by Walker and colleagues, former extremely preterm NICU patients (n=43) appeared to be less sensitive for the detection of cold and warmth stimuli and had higher cold and heat pain thresholds compared to controls (n=44).[10] In each study, subjects were compared with healthy controls. However, comparison

between different studies is hampered by the lack of uniform testing protocols and reference values. Some studies measured a thermal threshold for actual pain,[11] while others measured a thermal threshold for unpleasantness rather than for pain.[7] Therefore, the aim of the present study is to provide reference values for 8-17-year-old children and adolescents and to present a standard thermal QST testing protocol which is not time consuming and useful for repeated evaluation over time.

METHODS

Participants

Participants were recruited as healthy controls for a neuroimaging study regarding the long-term effects of early pain.[12] Besides Magnetic Resonance Imaging (MRI) scans, thermal QST tests were performed and the results are used for this current study. The healthy subjects were recruited through two different mechanisms. First, all included participants were asked whether they could recommend someone else in the age range of 8-18 years who would also be interested in volunteering. Potential candidates were sent an invitation letter and were contacted two weeks later by phone to ask if they were interested in participation. Invitations were also sent to parents of children of three primary schools in Rotterdam. Parents were asked to contact the researcher to make an appointment for the study. Only term born children and adolescents aged 8 years up to and including 17 years old were included. Exclusion criteria were the following: a history of severe early pain such as surgery in the neonatal period, preterm birth, intellectual disabilities, or gross motor or sensory disabilities.

This study was performed at the Erasmus University Medical Center (Erasmus MC) in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board of Erasmus MC. Informed consent was obtained from the parents of each subject prior to participation. According to Dutch law informed assent was also obtained from children 12 years of age and older prior to participation. Recruitment into the study took place from June 2011 to March 2013.

Materials

QST tests were performed with the computer-controlled Thermal Sensory Analyzer (TSA type II, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel) (Figure 1) with a Peltier-based contact thermode (30 x 30 mm) (Figure 2). WinTSA software (version 5.35) served to determine the detection- and pain thresholds, and a subtest of the Amsterdam Neuropsychological Tasks (ANT)[13] was used to measure visual-motor reaction time.

Figure 1 - Thermal Sensory Analyzer-II
(Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel)

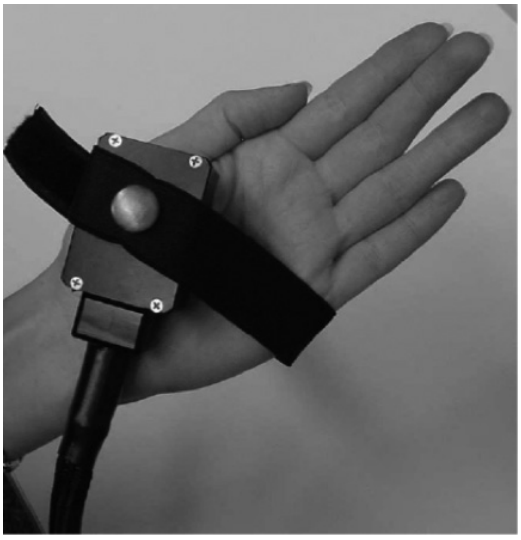

Figure 2 - Peltier-based contact thermode (30 x 30 mm)

Test protocol

In previous QST studies at our department we used the same standardized TSA-II test protocol to determine detection- and pain thresholds.[8,14] The protocol is structured as follows: explaining the procedure to the subject in less than a minute, determining visual-motor reaction time since one of the QST subtests is reaction time dependent (2-3 minutes),[13] determining detection- and pain thresholds using the reaction time depen-

dent Method of Limits (MLI) (8-10 minutes), and determining detection thresholds using the reaction time independent Method of Levels (MLE) (4-5 minutes). Thus, the entire protocol takes approximately 14-18 minutes. The entire TSA-II thermode-stimulating surface was placed in contact with the skin of the thenar eminence of the non-dominant hand and was firmly secured by a Velcro band. The non-dominant hand was chosen so as to allow the subject to use the dominant hand for clicking the button during the MLI subtest. Detection thresholds were measured with two methods, MLI and MLE, as these are both commonly used in the literature.[7-10,14,15] Furthermore, a previous study in 5-year-old children demonstrated significant differences between both methods in which the MLE established more sensitive detection thresholds compared to the MLI.[8] Another study in 6 to 17-year-old subjects also found more sensitive detection thresholds using the MLE compared to the MLI technique.[15] All QST tests in this study were conducted by the same researcher (GB).

Preparation

Skin temperature of the thenar eminence was measured with a skin thermometer. Room temperature was measured to ensure that the test environment was the same for every subject. After this, the protocol was explained to the child and his or her parents. It was emphasized that testing could not harm the hand, and parents were asked not to interact with their child during the assessment.

Visual-motor reaction time

After preparation, the child's reaction time was determined with the short *base-line speed task* of the Amsterdam Neuropsychological Tasks (ANT).[13] In case of differences in reaction time between groups, it is possible to correct for reaction time in the MLI group analysis.

MLI

Next, detection thresholds for cold and warmth were determined using the MLI technique. The baseline temperature of the thermode was set at the standard temperature of 32°C (centre of neutral range). From baseline, the temperature was steadily lowered at a rate of 1°C/sec. The researcher instructed the participant as follows: "The thermode is going to become cold, press the button as soon as you feel the temperature changing". After the button was pressed, the temperature returned to 32°C at a rate of 1.0°C/sec. This was repeated five times with 6 seconds between each stimulus. The first two stimuli served as rehearsal stimuli. The detection threshold was calculated as the mean value of the last four temperatures. Next, the temperature was steadily increased at a rate of 1°C/sec to determine the detection threshold for warmth using the same technique.

Subsequently, the MLI technique was applied to determine pain thresholds for cold and heat. Starting again from the baseline temperature of 32°C, the temperature was steadily

lowered at a rate of 1.5°C/sec. The child was asked to press the button when the cold sensation started to feel painful. After the button was pressed, the temperature returned to 32°C at a rate of 10.0°C/sec. This was repeated four times with 10 seconds between each stimulus. The first stimulus served as a rehearsal stimulus and the cold pain threshold was calculated as the mean value of the last four temperatures. Next, the pain threshold for heat was determined in the same manner. When the child did not press the button before the minimum temperature of 0°C or the maximum temperature of 50°C, the test automatically terminated. In that case, the cut-off temperature of 0°C or 50°C was used in the calculation of the mean threshold and the fact that the participant did not reach his or her pain threshold was made note of.

MLE

Next, detection thresholds for cold and warmth were determined with the MLE technique to obtain thresholds without the possible influence of reaction time. The researcher told the child that the thermode would either become colder, or would not change in temperature. The first thermal stimulus was 3.0°C below the baseline temperature of 32.0°C. Following each thermal stimulus the researcher asked "Did the thermode become cold or not?" The researcher pressed the 'yes' or 'no' button of the mouse depending on the answer. The next stimulus decreased with half of the previous step size from baseline, or decreased with the same step size estimated from the prior temperature depending on the answer of the child. The test terminated when the step size had decreased to a level of 0.1°C. The number of stimuli needed to decrease the step size to 0.1°C was registered as well. The warm detection threshold was determined in the same manner starting with a stimulus temperature of 3.0°C above the baseline temperature.

Statistical analysis

Normally distributed variables are presented as mean (standard deviation) and non-normally distributed variables as median (range). We defined four age groups: 8-9 years, 10-11 years, 12-13 years, and 14-17 years old. Differences in demographic characteristics between those age groups and between gender groups were determined with independent samples t-test for two groups or ANOVA for more than two groups (with post hoc Bonferroni correction) for continuous data and chi square tests for categorical data. Detection thresholds obtained by the MLI and MLE, and pain thresholds obtained by the MLI were compared between age groups and gender groups using an independent samples t-test or ANOVA (with post hoc Bonferroni correction). Additionally, linear regression analyses (which are in essence the same as ANCOVA tests but nowadays more often applied) served to correct for the mean reaction time. Numbers of children who did not reach a pain threshold during the MLI were compared between groups using a chi square test. Correlations between detection thresholds obtained with the MLI and the

MLE, and between reaction time and thresholds obtained with the MLI, were determined using Pearson product moment correlation coefficients. A p-value of 0.05 or less was considered statistically significant. Analyses were conducted using SPSS 20.0.

RESULTS

Demographic data

Seventy-five eligible subjects were recruited. Two children (8 and 9 years old) who were not able to reliably conduct the test due to attention deficits were excluded. One of them had already been diagnosed with attention deficit hyperactivity disorder (ADHD) prior to the study. Furthermore, four children were preterm born and were therefore excluded from the analyses afterwards. All the 69 remaining subjects successfully completed the entire QST test in approximately 14-18 minutes (including explanation). The subjects were aged 8 to 17 years with a median age of 11.2 years (IQR 10.2 to 12.6 years). Twenty-eight were males (40.6%; Table 1). Demographic characteristics per age group are presented in Table 1. Moreover, skin temperature and room temperature did not significantly differ between the age groups (p=0.72 and p=0.47, respectively). Reaction time differed significantly between age groups (p=0.02; post-hoc Bonferroni correction: 10-11 year versus 14-17 years; p=0.02), indicating a faster reaction time in the oldest subjects. These values are presented in Table 2. There were no statistically significant differences in age, skin temperature, room temperature, or reaction time between males and females.

Table 1 - Demographic characteristics

Control group (n=69)	Total group (n=69)	8-9 years (n=14)	10-11 years (n=31)	12-13 years (n=12)	14-17 years (n=12)
Age Years, Median (IQR)	11.2 (10.2 to 12.6)	9.0 (8.7 to 9.4)	11.1 (10.6 to 11.3)	12.5 (12.5 to 13.0)	16.5 (14.7 to 17.6)
Sex n (%) Male	28 (40.6)	6 (42.9)	13 (41.9)	4 (33.3)	5 (41.7)
Ethnicity n (%) Western European	47 (68.1)	7 (50.0)	20 (64.5)	9 (75.0)	11 (91.7)
Handedness n (%) Right	66 (95.7)	13 (92.9)	31 (100)	11 (91.7)	11 (91.7)
Reaction time ms, Median (IQR)	297 (274 to 327)	313 (290 to 335)	307 (280 to 357)	300 (260 to 310)	259 (238 to 294)

QST reference data

Total group MLI and MLE

Mean values and standard deviations of the detection- and pain thresholds are presented in the left-hand column of Table 2. Regarding the pain thresholds for cold and warmth, around 40% of the participants did not reach their pain threshold at least one

Table 2 - Detection- and pain thresholds per age group

Control group (n=69)	Total group (n=69)	8-9 years (n=14)	10-11 years (n=31)	12-13 years (n=12)	14-17 years (n=12)	P-value
Method of Limits (MLI)						
Cold detection threshold °C, mean (SD)	30.7 (0.7)	30.6 (0.9)	30.6 (0.8)	30.8 (0.5)	31.0 (0.4)	0.43
Warm detection threshold °C, mean (SD)	33.9 (1.2)	34.6 (1.7)	33.8 (0.9)	34.1 (1.1)	33.2 (0.5)	**0.01***
Cold pain threshold °C, mean (SD)	10.0 (9.1)	9.7 (10.8)	9.2 (9.4)	12.3 (9.0)	10.0 (6.7)	0.81
Threshold not reached n (%)	27 (39)	8 (57.1)	14 (45.2)	3 (25.0)	2 (16.7)	0.12
Heat pain threshold °C, mean (SD)	45.9 (4.2)	43.2 (5.4)	46.9 (3.7)	45.9 (4.0)	46.2 (3.2)	**0.05****
Threshold not reached n (%)	28 (41)	6 (42.9)	16 (51.6)	4 (33.3)	2 (16.7)	0.20
Method of Levels (MLE)						
Cold detection threshold °C, mean (SD)	30.8 (1.2)	30.5 (1.4)	30.6 (1.4)	31.0 (0.6)	31.2 (0.4)	0.29
Number of stimuli mean (SD)	11 (3)	11 (4)	11 (3)	10 (3)	12 (3)	0.24
Warm detection threshold °C, mean (SD)	33.6 (1.0)	33.7 (1.1)	33.7 (0.9)	33.6 (1.2)	33.1 (0.7)	0.21
Number of stimuli mean (SD)	9 (3)	10 (3)	9 (3)	9 (2)	10 (2)	0.25

ANOVA test for continuous data and Chi squared test for categorical data were used to test differences between the four age groups
** Post-hoc Bonferroni correction: 8-9 year old versus 14-17 years old; p=0.01*
*** Post-hoc Bonferroni correction: 8-9 year old versus 10-11 years old; p=0.04*

time during the test (out of the four stimuli). The detection thresholds obtained with the MLI were highly correlated to the detection thresholds obtained with the MLE (p<0.01). The reaction time obtained with the ANT was not correlated to the four MLI modalities (detection threshold cold: p=0.16, detection threshold warm: p=0.12, pain threshold cold: p=0.28, and pain threshold heat: p=0.94).

Age effects

Age effects were found in the warm detection threshold obtained with the MLI, indicating a higher detection threshold for warmth in the youngest children (34.6 SD 1.7) compared to the oldest group (33.2 SD 0.5) (p=0.01). No significant differences were found in the detection threshold for warmth obtained with the MLE, and in detection thresholds for cold obtained with both the MLI of the MLE technique. Furthermore, a significant age effect in the heat pain threshold was found, indicating a lower threshold in age group 8-9 years (43.2 SD 5.4) compared to age group 10-11 years (46.9 SD 3.7; p=0.05). These were the only significant age effects (Table 2). After additional correction for the mean reaction time, they remained significant (warm detection threshold p=0.02; heat pain threshold p=0.05).

Gender effects

No statistical significant differences in detection thresholds obtained with both the MLI and the MLE technique were found between males and females. Regarding pain thresh-

olds, females were statistically significantly more sensitive for both cold (females 12.0 SD 9.4, males 7.0 SD 7.9; p=0.03) and heat pain (females 44.9 SD 4.3, males 47.3 SD 3.7; p=0.02) compared to males. Furthermore, more than twice as many males did not reach their pain threshold for cold (males 57.1%, females 26.8; p=0.01) and for heat (males 60.7%, females 26.8; p=0.01), compared to females.

DISCUSSION

The aim of this study was to provide Dutch reference values and a standardized testing protocol for thermal quantitative sensory testing in children and adolescents. Through the years, we have gained much experience with this testing protocol and noticed that it is very easy to conduct in children.[8,14] In this current study we obtained reliable QST data from almost all participants. Only two subjects could not complete the protocol successfully due to attention deficits. One of them was already diagnosed with ADHD. Furthermore, the testing protocol is not time consuming since it only takes 14-18 minutes to complete.

Two other studies have provided protocols and reference values for thermal quantitative sensory testing in children with the use of the TSA-II.[7,15] The protocol of Meier and colleagues (2001) is comparable to our protocol. However, they do not specify when the child had to press the button during the determination of the pain thresholds and state that the quality of thermal pain perception (burning versus pricking etcetera) was not assessed.[15] Furthermore, gender- or age differences were not described and individual reaction time was not assessed in that study. Valid comparison with our reference values is not possible. Yet, the detection thresholds obtained with the MLI are roughly the same, while the pain thresholds differ more than 4°C, suggesting a higher sensitivity for both cold and heat pain in the study by Meier and colleagues.[15] However, these differences in reference values could have been caused by different instructions given to the subjects rather than actual differences in pain sensitivity between children in both studies, since we do not know which instructions were given in this previous study. In the recent study by Blankenburg and colleagues, children were instructed to press the button of the TSA-II as soon as the thermode started to stitch, ache or burn.[7] In our study children were asked to press the button during the MLI pain subtests as soon as the temperature started to feel painful. Therefore our reference values represent actual pain thresholds. This may probably explain why our values are much higher than in the study by Blankenburg and colleagues (6°C or more difference for cold pain and 2 or more for heat pain depending on age and gender).[7] The fact that Blankenburg and colleagues measured thresholds on the dorsal side of the hand instead of the thenar eminence could also have been a reason

for differences between their study and ours. Furthermore, Blankenburg and colleagues used a logarithmic data transformation for their detection thresholds since the data were not normally distributed, which distorts comparison to our reference values. Previous clinical studies in children did not present logarithmic transformed data, in line with our study.

We found only small age effects with respect to the detection threshold for warmth and the pain threshold for heat measured with the MLI, in which the youngest children were less sensitive to detect a warm stimulus but – interestingly – more sensitive to heat pain in comparison to older participants. This is in line with a previous study that found that 6 to 8-year-old children (24 boys and 24 girls) were generally less sensitive to thermal and mechanical detection stimuli but more sensitive to all pain stimuli than 9 to 12-year-old children (32 boys and 32 girls), whereas the differences between these older children and adolescents (13–17 years; 32 boys and 32 girls) were slight.[7] However, neither the detection thresholds obtained with the MLE nor detection and pain thresholds for cold differed between our age groups. Although reaction time was not significantly correlated to the MLI thresholds, differences in attention among age groups during the MLI tests could possibly have influenced the results. Reaction time was measured at the start of the test protocol when the attention of the subject was probable the highest. Since attention deficits have less influence on MLE results, this could explain the absence of age group differences using the MLE technique. Moreover, the variance in pain thresholds for heat is smaller in comparison with the variance for cold pain thresholds, therefore significant differences between age groups are easier to detect with respect to heat pain thresholds.

Furthermore, girls proved more sensitive than boys to both cold and heat pain stimuli. This is also in line with other studies.[7] Therefore we recommend same gender distributions in case-control studies. Additionally, boys statistically significantly reached their pain threshold for both cold and heat less often than girls. A previous version of the TSA permitted to lower the minimum temperature of the TSA-II to −10°C, instead of 0°C. This can be a solution to avoid participants not reaching their pain threshold for cold, however the question arises whether this is ethical justifiable for studies in children. Moreover, we recommend measuring every participant's reaction time even though in the present study it was not significantly correlated to the reaction time dependent MLI subtests. In a previous study of our research group in younger children, however, the detection thresholds obtained in a reaction time dependent fashion were significantly correlated to IQ, while the detection thresholds obtained in a reaction time independent fashion were not.[8] Unfortunately reaction time was not tested in this previous study.[8]

We chose to measure the detection- and pain thresholds with thermal stimuli using the TSA-II because it is feasible and therefore often used in experimental pain research in children.[8-10] Since the device is MRI compatible, it also gains popularity in functional MRI studies measuring brain activation during pain.[11,16] To be able to compare our results with previous studies, we chose to obtain detection- and pain thresholds with the TSA-II as well. However, a few features speak against its use: it is an expensive device, and instructions need to be standard and unambiguous to avoid that one child during the MLI pain test will press the button when the temperature starts to hurt and another when it starts to itch for example. Future studies that will test the inter-instructor variability would be valuable.

Possible alternatives are techniques using cold water or electrical stimuli, which are also often used in children. A popular test to determine pain intensity and tolerance is the cold pressor task[5,17] in which children immerse a hand or forearm in cold water and give pain scores for the duration of the test. These scores are thought to reflect the pain intensity experienced. Furthermore, the immersion time gives information about pain tolerance.[17] A disadvantage is that it is a qualitative test instead of a quantitative sensory test since children have to give pain scores on a 0-10 scale. The Neurometer (Neurotron, Inc., Baltimore, MD, USA) allows for electrodiagnostic sensory nerve testing[18] but is very painful and will therefore probably frighten children. Furthermore, it is less used in previous studies compared to the other techniques mentioned above

Our standardized protocol only takes 14-18 minutes to complete and is therefore also useful in clinical practice for diagnostic purposes.[14] In a child with congenital pain insensitivity syndrome we found elevated detection- and pain thresholds measured with both the MLI and MLE technique.[14] The TSA-II is also used for the detection of neuropathies in adults.[19] This study found that the TSA-II had a sensitivity of 72% for the diagnosis of small fiber neuropathy and authors recommended the measurement of both cold and warmth detection thresholds.[19] Since our protocol includes both the MLI and the MLE technique, based on our findings it can be shortened by only using the MLI technique for both the determination of the detection- and pain thresholds in children from 8 years onwards instead of using the MLE technique. Since the MLI technique is preferred for the determination of pain thresholds in children, we advise to use the MLI also for the determination of the detection thresholds in order to be consistent in all the different modalities, even though the MLE technique appears to be a bit more sensitive for the determination of detection thresholds in children.[8,15] In adults MLE is used for the determination of pain thresholds,[11] but the disadvantage is that it is more time-consuming than the MLI pain test and that temperatures above the pain threshold are reached. For specific groups such as for younger children, however, the MLE technique is preferred rather than the MLI technique with respect to detection threshold measurements.[8]

The strength of our reference values is that they are easy to interpret and may serve as normative values for future studies. The sample size was relatively small, however, although it is larger than control groups in previous studies.[8,9,14] Other possible limitations are the testing at only one body site and the application of thermal quantitative sensory testing only. However, the positive side is that this design enabled us to complete the entire protocol in no more than 14-18 minutes, which decreases the risk for fatigue and distraction in children.

CONCLUSION

We conclude that this study protocol is applicable for children from 8 years onwards, not time consuming and feasible even for daily practice. Furthermore, we provide easy interpretable thermal detection and pain reference values for 8 to 17-year-old children and adolescents.

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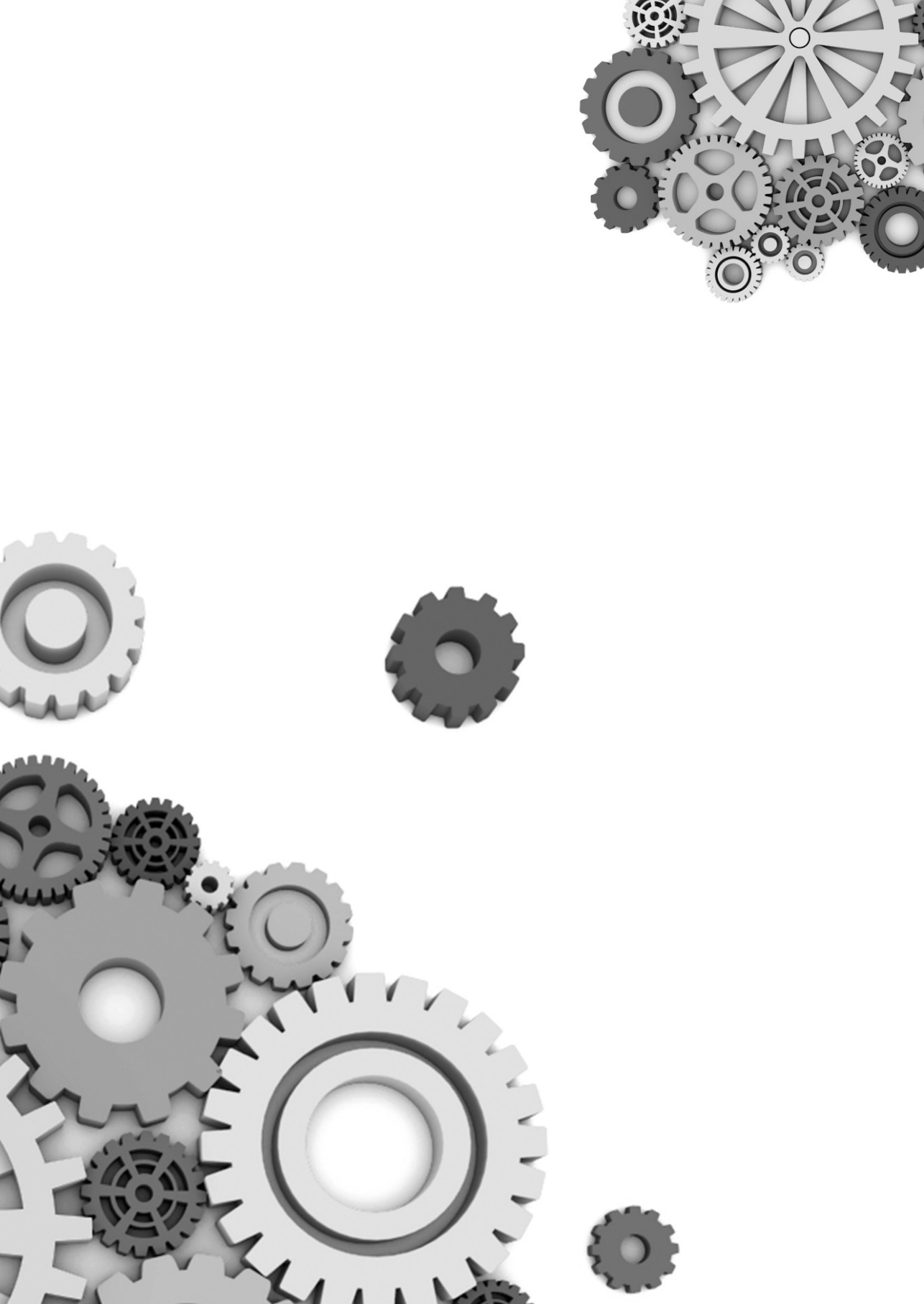

Chapter 4

Functional MRI pain studies in children? Yes, we (s)can!

Gerbrich E. van den Bosch, Tonya White, Dick Tibboel, Monique van Dijk

Pediatric Radiology 2013; Sep 43(9):1235-6

In general, clinical research in children continues to generate ethical and regulatory issues.[1] Magnetic resonance imaging (MRI) is an accepted technique for scientific research in adults, but has been suggested to be more frightening for children[2] and to present more ethical dilemmas.[3] Westra et al. studied discomfort in 5-to 12-year-old children undergoing a clinical MRI, and found that 44% of the children rated the procedure as unpleasant.[4] Thus, functional MRI (fMRI) during which children receive a pain stimulus may be considered even more frightening.

We conducted non-clinical structural and functional brain MRI scans in 98 children (median 10 years, range 8-16 years old) to determine the possible effects of neonatal pain on pain processing later in life. The study was performed at the Erasmus MC in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board at the Erasmus MC. Informed consent was obtained from the parents of each child prior to participation.

Before undergoing the MRI procedure, the children were first placed in a mock scanner to help them adjust to the MRI environment. Second, we measured warm and cold detection- and pain-thresholds outside the scanner, using the Thermal Sensory Analyzer-II (Medoc Advanced Medical Systems, St. Ramat, Israel). Third, the children underwent five MRI scans without sedation (total 45 min). During the last two scans we applied the Thermal Sensory Analyzer-II: eight warm (41°C) and eight potentially painful hot stimuli (46°C) on the thenar eminence of their non-dominant hand. At four time points, i.e. before and after the mock practice session, and before and after the real MRI scans, we asked the child, the parent, and the researcher to report the child's level of "fun" and "fear" using the Wong-Baker faces rating scale (0 = most fun/not at all fearful and 5 = not at all fun/very frightening).[5]

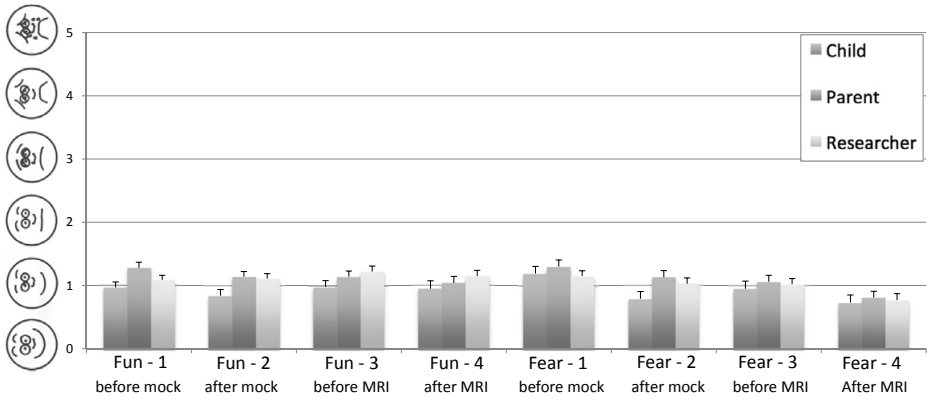

Figure 1 – Mean Wong-Baker faces ratings
Lower scores on the Wong-Baker faces scale indicate a higher level of fun and a lower level of fear. The error bars represent the standard error of the mean.

All 98 children completed the mock procedure, and only two children (11 and 12 years old) refused to undergo the real scans. Thirteen children (14%) did not complete the entire scanning protocol although their ratings were low; "fun" median 1 (interquartile range (IQR) 0 to 2), and "fear"; median 1 (IQR 1 to 3). One of the reasons was pressure discomfort caused by the headphones. Figure 1 shows the mean ratings for fun and fear of the child, parent and researcher for all 98 children. Robust regression analysis (SAS 9.2) revealed that higher age was associated with slightly lower fear scores rated by the child (estimate −0.07, 95% confidence interval −0.13 to −0.01, p=0.02).

From these findings we conclude that unsedated MRI research is well tolerated and not harmful or frightening for children. In contrast, it can even be fun!

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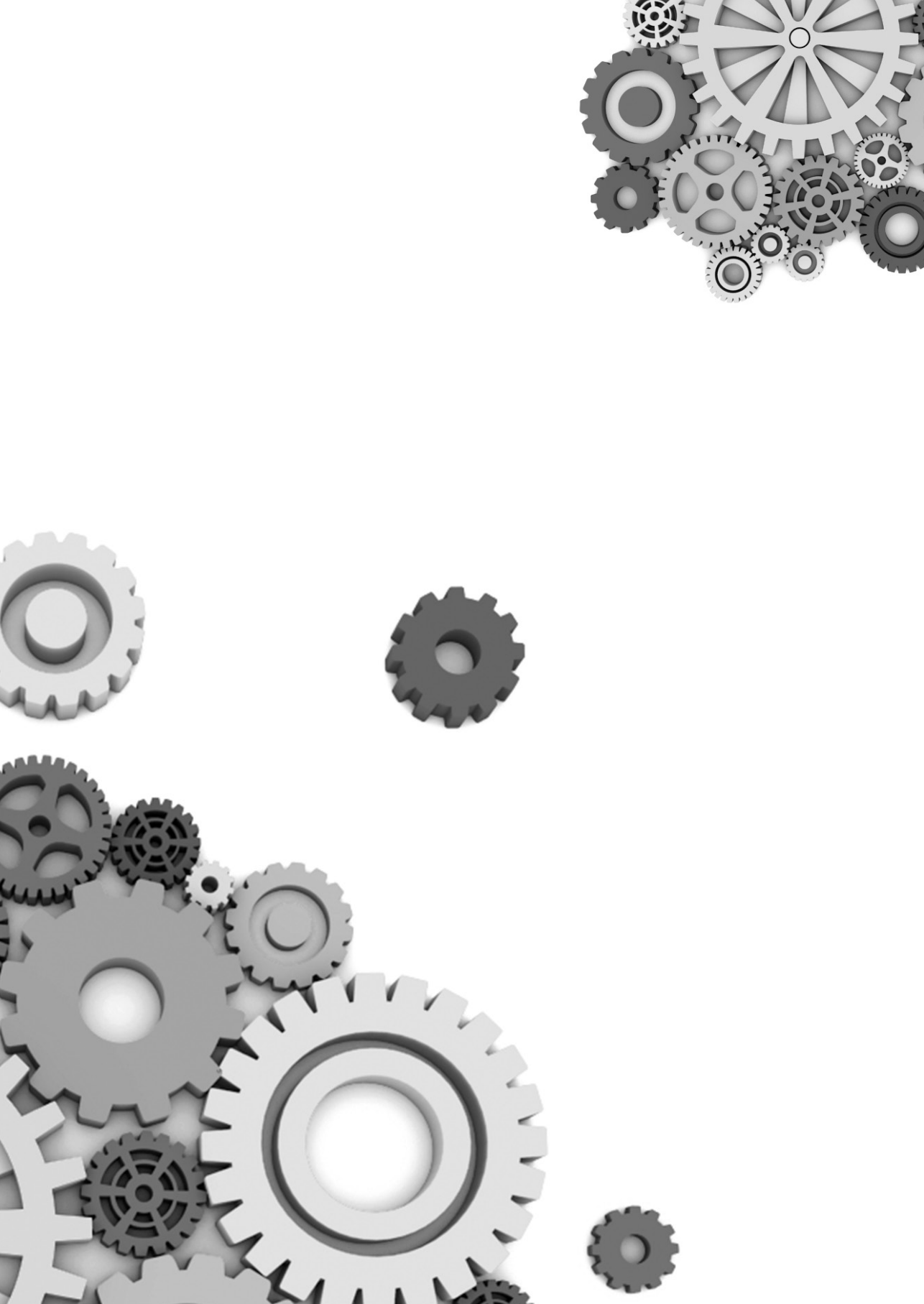

Chapter 5

Brain connectivity during verbal working memory in children and adolescents

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ABSTRACT

Working memory (WkM) is a fundamental cognitive process that serves as a building block for higher order cognitive functions. While studies have shown that children and adolescents utilize similar brain regions during verbal WkM, there have been few studies that evaluate the developmental differences in brain connectivity. Our goal was to study the development of brain connectivity related to verbal WkM in typically developing children and adolescents.

Thirty-five healthy children and adolescents, divided into three groups: 9-12 (children), 13-16 (young adolescents), and 17-19 (older adolescents) years, were included in this functional magnetic resonance imaging (fMRI) study. The verbal WkM task involved a modified Sternberg item recognition paradigm using three different loads. Brain connectivity analysis was performed using independent component analyses and regressing the components with the design matrix to determine task-related networks.

Connectivity analyses resulted in four components associated solely with encoding, four solely with recognition and two with both. Two networks demonstrated age-related differences with respect to load, 1) the left motor area and right cerebellum, and 2) the left prefrontal cortex, left parietal lobe, and right cerebellum. Post hoc analyses revealed that the first network showed significant effects of age between children and the two older groups. There was increasing connectivity with increasing load for adolescents. The second network demonstrated age-related differences between children and older adolescents. Children have higher task-related connectivity at lower loads, but they tend to equalize with the adolescents with higher loads. Finally, a non-load related network involving the orbital frontal and anterior cingulate cortices showed less connectivity in children.

INTRODUCTION

Working memory (WkM) is considered to be one of the building blocks for higher cognitive functioning. It provides an essential interface between perception, attention, memory and action.[1] WkM involves three primary processes: encoding information, actively maintaining this information on-line in memory, and finally, using the information to guide behavior. During encoding, individuals actively attend and construct an internal representation of the information in memory. This mental representation of the information is maintained during a delay period, during which the information is actively prevented from decaying due to interfering or competing stimuli. Finally, the information is retrieved from the memory buffer and conveyed through a motor response (e.g. verbal, oculomotor or manual response). The processes involving WkM are crucial for completing higher-order cognitive tasks,[1] and is one of the main reasons for the exponential rise in studies utilizing WkM paradigms in both health and illnesses.

One important WkM paradigm emerged in the late 1960's, carrying the name of its founder, is known as the Sternberg Item Recognition Paradigm (SIRP).[2] This task is interesting for several reasons. First, it allows for the separation of the motor component and the speed of mental scanning, thus allow for the measurement of both WkM and non-WkM components.[2] The SIRP has been shown to be relatively free from practice effects.[3] In addition, the SIRP allows separation of the encoding, maintenance, and the retrieval phase of WkM. This is particularly useful in imaging studies focusing on separate phases of WkM and also allows for comparisons with non-human primate studies mapping the neural architecture of WkM networks.[4] Finally, the SIRP allows the testing for developmental differences within the different components of WkM.[5]

It is known from behavioral studies that WkM performance continues to improve from childhood, through adolescence and into early adulthood.[6-8] In addition, different trajectories of WkM development are present for different components and forms (verbal, spatial, objects) of WkM.[9-12] A number of studies of verbal WkM have shown load-related developmental differences.[5,13] In addition, there have been studies showing developmental differences in WkM maintenance, especially when information is manipulated during the delay period.[13,14] The transition of passive maintenance into active verbal rehearsal or active refreshment emerges during childhood.[15,16] Active verbal rehearsal is an important component during maintenance to efficiently retain information in WkM and this becomes more difficult with increasing loads. The developmental behavioral differences in WkM provide a framework for understanding developmental differences in neuroimaging studies of WkM.

There have been a number of functional imaging studies evaluating WkM in children and adolescents.[14,17-21] While children have been shown to activate similar brain regions as adults[22,23] there are several distinct developmental differences, although the findings are inconsistent.

O'Hare et al. evaluated developmental differences in 12 children (7-10 years), 10 adolescents (11-15 years), and eight young adults (20-28 years) during an fMRI Sternberg task.[18] They found increasing activation with increasing load in frontal, parietal and cerebellar regions in adolescents and adults, while children recruited only the left ventral prefrontal cortex with increasing WkM load. Crone and colleagues also compared three age groups (8-12 years; n=14, 13-17 years; n=12, and 18-25 years old; n=18) and found that while children had poorer performance on an object-WkM task with separate maintenance and manipulation conditions compared with adolescents and adults, they found no differences in the activation profile of the ventrolateral prefrontal cortex,[17] a region associated with online maintenance. Finn and colleagues followed ten female adolescents in their longitudinal fMRI study and found that younger adolescents have more activation in the hippocampus and older adolescents have a stronger relationship between behavioral performance and functional activity in the prefrontal cortex during a match-to-sample Sternberg task.[21] Klingberg used functional MRI to measure brain activity during a WkM task in 13 participants between 9-18 years of age, and found a positive correlation between age-related increases in WkM capacity and brain activity in the superior frontal and intraparietal cortex.[19] While a summary of these studies that utilized different age groups, methodologies, and regions of interest is challenging, nearly all studies show that there are age-related increases in specific areas associated with adolescent development.

WkM is disrupted in a number of psychiatric and neurological disorders, such as schizophrenia and Attention-Deficit/Hyperactivity Disorder.[24-26] Therefore, understanding the normal developmental trajectories of WkM is important to better understand when trajectories go awry. It is often unclear when during the course of development these abnormalities in WkM occur. Thus, having a good understanding of the normal development of WkM will help determine when in the course of development abnormal trajectories diverge from the normal trajectories.

Since brain function involves distributed neural networks, approaches that measure functional connectivity are well suited to study age-related network differences between childhood and late adolescence. Since the prefrontal cortex has a protracted development, our hypothesis was that connections between the prefrontal cortex and outlying brain regions would strengthen from childhood through adolescence. Therefore our aim

was to determine specific connections between the prefrontal cortex with other brain regions while performing a modified Sternberg WkM task. We were particularly interested in studying connectivity differences related to WkM load, as significant developmental differences have been identified from behavioral and neuroimaging studies. In addition, fMRI studies using the SIRP have seen activation in the dorsolateral prefrontal cortex only during retrieval and not during encoding or maintenance.[27] Since the development of passive maintenance techniques into active techniques occurs during early childhood,[15,16] we choose to focus our study on the developmental differences during encoding and retrieval and not during the maintenance phase of the SIRP.

Our primary hypothesis involved age-related differences in the prefrontal cortex. However, the application of a data driven approach (Independent Component Analysis; ICA), allowed us to test other networks that contribute to verbal WkM. Therefore, our secondary aim was to assess alternative networks that show age-related differences in brain connectivity during verbal WkM tasks in typically developing children and adolescents. To our knowledge no other studies have examined developmental differences in functional connectivity associated with WkM performance in typically developing children and adolescents. However, there has been one recent study evaluating functional connectivity in adolescents.[21]

METHODS

Participants

Our participants consisted of typically developing children and adolescents between the ages of 9 and 19 years. To evaluate age-related differences, these participants were divided into three groups consisting of children (between the ages of 9 and 12 years; n=10), young adolescents (between the ages of 13 and 16 years; n=12), and older adolescents (between the ages of 17 and 19 years; n=13). Participants were recruited from advertisements in the local community, and via families who had participated in other MRI studies from our research group.[26,28] Participants were excluded if they were pregnant, had a history of any psychiatric disorder, including a history of substance dependence or on-going substance abuse (within the past month), neurological disorders, head injuries, or a medical illness that involved the brain. Participants were also screened to assure that they had no contraindications for participation in an MRI study such as metal implants or claustrophobia. All participants underwent a thorough diagnostic assessment using the Kiddie-SADS-PL.[29] Their socioeconomic status (SES) was measured by using the Hollingshead SES scale.[30] This study was performed at the University of Minnesota in compliance with the Code of Ethics of the World Medical

Association (Declaration of Helsinki) and was approved by the Institutional Review Board at the University of Minnesota. Informed consent and assent was obtained prior to participation.

Working Memory Paradigm

Verbal WkM was tested using a modified Sternberg Item Recognition Paradigm (SIRP) using three WkM loads[2] (Figure 1). The modified SIRP targeted encoding and retrieval of information separately and was easy enough to be performed well by children. The stimuli were designed as an integrated block and event-related paradigm and each run consisted of two blocks for each WkM Load (total = 6 blocks per run).[26,31] During a WkM block, participants were initially presented with the word 'Learn'. This was followed by the simultaneous presentation of one, three, or five digits for seven seconds ('Encode'). After a short delay of 2.5 seconds, 16 single digits were presented sequentially at a rate of 2.7 seconds for each digit ('Recognition'). The participants pushed their right thumb if the digit was a member of the memorized set ('Target'), or their left thumb if the digit was not a member of the memorized set ('Foil'). Accuracy and response time were measured for each response. All the participants who participated in this study had two practice sessions prior to the fMRI session. During the first practice session, participants were seated in a chair in front of a monitor and performed the WkM task with a team member describing the task. The second practice session was performed inside a mock scanner with stimuli identical to that used during the fMRI session. The participants practiced until they understood and were comfortable performing the task. Participants were told to respond as quickly as possible without making mistakes. During the fMRI session, a vacuum bag was placed around the back of the head to reduce head

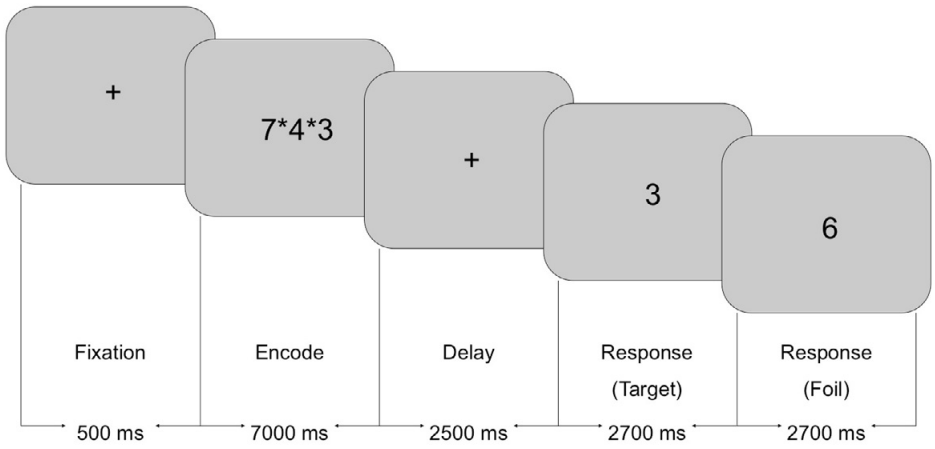

Figure 1 - Sternberg Item Recognition Paradigm

motion. The paradigm was programmed using E-Prime (Psychology Software Tools, Inc.) The participants wore a set of fMRI compatible gloves with buttons associated with each finger and thumb. There were three runs, each lasting five minutes and 58 seconds.

MRI Sequence

The MRI images were acquired with a 3T Siemens MR system (Erlangen, Germany) located at the Center for Magnetic Resonance Research at the University of Minnesota. After an initial localizer scan was obtained, a coronal scout image (12 slices; field of view (FoV) 224 mm, TR 2000 ms; TE 72 ms; resolution 2.3 × 1.8 × 2 mm) was obtained to locate the coronal midline. A second scout image was then attained using sagittal images acquired along the coronal midline (12 slices; FoV 224 mm; TR 2040 ms, TE 62 ms; resolution 1.2 × 0.9 × 2 mm). These sagittal slices were used to orient the volume along the anterior/posterior commissure (ACPC) plane. Functional images were obtained using a gradient echo sequence with 27 axial slices and an in-plane resolution of 3.4 × 3.4 mm, 4 mm slice thickness, and a 1 mm gap. Additional sequence parameters included: TE = 30 ms, TR = 2000 ms, flip angle = 90 degrees and FoV = 220 mm. A total of 177 volumes were obtained for each of the three runs (531 volumes in total).

Image Processing

All the functional images were preprocessed using a combination of Analysis of Functional NeuroImages (AFNI, http://afni.nimh.nih.gov/)[32] and FMRIB's Software Library (FSL, FMRIB Software Library; FMRIB, Functional Magnetic Resonance Imaging of the Brain;http://www.fmrib.ox.ac.uk/fsl/).[33] Following the conversion from DICOM to the Nifti format, slice timing correction and motion correction were performed using AFNI.[32] Participants who were unable to complete three runs of the SIRP or participants who had greater than 2.5 mm of motion in the x, y, or z directions were excluded from the analyses. Images were oriented to standard Montreal Neurological Institute (MNI) space utilizing FSL in a 3-stage process. First, for each individual a mean echo planar imaging (EPI) image was generated from the fMRI time series. This mean EPI image was registered to an EPI template in standard space using a 12-parameter transformation.[34,35] Finally, the 12-parameter transformation was applied to the entire fMRI time series for each individual and each run. The data were spatially smoothed using an 8-mm full width at half-maximum Gaussian kernel.[36]

Independent Component Analysis

Following the preprocessing steps, a group Independent Component Analysis (ICA) was performed on the preprocessed data.[37,38] The methods prescribed by this process were performed using GIFT (Matlab toolbox version 1.3c http://icatb.sourceforge.net). ICA allows a model free analyses of the data and thus was well suited as an initial step to

derive specific brain networks. From this, we were able to test which of these networks were associated with our WkM task. We chose to use this approach, as it was our intent to initially extract network information and to use these networks to assess age-related differences in connectivity during WkM. ICA is a statistical and computational data-driven technique that is designed to extract temporally related signals that are hidden within sets of random or unrelated variables. It assumes that the fMRI time series are linear mixtures of independent source signals that are buried within noise. The algorithm (infomax) was designed to extract maximally independent signals and their mixing coefficients. The principle behind ICA is that these maximally independent source signals represent temporally coherent groupings of BOLD signal change, often referred to as component maps. These components map the functional connectivity between different brain regions. Since ICA is a data-driven approach, the functional networks are generated without any assumptions about the shape of the hemodynamic time courses. The spatial maps generated by ICA were averaged together across the three scan sessions and the dimensionality was not constrained. This resulted in 26 independent component (IC) spatial maps for every participant. These IC spatial maps represent the regions of the brain related to a specific time course. Every voxel within a component spatial map contains a z score, with high z scores reflecting a greater contribution to the associated time course.

Component Selection

One of the strengths of ICA is its ability to detect noise-related components that represent signal artifacts such as head motion and eye movement. Thus, we first evaluated each of the spatial maps and eliminated those with motion or other artifacts. These were readily identified by symmetric activations on the opposite sides of the skull, activations within the ventricles, or activation within the eye itself. The second phase consisted of identifying and limiting the components to only those that were task-related. The SIRP has the advantage to be able to parse out the encoding, maintenance, and retrieval phases as separate time series. We did not calculate connectivity during the maintenance phase of the task, as the optimum method would be to parametrically alter the delay period to assess for effects of delay. Adding this additional measure would also have significantly increased the acquisition time, which would have been difficult especially for the younger children. The effect of load was determined via a mixed-model repeated measures ANCOVA using the beta weights that reflect task modulation at the different loads. The ICA component time courses were regressed against the design matrix for the working memory task in GIFT using a SPM5 general linear model (GLM) to obtain the beta weights for each load of the working memory task. The design matrix included columns for both encoding and recognition for each of the three WkM loads. The resulting beta weights from this regression analysis represent the degree to which each component

was associated with the WkM task relative to the fixation baseline (i.e., a high beta weight represents a large task-related modulation of a component for a given regressor). The components that showed a statistically significant effect of load or age-related differences for either encoding, recognition or both were included in the study. These components were used to assess group differences using a mixed-model repeated-measures analysis of variance (ANOVA).

Statistical Analyses

The demographic data was assessed using chi-square for categorical data and ANOVA for normally distributed continuous data. We used the Kruskal-Wallis test for non-normally distributed continuous data. A 3 (age group) by 2 (encode/recognition) by 3 (load) by 3 (run) mixed-model repeated measures ANOVA was performed using age group, task, and load as the fixed effects, and subject as the random variable. We also used repeated measures ANOVA for post-hoc analysis comparing the three different age groups. The task-related beta-weights for each of the individual components were entered into a 3 (age group) by 3 (load) mixed-model repeated-measures ANOVA. To examine performance differences between the different age groups, a 3 (age group) by 3 (run) by 3 (load) mixed-model analysis of covariance (ANCOVA) was performed using response time (RT) and accuracy as covariates. We also analyzed age as a continuous variable using a mixed-model regression analysis. We examined differences in head motion during scanning using a 3 (age group) by 3 (run) repeated measures ANOVA. A Bonferroni correction was conducted to correct for multiple testing. The analyses were performed using SAS version 9.2 (Institute Inc., Cary, NC, USA).

RESULTS

Study Population

From a total of 41 participants who completed scanning, six children were excluded due to significant motion. The 35 participants included in the study were between 9 and 19 years of age with a mean age ± S.D. of 15.0 ± 3.0. The total group included 16 girls and 19 boys. Age group subsamples included 10 children aged 9-12 (10.9 ± 0.9), 12 young adolescents aged 13-16 (15.2 ± 1.0) and 13 older adolescents aged 17-19 years old (18.1 ± 0.9). No significant differences in gender, socioeconomic status or handedness were found between these subgroups (Table 1). There were no significant differences in movement across age groups using both the maximum ($F_{1,101} = 1.74$, $p = 0.190$) and mean movement parameters derived from AFNI ($F_{1,101} = 0.02$, $p = 0.903$). All participants were debriefed after the task and were asked what strategy that they used to remember the numbers. All participants used the same strategy of repeating the numbers sequentially

Table 1- Demographic characteristics per age group

		Age group			
		Children (9-12 years)	Young adolescents (13-16 years)	Older adolescents (17-19 years)	p-value
Total (n=35)		10	12	13	NA
Age (mean ±SD)		10.9 ± 0.9	15.2 ± 1.0	18.1 ± 0.9	NA
Gender (male %)		70.0	50.0	46.2	NS
Handedness (%)	Right	80.0	66.7	84.6	NS
	Left	0	0	7.7	
	Both	10.0	16.7	0	
	No measurement	10.0	16.7	7.7	
SES (mean ±SD)		58.0 ± 7.6	54.0 ± 6.8	50.9 ± 6.6	NS

Table note: NA = Not Applicable, NS = Not Significant
P-values were derived from ANOVAs for normally distributed continuous variables, Kruskal-Wallis test for non-normally distributed continuous variables and χ^2-tests for categorical variables

in their mind. They did this in the order that the numbers were presented, thus, without reordering and none of the subjects reported using a visual spatial strategy.

Behavioral Results

Probe response time and probe accuracy

A mixed-model repeated-measures ANOVA found that both age group ($F_{2,68}$ = 8.24, p < 0.001) and WkM load ($F_{2,513}$ = 160.0, p < 0.0001) significantly affected probe response time (probe RT), and these factors did not interact. Children responded more slowly than older participants, and in all groups and the RT increased with increasing WkM load. For the probe accuracy there were significant main effects of run ($F_{2,515}$ = 8.00, p < 0.001), age group ($F_{2,63}$ = 5.0, p < 0.001), and load ($F_{2,508}$ = 45.49, p < 0.0001). There was also an interaction between age group and load ($F_{4,508}$ = 5.42, p < 0.001) for probe accuracy. With increasing loads and successive runs, accuracy decreased. Thus, children between 9-12 years had longer response times and were less accurate for both probes and foils compared to the older participants (Figure 2).

Comparing the children and the younger adolescents in the post-hoc analysis showed that there were significant main effects of age group ($F_{2,46}$ = 12.47, p < 0.001), load ($F_{2,336}$ = 105.1, p < 0.0001), and run (($F_{2,341}$ = 3.78, p = 0.02) for the probe RT using the mixed model repeated measures. In addition, there was an interaction effect of run by load ($F_{4,336}$ = 2.50, p = 0.04). There were significant main effects of age group ($F_{1,42}$ = 6.30, p < 0.02), load ($F_{2,331}$ = 40.9, p < 0.0001), and run (($F_{2,338}$ = 7.29, p < 0.001) for the probe accuracy using the mixed model repeated measures analysis. In addition, there was also an interaction effect of age group by load ($F_{2,331}$ = 5.22, p = 0.006).

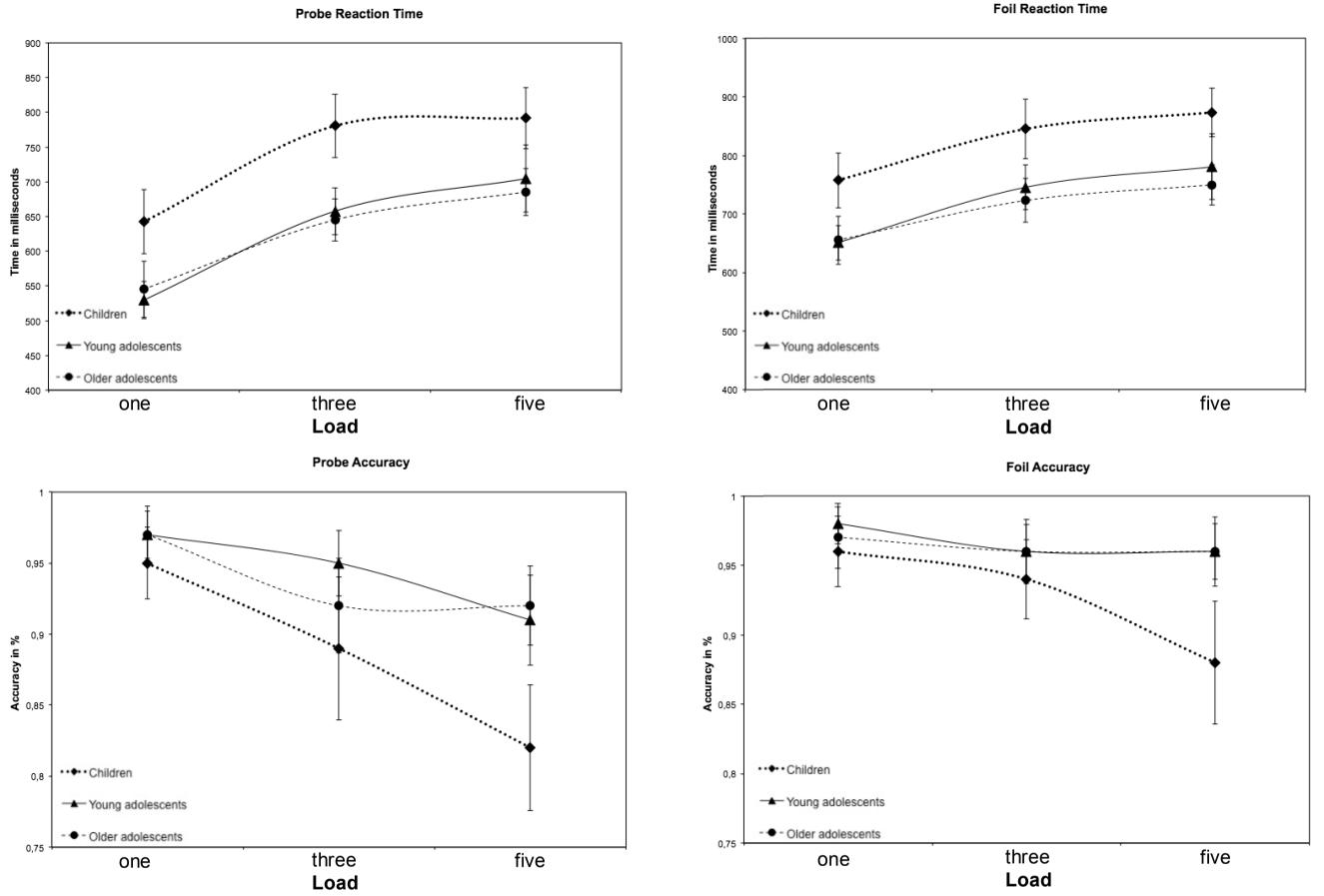

Figure 2 - Behavioral results

When comparing the children with the older adolescents, we found significant main effects of both age group ($F_{2,43}$ = 12.03, p = 0.001) and load ($F_{2,331}$ = 97.7, p < 0.0001) for the probe RT. No interaction effects were observed. There were significant main effects of age group ($F_{1,40}$ = 6.40, p = 0.02), load ($F_{2,327}$ = 35.2, p < 0.0001), and run (($F_{2,331}$ = 5.09, p = 0.006) for the probe accuracy. In addition, there was also an interaction effect of age group by load ($F_{2,327}$ = 9.15, p < 0.001) and run by load ($F_{4,327}$ = 3.43, p = 0.009).

Finally, when comparing the younger adolescents with the older adolescents in the post-hoc analysis, the results showed a significant main effect of load ($F_{2,335}$ = 118.5, p < 0.0001) for the probe RT using the mixed model repeated measures. No interaction effects were observed. There were significant main effects of both load ($F_{2,358}$ = 17.2, p < 0.0001) and run (($F_{2,358}$ = 4.17, p = 0.02) for the probe accuracy using the mixed model repeated measures. No interaction effects were observed.

Foil response time and foil accuracy

The mixed-model repeated-measures ANOVA showed that there were significant main effects for run ($F_{2,517}$ = 3.56, p < 0.05), age group ($F_{2,68}$ = 6.83, p < 0.001), and load ($F_{2,512}$ = 76.82, p < 0.0001) for foil response times. The response time for the foils (foil RT), decreased with successive runs. There was also a run by load interaction ($F_{4,512}$ = 5.51, p < 0.001) with shorter response times associated with lower loads. The accuracy of the foil conditions showed main effects for both age group ($F_{2,64}$ = 3.49, p < 0.05), and load ($F_{2,508}$ = 14.49, p < 0.001). In addition, the accuracy of the foil condition also had an age group by load interaction ($F_{4,508}$ = 7.19, p < 0.001) (Figure 2).

In the post-hoc analysis we found significant main effects when comparing the children with the younger adolescents for age group ($F_{2,45}$ = 8.62, p = 0.005), and load ($F_{2,335}$ = 48.82, p < 0.0001) for the foil RT. In addition, there was an interaction effect of run by load ($F_{4,335}$ = 3.26, p = 0.01). There were significant main effects for both age group ($F_{2,42}$ = 4.87, p = 0.03) and load ($F_{2,331}$ = 16.73, p < 0.0001) for the foil accuracy. There was also an interaction effect of age group by load ($F_{2,331}$ = 8.44, p < 0.001).

In the comparison between the children and the older adolescents we found significant main effects for run ($F_{1,43}$ = 12.29, p = 0.001) and load ($F_{2,330}$ = 49.15, p < 0.0001) for the foil RT. In addition, there was an interaction effect of run by load ($F_{4,330}$ = 6.47, p < 0.0001). There were significant main effects for both age group ($F_{2,40}$ = 4.46, p = 0.04), and load ($F_{2,327}$ = 12.65, p < 0.0001) for the foil accuracy. There was also an interaction effect of age group by load ($F_{2,327}$ = 10.52, p < 0.001) and an interaction effect of run by age group ($F_{2,330}$ = 3.42, p = 0.03).

Finally, we compared the younger adolescents with the older adolescents and found a significant main effect for load ($F_{2,355}$ = 56.7, p < 0.0001) for the foil RT. In addition, we found an interaction effect of run by load ($F_{4,355}$ = 2.70, p = 0.03). There were no significant main effects for the foil accuracy using the mixed model repeated measures. No interaction effects were observed.

Imaging Results

Out of a total of 26 components, 7 components were related to motion or other artifacts and were removed. We first evaluated networks that were related to load. Ten load-related components were grouped depending on whether they were significantly related to the encoding phase, recognition phase, or both using a mixed-model repeated-measures ANOVA; four ICs were associated solely with encoding, four solely with recognition, and two with both (Table 2 and Figure 3). Two IC networks demonstrated age-related differences with respect to load. A network involving the left motor area and the right cerebellum demonstrated age-related differences during encoding ($F_{2,273}$ = 6.3, p = 0.002). This same network also showed an age group by run interaction ($F_{2,269}$ = 4.8, p = 0.009). A network involving the left prefrontal cortex, the left parietal lobe, and the right cerebellum demonstrated age-related differences during recognition ($F_{2,245}$ = 4.4, p = 0.013) (Table 2 and Figure 3).

Post hoc analyses were performed to assess differences between each of the three different age groups. We found that the left motor/right cerebellar network showed a significant effect of age between the child group compared with both the younger adolescent group ($F_{1,170}$ = 4.9, p = 0.029) and the older adolescent group ($F_{1,188}$ = 11.0, p = 0.001). With greater load, adolescents showed greater functional connectivity within this network compared to the children (Figure 4a). There were no significant differences between the younger adolescent group and the older adolescent group. The interaction between age group and run showed a significant difference between the child group and the older adolescent group ($F_{1,176}$ = 8.3, p = 0.005) (Figure 5a). These analyses were repeated using a mixed-model repeated measures ANCOVA with each of the behavioral measures (response time and accuracy) as covariates. None of the findings remained significant when performance was used as a covariate. When performing a separate analysis in which we compared the lowest load of the children with the highest load of the younger and older adolescents, we found significant differences during encoding (p = 0.024) for this network.

The left prefrontal/left parietal/right cerebellar network showed age-related differences only between the child group and the older adolescent group ($F_{1,185}$ = 9.2, p = 0.003). There were no significant differences between the child and young adolescent

Table 2 – Independent Components related to load

Brain network	Effect of load	
Encoding	***NumDF/DenDF/F/P***	
Left motor area, right cerebellum	2/269/18.71/<0.0001	
Right pre-frontal and parietal cortex, left cerebellum	2/269/4.81/0.0089	
Occipital lobe	2/301/24.41/<0.0001	
Occipital lobe	2/269/12.91/<0.0001	
Recognition	***NumDF/DenDF/F/P***	
Posterior cingulate cortex	2/269/7.54/0.0006	
Right motor area, left cerebellum	2/269/7.08/0.0010	
Left parietal and pre-frontal cortex, right cerebellum	2/269/3.07/0.0479	
Anterior and posterior cingulate cortex, medial cerebellum	2/269/8.55/0.0003	
Encoding and Recognition	***NumDF/DenDF/F/P Encoding***	***NumDF/DenDF/F/P Recognition***
Bilateral cerebellum, pre-frontal and parietal cortex	2/305/14.40/<0.0001	2/272/16.34/<0.0001
Right cerebellum, bilateral motor areas	2/301/7.72/0.0005	2/269/16.98/<0.0001
Age-related Differences	***NumDF/DenDF/F/P Encoding***	***NumDF/DenDF/F/P Recognition***
Left motor area, right cerebellum	2/273/6.27/0.0022	-
Left parietal and pre-frontal cortex, right cerebellum	-	2/245/4.40/0.0133

NumDF = Numerator degrees of freedom, DenDF = Denominator degrees of freedom, F = F value

group, nor between the young adolescent and older adolescent groups (Figure 4b). There was also an age group by run interaction between the child group and the older adolescent group ($F_{1,176}$ = 4.1, p = 0.043) (Figure 5b). None of the findings remained significant when the analyses were repeated using a mixed-model repeated measures ANCOVA with each of the behavioral measures (response time and accuracy) as covariates. The comparison of the lowest load of the children with the highest load of the younger and older adolescents, showed no significant differences during recognition (p = 0.476).

Age-related Differences Unrelated to Load

A network involving the anterior cingulate cortex and orbital frontal cortex demonstrated age-related differences during encoding ($F_{2,301}$ = 3.1, p = 0.047). This network was related to the overall working memory task, but was not related to WkM load. Using post-hoc analysis we found that the anterior cingulate cortex and the orbital frontal cortex showed age-related differences only between the child group and the older adolescent group ($F_{1,197}$ = 5.7, p = 0.018), although there was a trend between the younger and older adolescents ($F_{1,215}$ = 3.0, p = 0.086, Figure 6). We also found an age group by run interaction between the child group and the older adolescent group ($F_{1,197}$ = 3.9, p = 0.050). None

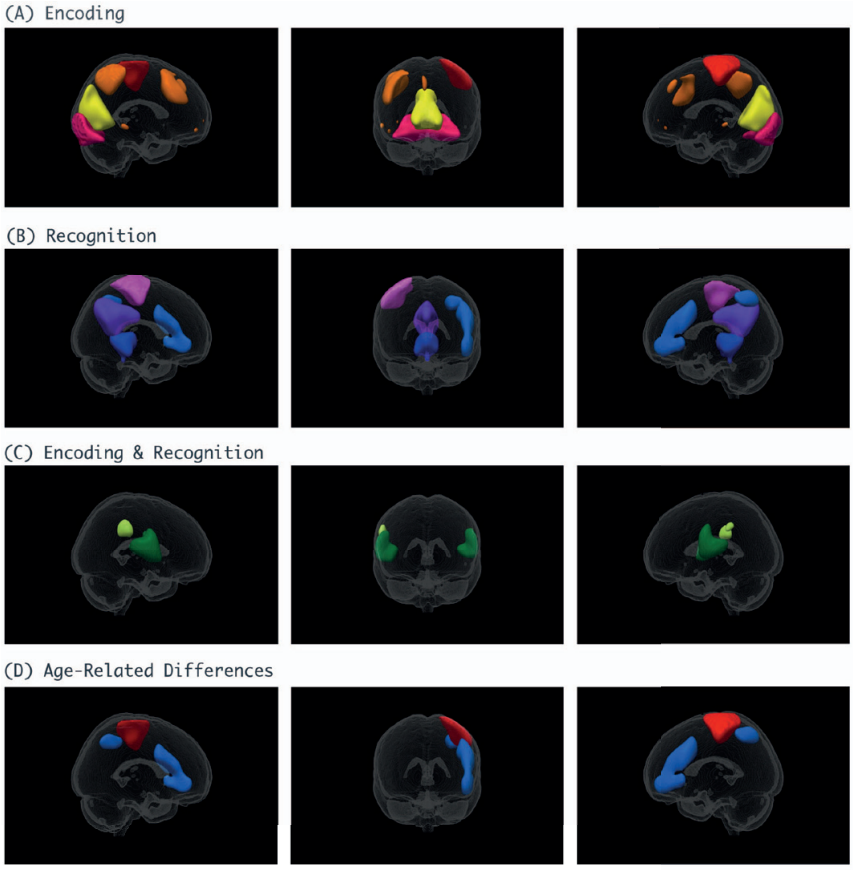

Figure 3 - Independent components related to load

of these findings remained significant when we used performance as a covariate. When comparing the lowest load of the children with the highest load of the younger and older adolescents, we found significant differences during encoding ($p < 0.0001$) in this network.

Age as a Continuous Variable

To confirm the age-related differences found in the three above described networks, we ran a mixed-model regression analysis with age as the random variable and load and run as fixed effects. The left motor area and right cerebellum network showed significant differences during encoding ($F_{1,99} = 4.7$, $p = 0.032$) and the left prefrontal, left parietal cortex, and the right cerebellum network showed significant differences during recognition ($F_{1,99} = 5.1$, $p = 0.026$). The third network involving the anterior cingulate cortex and the orbital frontal cortex, however, did not show significant differences during encoding using the mixed-model regression.

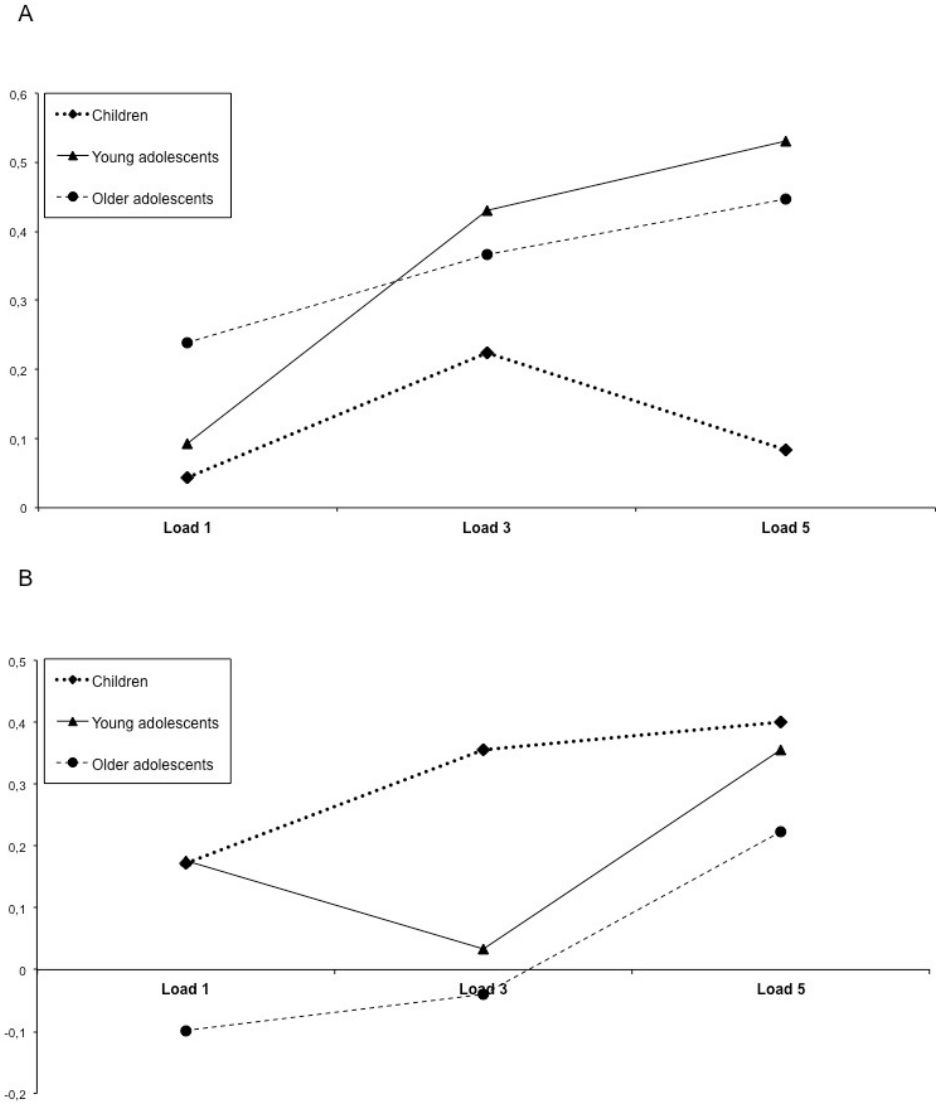

Figure 4 - Beta weights for connectivity a) left motor area, right cerebellum, b) left parietal and prefrontal cortex, right cerebellum

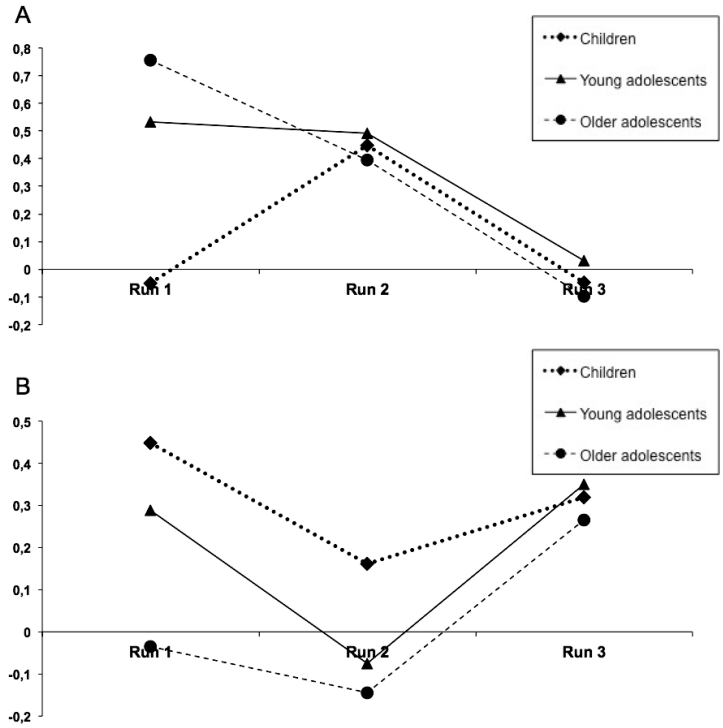

Figure 5 - Beta weights for connectivity per run a) left motor area, right cerebellum, b) left parietal and pre-frontal cortex, right cerebellum

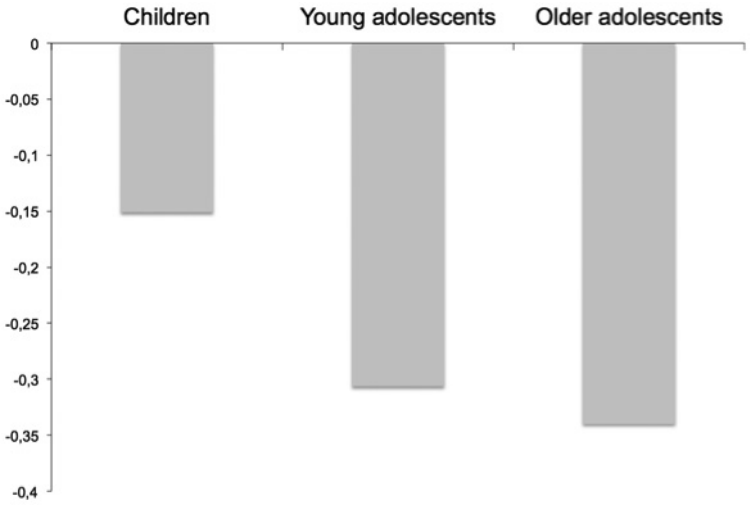

Figure 6 - Mean beta weights for connectivity of the anterior cingulate cortex and orbital frontal cortex

DISCUSSION

In this fMRI study of typically developing children and adolescents, we demonstrated age-related differences between brain connectivity and verbal WkM in several distinct brain networks. These networks can be sub grouped into load-dependent and load independent networks. The age-related differences related to load were found in two specific brain networks involving 1) the left motor area and right cerebellum, and 2) the left prefrontal cortex, left parietal lobe, and right cerebellum. The first network is associated with motor functioning and the second network involves brain regions shown in prior studies to be involved in WkM performance.[20,22,23] Activations in the cerebellum have also been found in previous fMRI studies on WkM.[39]

There have been several fMRI studies that evaluate developmental differences in working memory,[17,19,20,40] although to our knowledge only one study has evaluated functional connectivity within working memory networks and found developmental differences in prefrontal and hippocampal connectivity.[21] A major strength of this study was the longitudinal design and the homogeneous population of 10 females. However, they evaluated changes between mid- (mean age 15.1 years) to late adolescence (mean age 18.3 years), where we notice the major differences taking place between the children and mid- to late-adolescents. Thus, while there is clear overlap between our studies within the prefrontal cortex, the differences in motor networks could be attributed to the age of the sample or methodological differences between the two studies (data driven approach versus a region of interest approach). Studies using traditional GLM analyses have shown age-related increases in activity in several brain regions: focal regions of the left and right dorsolateral prefrontal cortex, left ventrolateral prefrontal cortex, left premotor cortex and the left and right posterior parietal cortex. [40] has shown that age was most predictive of brain activity. Klingberg et al. found that older children showed higher activation in the superior frontal cortex and intraparietal cortex than younger children.[19] We found age-related differences in functional connectivity in regions overlapping with these prior studies.

Several studies have compared resting state activity or baseline epochs with brain activation during a WkM task.[41-45] Zou et al. found that resting state activity can predict the behavioral performance and brain activation during WkM.[41] Another study showed that connectivity during resting-state predicted the individual performance on a WkM task.[43] To our knowledge the relationship between resting state scans and brain activation during a WkM task has not been performed in children or adolescents. Since we did not collect resting-state fMRI scans as a part of this protocol, we are unable to test whether this relationship is also true during development. With the exponential rise in resting state studies, this is an important area for future research.

One network in which we found load- and age-related differences in functional connectivity between the child group and the older adolescent group was a left prefrontal, left parietal, and right cerebellar network. As this network has long been implicated in WkM function[46] it is not surprising that age-related differences would be identified within this network. Since performance suggests significant improvement with age, it is possible that the increased functional connectivity associated with age is tied to a better orchestration of brain function, translating to better performance. The fact that we found no differences between the child group and young adolescent group, or between the young adolescent and older adolescent group supports the idea of a developmental pathway in which young adolescents lie between children and older adolescents. The strength of the connectivity was stronger in children compared to the older adolescents, suggesting that children required greater coherence of neuronal activity with increasing WkM loads (Figure 4-b). This difference was no longer present when controlling for WkM performance, suggesting that performance differences were tied to the functional connectivity differences. This finding would be expected, given the strong relationship between task performance and age. This network does not survive stringent Bonferroni correction for multiple testing, thus it is possible that it is a Type II error. However, there is considerable evidence from prior studies as above described that would implicate that this network is associated with age-related differences in working memory.

In addition, we found age-related differences in a network associated with motor functioning (left motor area right cerebellum). In contrast with the above-mentioned network, this network showed differences between the child group compared with both the two older age groups. These differences in the motor network could possibly be a result of the prolonged developmental course of the cerebellum. It takes more time for the cerebellum to reach the peak volume in comparison with the cerebrum.[47] In this case there was greater functional connectivity in the adolescents compared to the children (Figure 4-a). Children had increasingly lower performance with increasing load compared to adolescents, and thus the differences could reflect less coherence with motor response networks in children. However, the age-related differences in this network were found during the encoding phase. Therefore, this age-related difference would be more difficult to explain by the manual motor response, as the participants did not press the button during the encoding phase.

The age-related differences that we found between children and adolescents performing a WkM task were not what we expected. In the cognitive network, involving the left prefrontal, left parietal, and right cerebellar network, the strength of the connectivity was stronger in children compared to the older adolescents, while in the motor network involving the left motor area and right cerebellum the functional connectivity was greater

in adolescents in comparing to the children. We would have predicted that connectivity strengthens with age, especially in the cognitive domain. However, the measurement of task-related connectivity may be different than resting-state or structural connectivity. For example, increased effort on a task may translate to greater measured connectivity between regions. Alternatively, different brain regions could have different developmental trajectories, and this mismatch in regional development could influence network connectivity. The network in which the connectivity is higher in adolescents is the network of the left motor area and the right cerebellum. This is the network that is specifically related to motor function. As mentioned above, this could be explained by the prolonged developmental course of the cerebellum, with the motor circuit in adolescents having more coherent connectivity due to better-developed cerebellar networks. The reason that the parietal/prefrontal/cerebellar network does not show the same pattern is perplexing. It may be that the children are exerting more effort for task completion, and thus there is greater connectivity within this network, including the cerebellar component. Another possibility is that adolescents are using alternate brain regions to complete the task, which results in more synchronous regions and greater noise in the system. This could have resulted in age-related differences in the strength of connections between the different regions. The network including the left motor area and right cerebellum showed age-related differences during the encoding phase, while the more cognitive network including the left prefrontal, left parietal, and right cerebellar network showed significant differences during the recognition phase. Marvel and colleagues found that the dorsal cerebellar dentate co-activated with the SMA during encoding and that this likely represents the activation of an articulatory motor trajectory.[48] During recognition they found that the ventral cerebellar dentate co-activated with prefrontal regions. These findings correspond very nicely with our results, as we found age-related motor differences during encoding and age-related cognitive differences during recognition. We can also distinguish between the motor and more cognitive pathways of the cerebellum during WkM.[48,49]

Interestingly, apart from the age-related differences, the cerebellum is involved in seven of the ten networks related to WkM in children (Table 2). This emphasizes the important role of the cerebellum in WkM tasks, which has been also documented from lesion[50] and transcranial magnetic stimulation studies.[51] A mixed-model regression analysis with age as the random variable and load and run as fixed effects was also performed on these two networks that were significantly related to load and age. We found that these two networks also showed age-related differences with age as a continuous variable in the model. These networks are strongly related with development along a linear trajectory.

A network involving the anterior cingulate cortex and orbital frontal cortex showed age-related differences that were not related to the load of the WkM task. Thus, this network showed age-related differences during encoding that was independent of the load. However, this network was not significant using age as a continuous variable, and thus it is possible that this network shows more non-linear effects, as evidenced in Figure 6.

Equally as interesting as the age-related differences in brain networks associated with WkM, is the fact that the majority of networks that we found were not different between the three age groups. This shows that the majority of functional brain networks associated with WkM show strong functional connectivity during the school age years and remain strong with development. We found four specific brain networks that were associated with encoding: 1) the right motor area and right cerebellum, 2) the right prefrontal and parietal cortex and left cerebellum and two networks involving both the occipital lobe (3 and 4). Four brain networks were associated with recognition: 1) the posterior cingulate cortex, 2) right motor area and left cerebellum, 3) left parietal and pre-frontal cortex and right cerebellum, and 4) a network involving the anterior and posterior cingulate cortex and medial cerebellum. We also demonstrated that the bilateral pre-frontal and parietal cortex and bilateral cerebellum and the right cerebellum and bilateral motor areas were associated with both encoding and recognition.

Nelson and colleagues found comparable associations between working memory in children and activations in the prefrontal, posterior parietal, and anterior cingulate cortex.[23] Olesen et al. also found fronto-parietal activation associated with WkM in children.[22] Thus, we provide evidence for mature functional connectivity patterns in children and adolescents within a number of WkM networks.

As expected, age-related differences were present in our behavioral data.[5,7] Children had a significantly longer response time for both probes and foils compared to adolescents. The accuracy of the working memory task was also lower for all the three working memory loads in children.

A limitation of the study is the relatively small sample size per subgroup. Nevertheless, literature describing the development of brain connectivity associated with WkM is sparse and our findings mesh well with the sample sizes of the GLM and connectivity studies in the literature. To confirm our results, we also analyzed the age-related differences using a mixed-model regression analysis. Age as a continuous variable effectively increased the sample size and provided support for developmental differences in two load-dependent networks. Larger sample sizes may identify additional brain regions with smaller effect sizes that show age group-related differences in WkM performance.

On the other hand, additional components could potentially be more prone to type II errors. Also, the test for age effects is certainly susceptible to type II error. However, when using Bonferroni correction, only the left motor area remains significant.

Another limitation of our study is that considerable scanning time was spent during the retrieval phase of the task. Therefore the encoding phase has less power in comparison with the retrieval phase. In addition, there was some blurring of maintenance and retrieval during the retrieval phase, as the information was held on-line during this period and was likely refreshed. An optimal design would have a balance between the encoding and retrieval time periods. However, we found significant age-related differences in connectivity in the left prefrontal cortex, left parietal lobe and right cerebellum during retrieval. Furthermore, there were as many load-related and age-related components during retrieval as during encoding. So the distribution of the networks during encoding and retrieval is the same, even with discrepancies in the duration of the encoding and retrieval phase. The question rises if the results would have been different if the study had been run with more even periods of encoding, maintaining and retrieval. Future studies could help to answer this question and possibly further optimize the design of the task. Another limitation is that we only used visually presented stimuli in this study. With auditory-presented stimuli, it is possible that we could have identified other age-related networks. As described by Kirschen et al. auditory presented stimuli during a WkM task are associated with greater medial cerebellar hemisphere activations while visual presented stimuli are associated with greater lateral hemisphere activations.[39] Another limitation is that fatigue could have occurred during such long WkM trials. However, as presented in figure 5, the age-related networks look more alike during run 3 than the earlier runs, which may mean that fatigue tends to create a situation in which even older adolescents fall back to more basic network strategies.

In conclusion, it is important to better understand the developmental trajectories in functional connectivity as children progress through adolescence into early adulthood. It is an age period where the risk for specific psychiatric disorders increases dramatically. We found age-related differences in performance and brain connectivity during WkM tasks in 9-19 year old typically developing children and adolescents. An important finding in this study is evidence for a developmental trajectory in the left prefrontal, left parietal and right cerebellar network. This is an important network that has been shown to be associated with WkM performance. Future neuroimaging studies should evaluate brain connectivity in larger populations, beginning at a younger age, and using longitudinal designs. These studies may help inform when in the course of development the trajectories go awry in children with emerging psychopathology.

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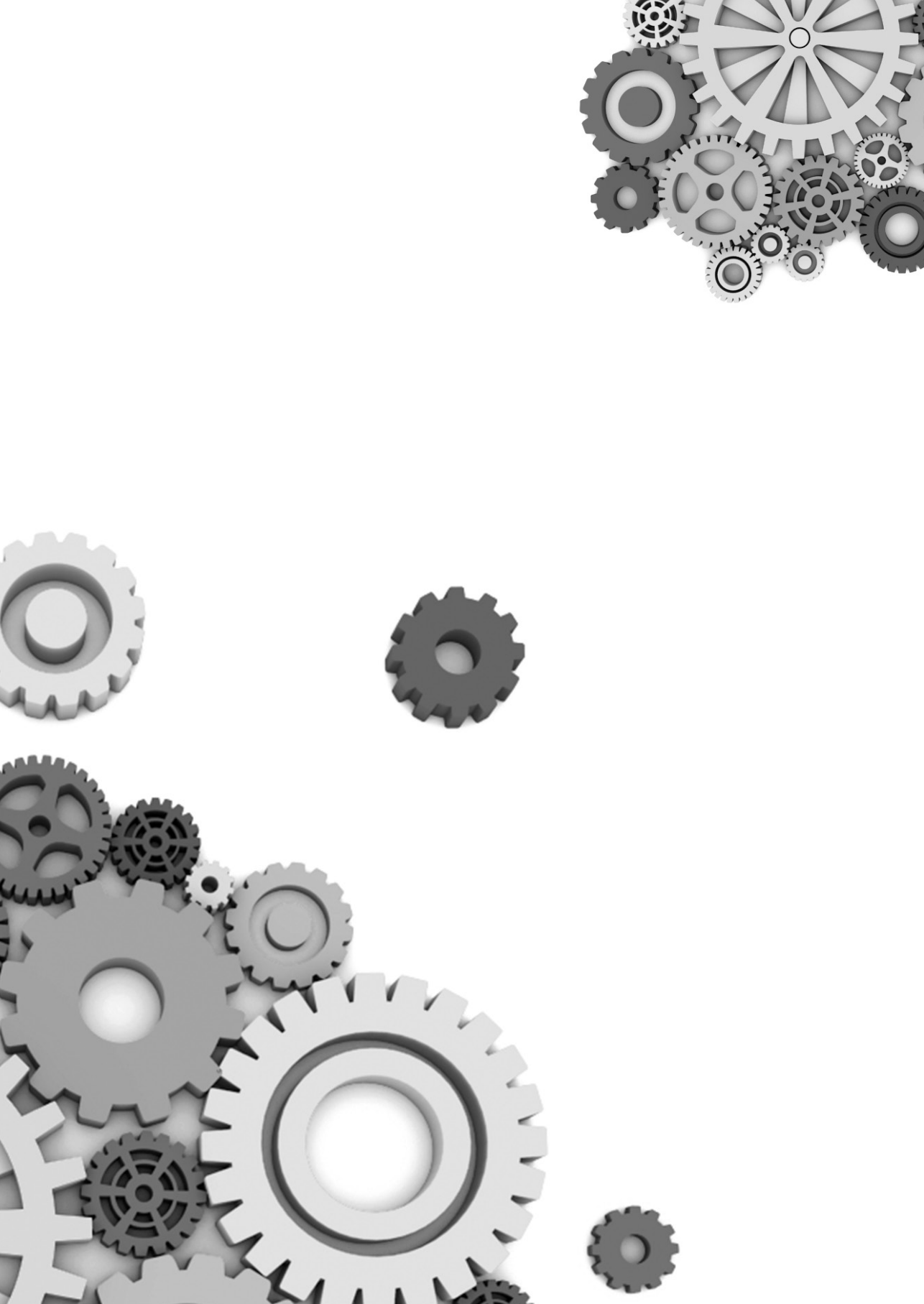
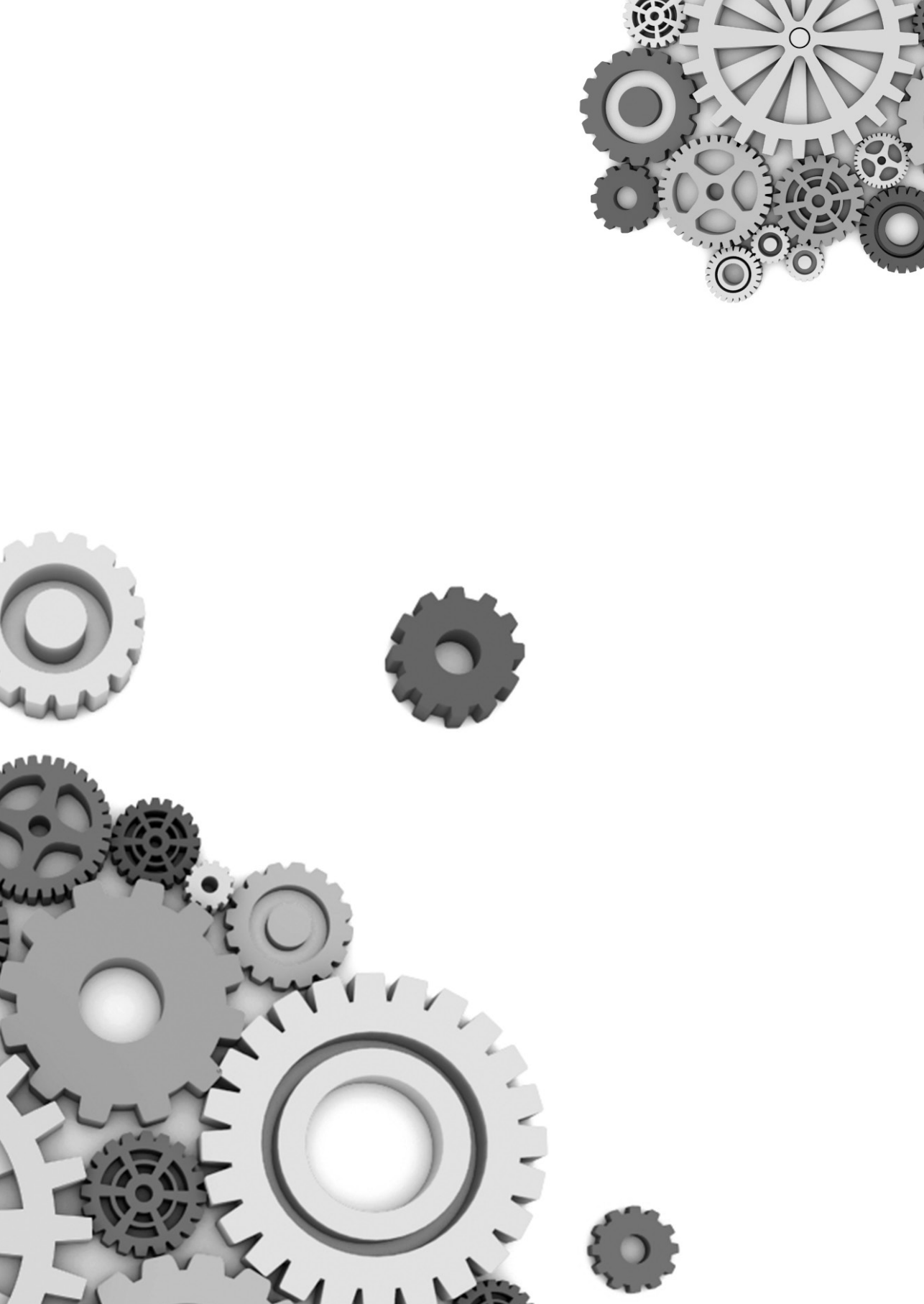


Part 2

Long-term consequences of early pain and opioid exposure



Chapter 6

Long-term neurobiological effects of extensive tissue damage in newborns and young infants

A neuroimaging study of children with giant congenital melanocytic naevi

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Submitted for publication

ABSTRACT

Background Both early pain and opioid exposure show neurotoxic effects in animal studies such as neuroapoptosis, impaired cognitive functioning, and alterations in pain sensitivity. We aimed to evaluate the long-term neurobiology of extensive tissue damage in children who received high doses of morphine. We hypothesised negative long-term effects.

Methods Children with surgical removal of giant congenital melanocytic naevi (GCMN) in early life, served as a homogeneous model for intense pain caused by extensive tissue damage in combination with high dosages of opioids. We compared 14 GCMN children (8-15 years) with 42 controls within the same age range. We conducted thermal sensory testing, structural and functional MRI during pain.

Results Greater parietal/occipital activation was seen during pain in cases compared to controls, suggesting alterations in sensory, but not pain specific brain regions. Furthermore, a thicker cortex was found in cases in the left rostral-middle-frontal cortex. We found no differences in brain volumes or in detection or pain thresholds between groups.

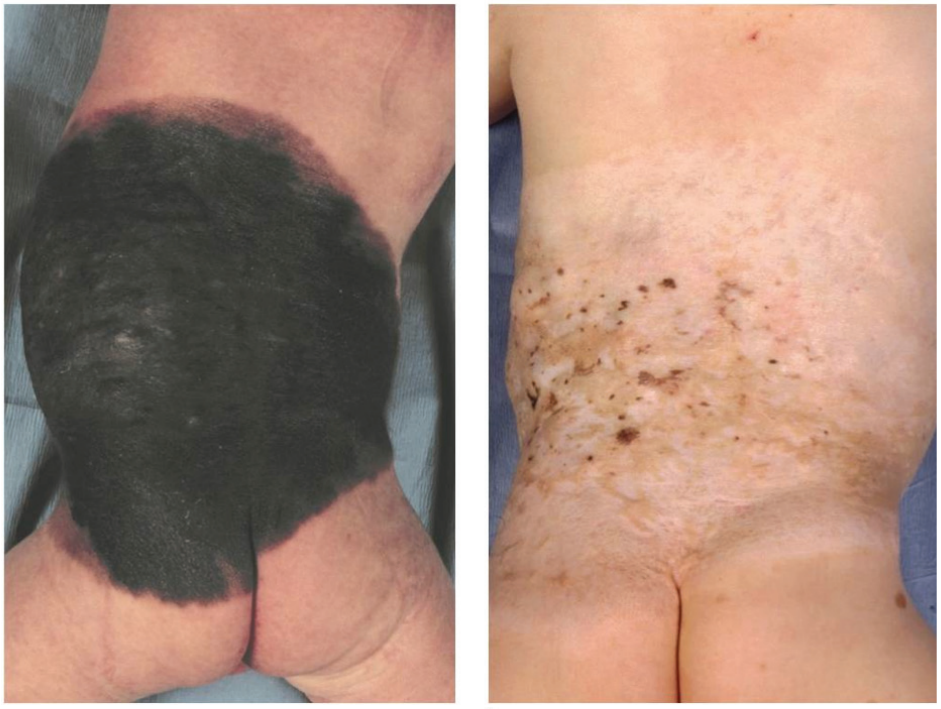
Conclusion The differences in brain activation during pain and in cortical thickness suggest a potential negative long-term effect of extensive tissue damage in combination with opioid treatment in early life. Future studies are needed to determine the implications for daily life of these neurobiological changes.

INTRODUCTION

Animal studies have provided evidence that neonatal pain and opioid use can have detrimental effects during early stages of neurodevelopment. Pain stimuli during neonatal life induced alterations in somatosensory thresholds[1] and neuroapoptosis[2] in rats. Supratherapeutical doses of opioids in the absence of pain also showed negative effects, such as neuronal degeneration, and these negative effects may contribute to cerebral dysfunction,[3] increased neuroapoptosis,[4] and impaired adult cognitive functioning.[5]

Consequences of early pain in humans include stronger pain responses during infancy,[6] long-term alterations in sensory and pain processing,[7] hyperalgesia to subsequent surgery after previous surgery in the first three months of life, especially if the tissue damage was in the same area[8] and more generalized hypoalgesia in preterm born children who received surgery in the neonatal period.[9] Neuroimaging studies in very prematurely born children showed altered neurodevelopment after repeated procedural pain, suggesting a relation between number of skin-breaking procedures and poorer corticospinal tract development,[10] reduced white and subcortical gray matter,[11] differences in functional brain activity,[12] and altered brain activation during pain.[13] This raises the question if such differences could be found in otherwise healthy children who receive large doses of opioids to reduce the pain from extensive tissue damage.

To answer this question we studied children born with a giant congenital melanocytic naevus (GCMN; Figure 1), which requires a very painful exchochleation procedure of the skin in the first weeks of life involving often more than one dermatome in otherwise healthy children. These children typically receive high dosages of opioids postoperatively according to standardized pain protocols.[8,14,15] This homogenous group of children serve as a model for extreme surgical pain and opioid exposure in early life. Since the brain develops considerably during this period, this intense pain and extensive tissue damage may have long-term effects on brain development. We performed structural and functional MRI (fMRI) to study the effects of early severe pain and opioid use on later brain morphology and functioning during pain processing, which is an important but yet largely understudied topic in humans. Our hypothesis, based on animal studies, was that extreme painful extensive tissue damage and associated exposure to opioids in early life would have negative long-term effects on pain sensitivity and brain development in humans as studies in rodents repetitively suggest.



a b

Figure 1 - Giant congenital melanocytic naevus
Before (a) and after surgery (b).

METHODS

Participants

Giant Congenital Melanocytic Naevus group

Most of the children with a GCMN in the Netherlands who require surgery are admitted and treated at the Erasmus MC-Sophia Children's Hospital in Rotterdam. The surgery is typically performed during the first six weeks of life, when the skin is more pliable. Eligible participants for this study were children between 8 and 18 years of age with a history of surgical removal of a GCMN during the first eight weeks of life. The postoperative analgesic treatment during intensive care admission was guided by earlier published pain management protocols[8,14,15] and started with dosages of 10 mcg/kg/hour in general. The electronic medical records showed that potentially thirty children could qualify. Exclusion criteria were no postoperative intensive care treatment, contra-indications for participation in an MRI study; brain abnormalities found on previous ultrasounds, CT, or MR scans (if available in the medical record), diagnosed neurologic disorders, or

gross motor or sensory disabilities (such as blindness or deafness) since these children could not properly understand the procedure and brain abnormalities would influence our structural and functional MRI results. Four patients who did not receive postoperative intensive care treatment were excluded, and two children hearing loss and a brain abnormality found on a previous MR scan (neurocutaneous melanosis around the amygdala) were also excluded. Twenty-four patients qualified for this study and received an informational letter. The families of five children declined participation. Two other children had permanent braces and could not participate in the MRI study. The families of these two children chose not to participate solely in the TSA test. Three children were lost to follow up. Thus, fourteen GCMN children were included in this study.

Control group

Healthy, normally developed children between 8 and 18 years of age were recruited through two different mechanisms. First, we asked all participants whether they could recommend someone in the age range of 8-18 years who would be interested in volunteering. In some cases, siblings or relatives of the GCMN group were invited as a control. Second, we mailed invitation letters to parents of children attending a primary school in Rotterdam. Parents were asked to contact the researcher in case of questions or to make an appointment for the study. Exclusion criteria were a history of severe early pain, mental disorders, monozygotic twins, diagnosed neurologic disorders, gross motor or sensory disabilities, or other specific contra-indications for an MRI study such as permanent braces. In the latter case, children were given the option to participate only in the behavioral component of the study. The use of psychoactive medication on the day of MRI scanning was a contraindication for the fMRI experiment since this could specifically influence brain activation. We included three times as many controls since oversampling the control group allows for a better modeling of the typical variation and decreasing the probability of type I errors. Thus, 42 controls were included.

The study was performed at the Erasmus MC in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board at the Erasmus MC. Informed consent was obtained from the parents of each subject prior to participation. Informed assent was also obtained from children 12 years of age and older prior to participation. Recruitment into the study took place from June 2011 to October 2012.

Procedure

First, all subjects completed a chronic pain questionnaire[16] and participated in a mock scanner session for approximately thirty minutes, allowing them to become accustomed to the noise and experience of a clinical MRI scanner. When the child successfully

completed this procedure, we determined the thermal detection- and pain thresholds. Hereafter, the MRI scans were obtained. The structural T_1 scan was acquired first, followed by two functional scans.

Examination of the individual pain thresholds

The individual detection- and pain thresholds were obtained using the MRI-compatible, computer-controlled Thermal Sensory Analyzer (TSA type II, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel) with a Peltier-based contact thermode (30 x 30 mm). The entire thermode-stimulating surface was placed in contact with the skin-testing site and was firmly secured by a Velcro band.

Before the detection- and pain thresholds were determined, skin temperature of the thenar eminence of the child's non-dominant hand was measured using a skin thermometer. We also measured room temperature to investigate if the test environment was the same for every subject and tested the child's reaction time with a subtest of the Amsterdam Neuropsychological Tasks (ANT).[17] After explaining the TSA test, we determined detection- and pain thresholds using a standardized protocol (see supplemental Methods 1).

Pain intensity and unpleasantness scores

Pain intensity of the thermal stimuli applied before and during the fMRI scans were measured using a numerical rating scale (NRS). In addition, perceived unpleasantness of the stimuli was measured during the fMRI scans. We asked the children to give a mean score for the painfully hot stimuli after each run. Once outside the MRI scanner, the subjects were again asked to rate the pain intensity of the painfully hot stimuli experienced during the fMRI (mean score for all the painful stimuli for both runs). Subjects were asked to verbally report a number between 0 (no pain at all / not unpleasant at all) to 10 (worst imaginable pain / extremely unpleasant) in response to the questions (presented in Dutch): 'How much pain did you experience?' and 'How unpleasant was the pain stimulus?' A pain intensity rating of 4 was considered to reflect pain of clinical concern.[18]

Chronic pain questionnaire

All participants filled out the Dutch chronic pain questionnaire,[16] measuring the incidence of chronic pain. Chronic pain is defined as recurrent or continuous pain for more than three months.

Image acquisition and structural and functional imaging analyses

MR images were acquired on a 3 Tesla scanner (Discovery MR750, General Electric, Milwaukee, MI, USA), and analyses were conducted using the Freesurfer image analysis suite version 5.1.0 for the structural MRI analyses (http://surfer.nmr.mgh.harvard.edu/)

and FMRIB's fMRI Expert Analysis Tool FEAT (http://www.fmrib.ox.ac.uk/fsl/feat5/index.html) for the fMRI analyses. The full description of the MRI analyses can be found in Supplemental Methods 2.

Statistical analysis

Normally distributed variables are presented as mean (standard deviation) and non-normally distributed variables as median (range). Differences in demographic characteristics, detection- and pain thresholds and NRS scores between cases and controls were determined with independent samples t-test for continuous data and Fisher's Exact tests for categorical data. Furthermore, all TSA outcome measures were also corrected for age, using an ANCOVA and logistic regression test. The correlation between total morphine exposure in the GCMN group and detection- and pain thresholds, and brain volumes was determined using Spearman rank order correlation coefficient. A p-value of less than 0.05 was considered statistically significant. Analyses were conducted using IBM SPSS 20.0.

RESULTS

Study Population

Fourteen GCMN children, nine boys and five girls with mean age 12.3 (SD 2.1) years participated in the study and were compared to twenty-two boys and twenty girls with a mean age of 11.6 (SD 2.4) (Table 1). The number of subjects included in each subtest are presented in Figure 2a,b. Demographic characteristics of all GCMN and control children are presented in Table 1. The median affected surface area was 18 percent of the total body. Furthermore, GCMN children received on average 26 mcg/kg/hour of morphine (range 5 - 146). Other clinical characteristics of the GCMN children at the time of surgery are presented in Table 2.

Detection and pain thresholds

Reliable data on detection and pain- thresholds were obtained from fourteen GCMN children and forty-one controls. Univariate analysis showed no differences in detection thresholds between cases and controls obtained using either the MLI or MLE. Pain thresholds obtained with the MLI were not statistically different between both groups, both corrected and uncorrected for age (Table 3).

No statistically significant correlations between total morphine exposure and detection thresholds (MLI and MLE) and pain thresholds were found in the GCMN group. Specifically, the positive and negative correlation coefficients indicated weak associations varying between 0.01 and 0.17. Mean reaction time (p=0.97), skin temperature (p=0.62) and

Table 1 - Demographic characteristics

		Surgical group (GCMN)	Control group	P value
Total group (N=56)		N=14	N=42	
Age (Mean (SD))		12.3 (2.1)	11.6 (2.4)	0.35
Gender (male %)		64.3	52.4	0.54
Ethnicity (Western European %)		100	76.2	0.05
Handedness (%)	Right	85.7	97.6	0.15
	Left	14.3	2.4	
Structural MRI analysis (n=43)		N=13	N=30	
Age (Mean (SD))		12.3 (2.1)	11.9 (2.4)	0.58
Gender (male %)		69.2	46.7	0.20
Ethnicity (Caucasian %)		100	80.0	0.16
Functional MRI analysis (n=35)		N=10	N=25	
Age (Mean (SD))		12.9 (1.9)	12.0 (2.7)	0.35
Gender (male %)		70.0	60.0	0.71
Ethnicity (Caucasian %)		100	76.0	0.15

P-values were derived from Independent samples T-test test for continuous variables and Fisher's Exact test for categorical variables
GCMN: Giant Congenital Melanocytic Naevus

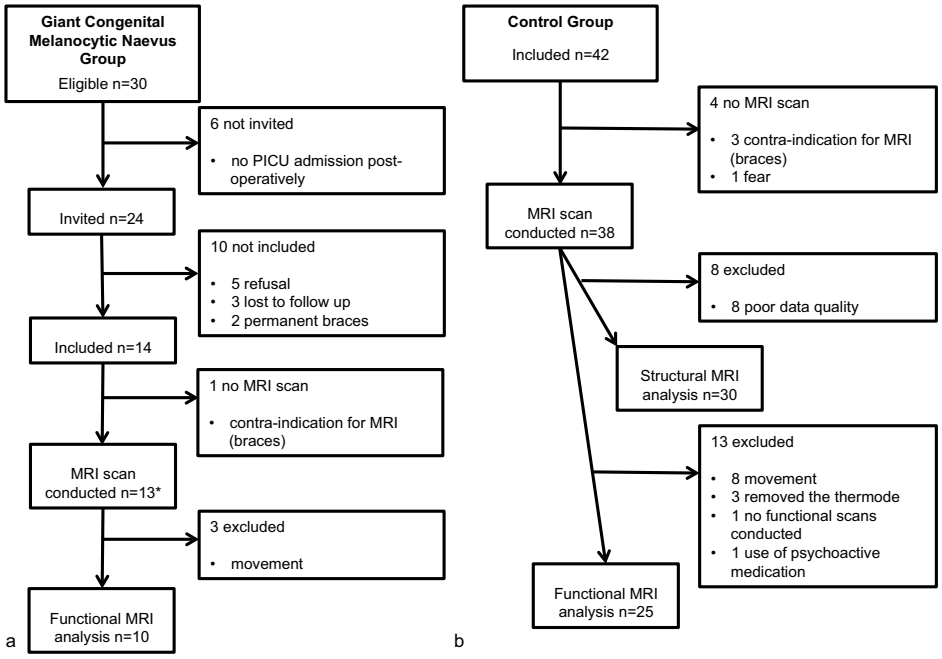


Figure 2a,b – Inclusion flowcharts
Inclusion flowchart of the Giant Congenital Melanocytic Naevus group (a) and the control group (b).
* All subjects were included in the structural analysis

Table 2 - Clinical characteristics of the surgical group

		Surgical group (GCMN) N=14
General characteristics		
Gestational age in weeks (median, range) *		40.4 (35.3 - 41.6)
Birth weight (grams, median, range) *		3540 (2500 - 5000)
Surgery		
Age at time of surgery in days (median, range)		31 (10 - 53)
Total body surface area in % (median, range) **		18 (5 - 30)
Location of the Tierfell Naevus (%)	Back	35.7
	Face or skull	28.6
	Chest and arm(s)	14.3
	Chest and leg(s)	14.3
	Legs	7.1
Postoperative phase		
Age at ICU admission in days (median, range)		31 (10 - 53)
Duration of ICU stay in days (median, range)		8 (2 - 36)
Total duration of hospital stay in days (median, range)		18 (7 - 46)
Postoperative need for mechanical ventilation (% yes)		64.3
Duration of mechanical ventilation in days (median, range)		6.5 (4 - 11)
Total use of IV morphine perioperative in mcg/kg (median, range) ***		2766 (241 - 14973)
Total use of IV midazolam postoperatively in mg/kg (median, range) ****		9.7 (0 - 58)

** Based on n=8 due to missing data*
*** Based on n=9 due to missing data*
**** In 4 children the medical record was incomplete and therefore the actual morphine dose could be higher than reported*
***** In 2 children the medical record was incomplete and therefore the actual midazolam dose could be higher than reported*
GCMN: Giant Congenital Melanocytic Naevus

room temperature did not differ between groups (p=0.74). Furthermore, there were no statistically significant differences in detection- and pain thresholds between boys and girls in both the GCMN and the control group.

Pain intensity and unpleasantness scores

All participants rated the pain intensity of the painful stimulus presented outside the MRI scanner (after the TSA test), even though some of these children did not participate in the MRI session. The mean score of the GCMN children (4.2 SD 2.7) and the controls (4.7 SD 3.8) did not significantly differ (p=0.63). The mean pain scores over two runs in children included in the fMRI analysis also did not significantly differ between cases (2.5 SD 2.8) and controls (3.5 SD 3.0; p=0.38). Forty-three percent of all subjects in the fMRI analysis (30% of the cases and 48% of the controls) described a mean pain score of 4 or

higher (suggestive of 'substantial pain'). Also the mean scores for unpleasantness were not significantly different between the groups (cases 1.7 SD 2.1, controls 2.9 SD 2.8; p=0.20). There were no significant differences in pain and unpleasantness scores for the runs that were excluded from the fMRI analysis (pain: p=0.90; unpleasantness: p=0.56). The pain scores afterwards also did not differ between cases (1.8 SD 2.6) and controls (3.3 SD 3.0; p=0.17).

Table 3 - Detection- and pain thresholds

	Surgical group (GCMN)	Control group	P value (uncorrected *)	P value (corrected for age **)
Method of Limits (MLI)	N=14	N=41		
Cold detection threshold (°C, mean (SD))	29.2 (3.7)	30.2 (3.0)	0.32	0.25
Warm detection threshold (°C, mean (SD))	35.2 (3.4)	34.0 (1.8)	0.24	0.08
Cold pain threshold (°C, mean (SD))	6.6 (7.2)	9.6 (8.6)	0.26	0.18
Threshold not reached (n, %)	5 (35.7)	18 (43.9)	0.76	0.99
Heat pain threshold (°C, mean (SD))	45.5 (4.4)	46.1 (4.0)	0.62	0.71
Threshold not reached (n, %)	4 (28.6)	19 (46.3)	0.35	0.59
Method of Levels (MLE)	N=14	N=41		
Cold detection threshold (°C, mean (SD))	30.5 (2.5)	30.7 (1.4)	0.65	0.52
Number of stimuli (mean (SD))	10 (3)	11 (3)	0.48	0.50
Warm detection threshold (°C, mean (SD))	33.7 (0.9)	33.6 (1.0)	0.77	0.58
Number of stimuli (mean (SD))	11 (5)	10 (3)	0.15	0.18

** P-values were derived from Independent samples T-test test for continuous variables and Fisher's Exact test for categorical variables*
*** P-values were derived using ANCOVAs correcting for age for continuous variables and logistic regression analyses for categorical variables*
GCMN: Giant Congenital Melanocytic Naevus

Chronic pain

Twelve (85.7%) of the children in the GCMN group experienced pain in the three months before the visit compared to 27 children (64.3%) in the control group (p=0.19). Three GCMN children (21.4%) had chronic pain (a duration longer than three months) compared to eight (19.0%) controls (p=1.0).

Structural imaging results

We found a significant thicker cortex in cases compared to controls in the left rostral-middle-frontal pole, corrected for age and gender (Figure 3). This difference was present after correcting for multiple testing and involved 954.52 mm^2. Total brain volume did not differ between cases (1250 cm^3 SD 127) and controls (1178 cm^3 SD 117; p=0.26) (Table 4).

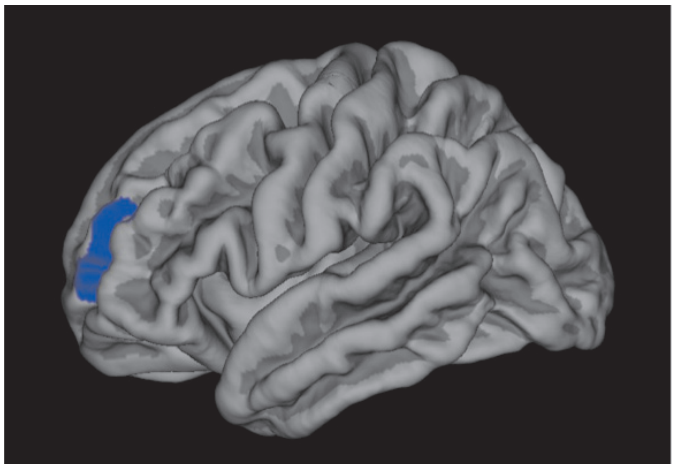


Figure 3 - Cortical thickness
Differences in cortical thickness in the left hemisphere in which cases have a statistically significant thicker cortex compared to controls in the rostral-middle-frontal pole.

Table 4 - Global brain volumes and volumes of pain related brain regions

		Surgical group (GCMN)	Control group	P value*	P value**
Global Brain Volumes		N=13	N=30		
Total Brain Volume (Mean (SD), cm^3)		1250 (127)	1178 (117)	0.26	NA
Cerebral White Matter (Mean (SD), cm^3)		439 (62)	406 (58)	0.28	0.94
Total Gray Volume (Mean (SD), cm^3)		761 (67)	728 (60)	0.38	0.59
Parietal lobe (Mean (SD), mm^3)	Left	78141 (5693)	73889 (7058)	0.15	0.37
	Right	79466 (7231)	76148 (6839)	0.40	0.77
Cerebellum (White Matter) (Mean (SD), mm^3)	Left	15989 (2359)	15288 (2179)	0.71	0.76
	Right	16402 (2195)	14912 (2063)	0.09	0.20
Cerebellum (Cortex) (Mean (SD), mm^3)	Left	59721 (9295)	57059 (5915)	0.81	0.70
	Right	59796 (8481)	57282 (6320)	0.91	0.66
Pain Related Brain Regions		N=13	N=30		
Thalamus (Mean (SD), mm^3)	Left	7530 (871)	7242 (875)	0.79	0.58
	Right	7757 (1143)	7269 (705)	0.27	0.58
Amygdala (Mean (SD), mm^3)	Left	1606 (315)	1639 (308)	0.34	0.13
	Right	1785 (285)	1795 (292)	0.35	0.13
Anterior Cingulate Cortex (Mean (SD), mm^3)	Left	2583 (485)	2379 (541)	0.43	0.75
	Right	3014 (931)	2543 (594)	0.09	0.19
Insula (Mean (SD), mm^3)	Left	7941 (816)	7592 (859)	0.56	0.85
	Right	7411 (979)	7564 (809)	0.16	**0.02**

** P-values were derived from ANCOVA test (correction for age and gender)*
*** P-values were derived from ANCOVA test (correction for total brain volume, age and gender)*
NA: Not applicable
GCMN: Giant Congenital Melanocytic Naevus

When specifically comparing volumes of pain related brain areas, only the right insula was significantly smaller in the GCMN group, after correction for total brain volume. This difference was modest (cases 7411 mm^3 SD 979, controls 7564 mm^3 SD 809; p=0.02), and disappeared after Bonferroni correction for multiple testing. Only the right anterior cingulate cortex was significantly correlated with total morphine exposure (Spearman rank coefficient 0.56, p=0.05), although it was insignificant after correction for multiple testing.

Functional imaging results

Nine cases and eighteen controls with two runs and one case and seven controls with one run were included in the fMRI analysis. The group analysis without correction for age and gender revealed that the painful stimulus of 46°C induced statistically significant activation in several brain areas in the GCMN group, including the right motor area and the insula (Figure 4-a). Painful stimuli also induced significant brain activation in the control group in the right motor area (Figure 4-b). A direct comparison revealed statistically significant increased activation bilaterally in the parietal and occipital lobe in cases (Figure 4-c and Table 5). After correction for gender and age the intensity of the activation was reduced in both groups and no longer significantly different. When excluding the one left-handed subject in the analysis (a case with two runs), we found comparable results as in the whole group presented in Figure 4.

Table 5 - Areas of activation - direct comparison

Cluster size (voxels)	P-value	MNI coordinates local maxima (mm)			Z-value	Anatomical area
		X	Y	Z		
2807	0.01	36	−64	58	4.84	Lateral Occipital Cortex (R)
		26	−60	68	3.87	
		20	−64	68	3.71	
		30	−54	56	4.57	Superior Parietal Lobule (R)
		32	−54	62	4.35	
		26	−48	44	4.04	
2073	0.04	−30	−72	60	3.82	Lateral Occipital Cortex (L)
		−28	−68	58	3.72	
		−26	−72	50	3.66	
		−36	−72	56	3.54	
		−38	−48	64	3.42	Superior Parietal Lobule (L)
		−36	−46	68	3.40	

Areas of activation (GCMN group > control group during pain) with cluster size, Z-values of the local maximum, Montreal Neurological Institute (MNI) coordinates, and the anatomical area of the local maximum (Harvard-Oxford Cortical Structural Atlas). R: Right, L: Left

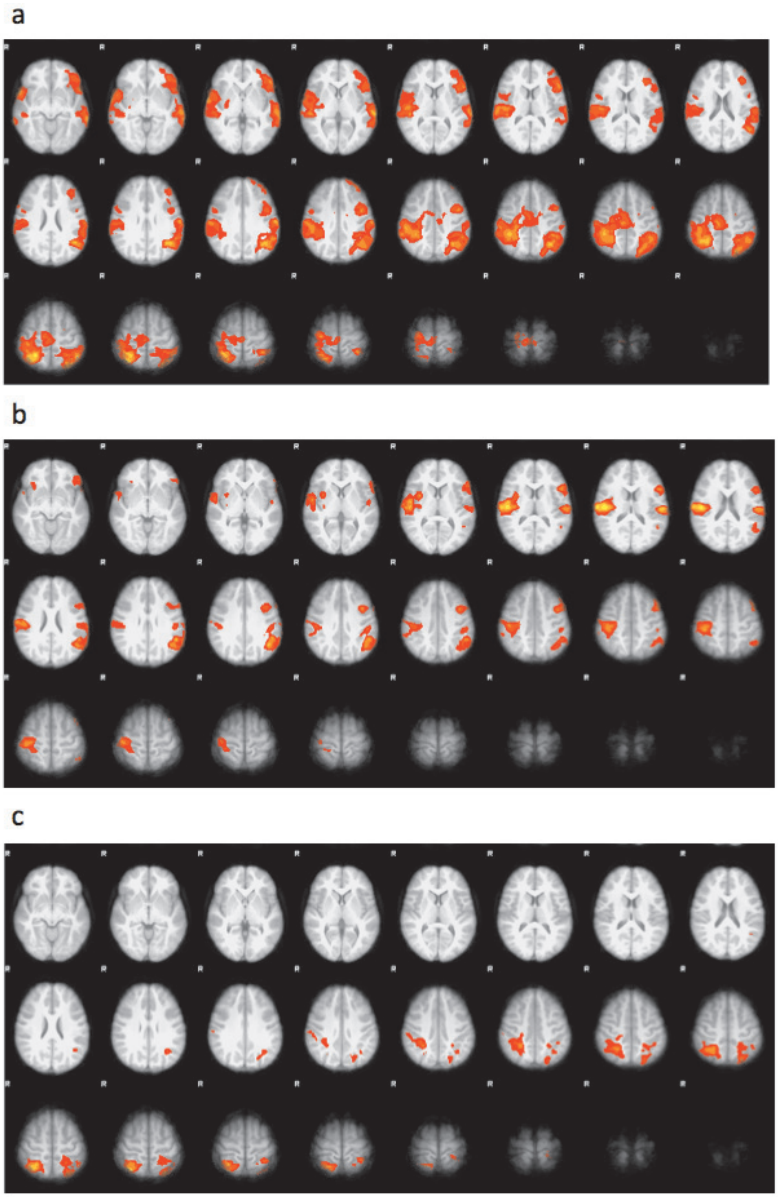


Figure 4 - Brain activation during pain
The axial slices show areas of activation during pain in the Giant Congenital Melanocytic Naevus group (a), the control group (b) and the direct comparison between both groups (GCMN>controls) (c) using a cluster significance threshold of $p<0.05$.

DISCUSSION

The overall purpose of this study was to determine the long-term effects of intense pain due to extensive tissue damage in the first weeks of life. Although children undergoing painful procedures currently receive adequate analgesic medications, most children are likely to experience major breakthrough pain. Thus, studying the combination of early intense pain and opioid use provides a scenario that is applicable to modern clinical care and takes into account present standards of pain management according to international guidelines and ethical principles. We found significantly greater brain activation during painful stimuli in cases, mainly in the parietal lobe, which may suggest subtle differences in sensory processing. However, these differences did not remain significant after correction for age and gender, possibly due to loss of power. Furthermore, a significant thicker cortex was found in the cases compared to controls in one specific brain region in the left hemisphere. No differences in brain volumes or in detection or pain thresholds were found between children with a history of surgical removal of a giant congenital melanocytic naevus 8 to 15 years earlier compared to healthy controls.

We found a thicker cortex in GCMN children in one brain region, namely the rostral-middle-frontal cortex compared to healthy controls. Since cortical thickness is associated with intelligence, in which a higher IQ is associated with faster thinning in childhood and a thicker cortex in adulthood this warrants further investigation.[19] However, no structural MRI differences in global brain morphology or in the volumes of pain related brain areas between both groups were observed and only cortical thickness of one brain region (right anterior cingulate cortex) was significantly positively correlated with total morphine exposure. The latter could possibly be explained because children who received more morphine experienced less breakthrough pain, which may have a less negative influence on brain morphology. Previous structural MRI studies in children with GCMN used qualitative approaches and found neurocutaneous melanosis.[20] One child in our study was excluded due to neurocutaneous melanosis. Our finding of a minor difference in cortical thickness and no differences in brain volumes in children with a history of severe pain is in line with adult studies that found a reversal of gray matter volume after pain relief.[21] The reversal of a decrease in gray matter volumes due to pain was found in patients who were successfully treated for chronic back pain and hip osteoarthritis.[22-25] Thus, it may be that differences in gray matter volume were present early in life, but resolved when there was no prolonged period of pain.

To our knowledge only one previous study utilized fMRI to determine the long-term consequences of early pain on brain function during pain,[13] and found significant differences in activation patterns in 11- to 16-year-old preterm born children with a history of NICU

admission compared to controls. They did not find the effect in former full term NICU children, suggesting a specific developmental window for the occurrence of long-term effects on pain processing. Another possibility is that the effects of prematurity rather than neonatal pain and opioid exposure caused the effect. We demonstrated subtle differences in brain activation between cases and controls, perhaps because the surgical pain was more intense than the procedural pain experienced by the full term NICU group of Hohmeister et al. It is interesting that the differences that we found between both groups were not specifically located in the pain centers of the brain, but rather in sensory regions. Since primary cortical areas typically develop earlier than secondary or tertiary brain regions,[26] it is possible that early pain and treatment with morphine resulted in activity dependent neuronal changes in the primary and secondary sensorimotor cortical regions. The difference in activation was not a result of volumetric differences, as we found no significant differences between the volumes of the parietal lobes. After correction for age and gender, the statistically significant difference disappeared, probably because of the loss of power in this specific analysis. Detection- and pain thresholds did not differ between groups, suggesting that pain sensitivity is not affected by early pain and opioid use, while three previous studies have shown contrasting findings. These findings included global hyposensitivity after cardiac surgery,[27] higher pain thresholds and greater perceptual sensitization after severe burn injuries,[7] and greater perceptual sensitization and elevated pain thresholds after NICU admission.[28] The occurrence of chronic pain in our study was comparable between cases (21.4%) and controls (19.0%) and slightly lower in comparison with Dutch reference values for chronic pain (23.7% in 8-11 years old, 35.7% in 12-15 years old, and 31.2% in 16-18 years old subjects).[16]

While histological animal studies suggest dramatic alterations in number of brain cells after early pain or supratherapeutic dosages of opioid administration, we found significant differences in cortical thickness in only one brain region and no differences in brain volumes in our study, although correlated histological studies in animals with MRI studies in humans should be performed with caution. Pain stimuli in neonatal rats induced hypersensitivity,[29] alterations in somatosensory thresholds[1] and neuroapoptosis.[2] Interestingly, preemptive morphine has been shown to decrease the neurological damage[2] and reduced the negative long-term effects of inflammatory injury.[1] Opioids given in the absence of pain are also associated with negative effects in animals such as apoptosis in brain regions associated with sensory and emotional memory functioning,[4] and hypersensitivity.[30] Extrapolating animal neurodevelopment to human neurodevelopment is complicated. In addition to histological studies being different from MRI studies, rats are born at a relatively early stage of brain maturation. Therefore, the brain of a neonatal rat pup roughly corresponds to that of a prematurely born child, rather than a term born child.[31] Furthermore, rodents receive supratherapeutic doses of opioids in the

absence of pain, or pain stimuli in the absence of opioids. The children in our study had been exposed to both intense pain and opioids; and the opioids may have ameliorated the negative effects of early pain. It would be very interesting to distinguish between the long-term effects of neonatal pain and opioid exposure in humans, but due to obvious ethical reasons, it is not feasible to study children with pain without treatment of analgesics. Moreover, the effects of pain in rodents are often measured shortly after the painful procedure and any changes in pain perception or brain morphology may have been only transient ones. Animal studies have shown age-dependent effects of pain on neurodegeneration.[2]

The strength of our study is that we provide a comprehensive view of the long-term effects of early pain and opioid use. This study also has limitations. First, as GCMN is rare, the case group is relatively small, but slightly larger than that in the only previous fMRI pain study who included 9 subjects in each group.[13] Even though the case group is small, it is a unique group without other confounding illness or pathology. Larger sample size would have been difficult to achieve since most of the patients with GCMN in the Netherlands are admitted to our hospital and therefore a multicenter approach was not possible. Two children were excluded due to a health condition. One had neurocutaneous melanosis, which is associated with GCMN and therefore not caused by pain or opioids. The second child had hearing loss, which may have been caused by opioid exposure since it is known that opium abuse has ototoxic effects.[32] Furthermore, we included children with a large age range that spans puberty. However, the age and gender distribution was not significantly different between cases and controls and we corrected for age in all analyses. Although NRS pain scores are widely used, we found that some children gave very different pain scores over the different time points for the same stimulus of 46°C. This could have been influenced by adaptation or environmental factors (before, during or after the scans, with or without the presence of parents). Our choice of 46°C was based on our prior study and was shown to provide adequate pain levels.[33] Higher temperatures would have probably caused more exclusion of subjects due to movement or fear.

In conclusion, we report greater brain activation in the parietal lobe during pain and a thicker cortex in the rostral-middle-frontal cortex in school-age children who in the first weeks of life underwent surgical removal of a GCMN. This may serve as a model for extensive tissue damage and associated severe pain and high dosing of opioids in term born children. However, their pain perception and brain volumes were not affected, perhaps due to the protective effects of opioids in the presence of pain. Our study provides information that is compatible with daily clinical practice. Future studies with larger sample sizes are needed to investigate the potential negative effects of slower thinning of the cortex in these children.

SUPPLEMENTARY DATA

Supplemental Methods 1

Detection thresholds were measured using both the reaction time dependent Method of Limits (MLI) and the reaction time independent Method of Levels (MLE). Detection- and pain thresholds for cold and warmth were first determined with the MLI technique. The thermode baseline temperature of 32°C was steadily lowered at a rate of 1°C/sec. The child was asked to press the button as soon as the cold stimulus was felt. We repeated this five times. The first two stimuli served as rehearsal stimuli. The detection threshold was calculated as the mean value of the last four temperatures. Next, the temperature was steadily increased at a rate of 1°C/sec to determine the detection threshold for warmth using the same technique.

This MLI technique was also applied to determine pain thresholds for cold and heat. Starting from the baseline temperature of 32°C, the temperature was steadily lowered at a rate of 1.5°C/sec. The child was asked to press the button when the cold sensation started to feel painful. After the child pressed the button, the temperature returned to 32°C at a rate of 10.0°C/sec. This was repeated four times. The first stimulus served as a rehearsal stimulus and the cold pain threshold was calculated as the mean value of the last four temperatures. Next, the pain threshold for heat was determined in the same manner. When the child did not press the button before the minimum temperature of 0°C or the maximum temperature of 50°C, the test automatically stopped. In that case, the cut-off temperature of 0°C or 50°C was used in the calculation of the mean threshold.

Next, we repeated the determination of the detection thresholds for cold and warmth with the MLE technique. The researcher told the child that the thermode would either become colder, or would not change in temperature. The first thermal stimulus was 3.0°C below the baseline temperature of 32.0°C. Following each thermal stimulus the researcher asked the child "Did the thermode become cold or not?" Depending on the answer, the next stimulus decreased with half of the previous step size from baseline, or decreased with the same step size estimated from the prior temperature. The test terminated when the step size had decreased to a level of 0.1°C. The number of stimuli needed to decrease the step size to 0.1°C was recorded. The warm detection threshold was determined in the same manner starting with a stimulus temperature of 3.0°C above the baseline temperature.

We finished the TSA-test by presenting one warm stimulus of 41°C and one potentially painful stimulus of 46°C. These temperatures were the same as the stimuli received during the fMRI scan, but the children were not informed of this. Children were asked to give a pain intensity score for both stimuli. The TSA tests were performed by the same researcher (GEvdB).

Supplemental Methods 2

Image acquisition

MR images were acquired on a 3 Tesla scanner (General Electric Discovery MR750, Milwaukee, MI, USA) using an 8-channel head coil for signal reception. A high-resolution structural T_1-weighted image was obtained using an inversion recovery fast spoiled gradient recalled (IR-FSPGR) sequence with the following parameters: TR = 10.3 ms, TE = 4.2 ms, TI = 350 ms, NEX = 1, flip angle = 16°, readout bandwidth= 20.8 kHz, matrix 256 x 256, imaging acceleration factor of 2, and an isotropic resolution of 0.9x0.9x0.9 mm^3. We conducted two runs of a fMRI paradigm using single-shot echo-planar imaging (EPI) T_2*-weighted sequences in transverse orientation sensitive to blood oxygen level dependent (BOLD) contrast (parameters: TR/TE 2000/30 ms, flip angle 85°, 64 x 64 matrix with a field-of-view of 260 x 260 mm^2; 39 slices and voxel sizes of 3.6 x 3.6 x 4.0 mm^3). Scan time was 6 min. 4 sec per run.

Functional MRI Block paradigm

The fMRI component consisted of two runs and utilized a block paradigm. During each of these two runs the TSA-II thermode was applied to the thenar eminence of the non-dominant hand and firmly secured with a Velcro band around the hand. Within each run, the temperature increased four times at a rate of 1.5°C/sec from the baseline temperature of 32°C to a warm temperature of 41°C and four times to a potentially painfully hot temperature of 46°C. These temperatures were derived from a previous study from our research group.[33] After each stimulus, the temperature decreased with 4.5°C/sec back to baseline and stayed at the baseline temperature for 15 seconds before the increasing to the next warm or pain stimulus. In order to prevent anticipation to the stimuli, the order and duration (8, 10, 12, 14 or 16 seconds) of the warm and hot stimuli was randomly determined at the beginning of the study and were different in both runs. However, the runs were the same for every subject. After each run we asked the child to provide a pain intensity and an unpleasantness score. During all scans, the children were monitored to assure that they followed our instructions.

Structural imaging analysis

Cortical reconstruction and volumetric segmentation was performed with the Freesurfer image analysis suite version 5.1.0 (http://surfer.nmr.mgh.harvard.edu/). Freesurfer computes these measures in an automated approach. Each image was visually inspected and subjects with poor quality data were excluded. In subjects with small errors in the gray/white segmentation, control points, and white matter edits were added to identify and correct misclassified white matter regions. When the segmentation improved, the corrected images were used. Evaluation of surface-based cortical thickness FreeSurfer was performed using the built-in program QDEC with a smoothing filter of 10 millimeter.

For the group analysis a general linear model (GLM) was fitted at each surface vertex. We corrected for age and gender and used a Monte Carlo correction ($p<0.05$) for multiple testing in the cortical thickness analyses. Total brain volume and volume of pain related brain regions, such as the thalamus, anterior cingulate cortex and insula,[34] were compared between cases and controls with ANCOVAs correcting for total brain volume, age, and gender using SPSS version 20.0. Furthermore, based on the functional imaging findings, we compared the volume of the parietal lobes between cases and controls and corrected again for age, gender and total brain volume. Bonferroni corrections for multiple testing were used in the analyses comparing brain volumes.

Functional imaging analysis

The functional images involved slice timing and motion correction using AFNI (http://afni.nimh.nih.gov/). Incomplete fMRI runs and runs with more than 6 mm of motion (maximum displacement) were excluded from the analyses. Runs of subjects who confessed that they pulled off the Velcro band with the thermode during the scans were also excluded. Functional images were co-registered to the structural image of the subject and both the functional and structural images were normalized using the Montreal Neurological Institute (MNI) 152 atlas using FSL's non-linear registration tool FNIRT. Finally, data were spatially smoothed using AFNI with an 8-mm full width at half-maximum Gaussian kernel. Following the preprocessing steps, single-subject analyses were performed using FMRIB's fMRI Expert Analysis Tool FEAT (http://www.fmrib.ox.ac.uk/fsl/feat5/index.html). The time series for the pain runs were modeled using a block design. Design matrices were created for both runs using the data from each subject's stimulus log file from the TSA. These matrices were created independently for each individual using an automated MATLAB program (MATLAB 7.1, The MathWorks Inc., Natick, MA, 2000). This modeled time series was convolved with the hemodynamic response function. Next, a general linear model was implemented using FMRIB's Improved Linear Model. The two within-subject runs were combined using a fixed effects model. The higher-level group analyses, which compared patients and controls for each of the contrasts; 46°C versus baseline, 41°C versus baseline, and 46°C versus 41°C, were performed using FMRIB's Local Analysis of Mixed Effects. We conducted the group analyses with and without correction for age and gender. We performed group analyses with and without the one left-handed case. Furthermore, we repeated the group analysis without children who rated a zero for pain during the stimulus of 46°C over the four time points; before the fMRI scans, during both runs and afterwards. Furthermore, we corrected for multiple comparisons using random Gaussian fields and significance was set at $p<0.05$ (two-tailed).

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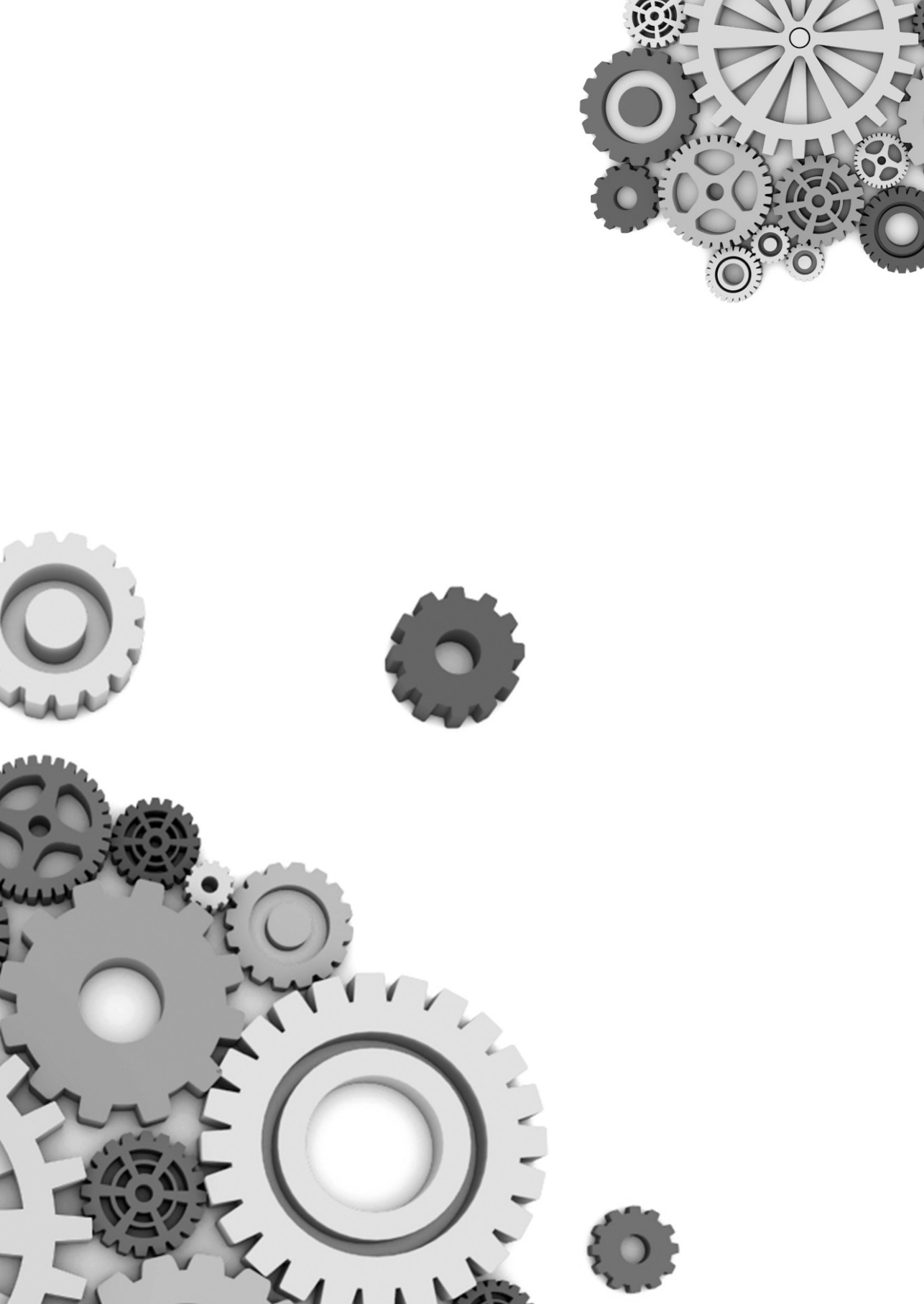

Chapter 7

Should we be concerned about exposure to anaesthetics and opioids in neonates?

A neuropsychological and neuroimaging exploratory study in humans

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ABSTRACT

Background Numerous studies in animals show neurotoxic effects of neonatal exposure to anaesthetic agents. In humans, however, the long-term effects of anaesthetics are largely unknown. In this neuroimaging study we studied signs of long-term effects of neonatal exposure to standardized amounts of anaesthetics and postoperative opioid exposure in humans.

Methods We included ten 14-17-year-old subjects who as neonates underwent major surgery and participated in a randomized-controlled trial comparing intermittent with continuous morphine administration. They were age-matched to ten healthy controls. Experimental thermal detection and pain thresholds were measured and neuropsychological functioning was assessed. Furthermore, we obtained high-resolution structural and functional Magnetic Resonance Imaging to measure brain morphology and functioning during pain.

Results No between-group differences in neuropsychological functioning and brain morphology were detected. However, cases were less sensitive to detect a warm stimulus compared to controls (mean detection threshold in cases 34.2 (1.4) versus 33.1 (0.6) in controls ($p=0.04$)). Furthermore, imaging showed significantly less brain activation in the occipital cortex in cases compared to controls during thermal pain stimuli.

Conclusions Besides thermal hyposensitivity and significantly less brain activation during pain, no other long-term effects of neonatal surgery and exposure to anaesthetics and opioids were found in this exploratory study. This suggests that, other than animal data imply, the neonatal surgery and exposure to anaesthetics in humans have only minor long-term effects. It is possible that the alarming findings in animals do not readily extrapolate to humans.

INTRODUCTION

Exposure to anesthetics and opioids in early life is suggested to be associated with negative long-term effects with as a result an ongoing debate with regard to postponing elective surgery in infants.[1-4] The fear for neurotoxic long-term effects is mainly based on rodent studies, which have reported neuroapoptosis, cognitive problems and abnormal social behavior after anesthesia with blockade of N-methyl-D-aspartate (NMDA) glutamate receptors as well as with gamma-amino butyric acid (GABA) receptor agonists.[5-10] In non-human primates, exposure to anesthetics induced neuroapoptosis as well.[11-13] Besides anesthetics, early opioid exposure is also associated with increased neuroapoptosis and impaired cognitive functioning in animals.[14,15] The same yields true for neonatal pain which has also negative long-term effects in the developing brain in rodents.[16] In human, exposure to anesthetics in infancy is associated with an increased rate of learning disabilities and behavioral problems,[17-19] although some studies did not find differences in cognitive and educational outcome.[20] With regards to pain sensitivity, surgery in the first months of life induced hyperalgesia.[21] In humans there is often a clinical need for operations, resulting in an unpreventable combination of anesthetics and preemptive analgesia. As a consequence studies conducted in humans are important, but so far scarce.

We conducted a prospective follow-up study among adolescents exposed to major surgery under general anesthesia as neonates and who participated in an RCT of postoperative opioid use.[22,23] In contrast to other studies, anesthetic exposure and opioid administration were standardized and thus well-quantified. We hypothesized that exposure to anesthetics and related exposure to pain and analgesics would negatively influence, brain volume, brain functioning and neuropsychological outcomes during adolescence.

METHODS

The original randomized controlled trial

The original double-blind RCT, in which the case subjects had been enrolled as neonate, was conducted between 1995 and 1998 in the Erasmus MC-Sophia Children's Hospital, Rotterdam, the Netherlands. We have published the full details previously.[22,23] The children were aged 0 to 3 years -old and received either 10μg kg^{-1} h^{-1} morphine continuous intravenous (IV) infusion or 30μg kg^{-1} every 3 hours in IV boluses (intermittent) for at least 24 hours after major abdominal or non-cardiac thoracic surgery. Both strategies were equally efficacious for children below one year.[20] The anesthetic treatment in cases was standardized according to the guidelines of the original RCT.[23] This included induction of anesthesia with IV thiopentone 3-5 mg kg^{-1} or by inhalation of halothane in

oxygen. Before orotracheal intubation, the neonates received 5 μg kg^{-1} fentanyl, which was facilitated with atracurium 0.5-1 mg kg^{-1} or suxamethonium 2mg kg^{-1}. The ventilation was controlled and the anesthesia was maintained with isoflurane 0.5 minimum alveolar concentrations in 60% nitrous oxide in oxygen or air in oxygen. Before surgical incision, the neonates received a second dose of 5 μg kg^{-1} fentanyl. Additional doses of fentanyl 2 μg kg^{-1} were given based on heart rate and mean arterial blood pressure. The neuromuscular block was antagonized at the end of surgery. Directly after surgery all patients received 100 μg kg^{-1} of morphine followed by either a morphine infusion of 10 μg kg^{-1} h^{-1} or three-hourly intravenous doses of 30 μg kg^{-1} starting with the first bolus three hours after surgery. Additional morphine was given in case of signs of pain.[23]

Follow-up study

Cases

Out of the 204 children enrolled the initial RCT, 19 died. Eligible for the present study were 62 of the remaining 185 children, i.e. the youngest age group who underwent major surgery in the first month of life. Seven of those 62 had been lost to follow-up, and 23 had a known contra-indication for participation in a neuroimaging and neuropsychological study. These contra-indications were mainly attributable to congenital problems or other medical factors that were not a potential effect of anesthesia. Thirty-two subjects received an information letter and were invited to participate (See Flowchart Figure 1).

Control group

Controls within an age range six months younger to six months older than the age range of the case group were recruited from a group of 75 healthy children and adolescents without a history of severe early pain who served as controls for this and other follow-up studies within our department. We mailed an invitation letter to potential candidates who were interested in our study and telephoned two weeks later to ask if they were still interested to participate in our study. We also mailed invitation letters to parents of healthy children attending schools in Rotterdam.

The study was performed at Erasmus MC in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board at the Erasmus MC. Informed consent was obtained from the parents of each adolescent, and assent was obtained from the participants themselves. Recruitment took place from January 2012 to March 2013. Children who had a contraindication for participation in the MRI study (i.e., pacemaker or permanent braces) were given the option to participate in all other assessments. The use of psychoactive medication on the day of MRI scanning was an exclusion criterion for the fMRI experiment.

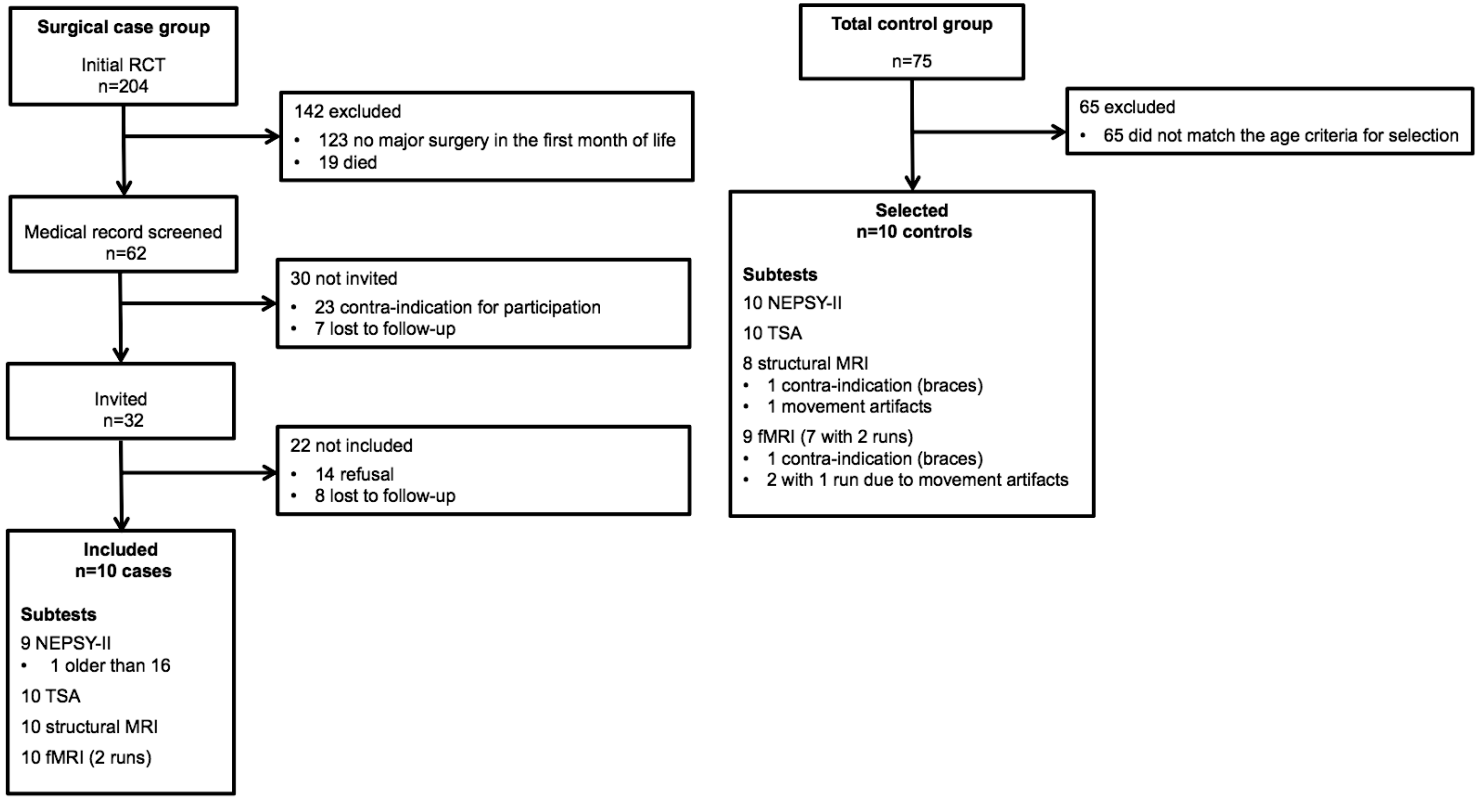

Figure 1 - Inclusion flowchart

Procedure

Neuropsychological testing

Participants under the age of 17 years first conducted a neuropsychological test, the NEPSY-II.[24] This test has been validated for children and adolescents between 3 and 16 years old, and therefore it was not administered to 17-year-old participants. Participants completed six subtests and one delayed test, which took approximately 30-45 minutes in total, and included several domains of cognitive functioning such as attention and executive functioning, language, memory and learning, and visuospatial processing.

Chronic pain questionnaire

The Dutch chronic pain questionnaire[25] addresses whether the participants are currently having pain and whether this pain has a duration of more than three months which is defined as chronic pain.[25]

Examination of the individual pain thresholds

Individual detection- and pain thresholds were obtained and pain stimuli were applied using the MRI-compatible, computer-controlled Thermal Sensory Analyzer (TSA type II, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel) with a Peltier-based contact thermode (30 x 30 mm). First we measured the skin temperature of the thenar eminence of the child's non-dominant hand and the room temperature to investigate if the test environment was the same for every subject. We also tested the child's reaction time with a subtest of the Amsterdam Neuropsychological Tasks (ANT)[26] since one of the thermal threshold subtests is reaction time dependent (Method of Limits; MLI). After explaining the thermal threshold test, we determined detection- and pain thresholds using a standardized protocol using both the reaction time dependent Method of Limits (MLI) and the reaction time independent Method of Levels (MLE). Furthermore, the pain intensity of a standardized thermal stimulus of 46°C was measured using a numerical rating scale (NRS), for more details see van den Bosch et al.[27]

Image acquisition and analyses

For detailed information with respect to image acquisition and analyses see the supplementary data (see also Figure 2).

Non-imaging statistical analysis

Normally distributed variables are presented as mean (standard deviation) and non-normally distributed variables as median (range). We used independent samples t-tests and Mann-Whitney U tests for continuous data and Fisher's exact tests for categorical

data. All tests were conducted with a two-sided significance level. Bonferroni correction was used to correct for multiple testing. A p-value of 0.05 or less was considered statistically significant. Analyses were conducted using IBM SPSS 20.0.

RESULTS

Study population

Thirty-two families received an information letter. Eight families could not be reached by phone, possibly because they had moved. Another fourteen families declined participation, mostly because the adolescent felt not inclined (Figure 1). Ten adolescents with a median age of 15.5 (range 14.5 - 17.0) years participated in the study. Ten controls were matched to the patient group based on age and had a median age of 15.1 (range 14.0 - 17.0) years (Table 1). One of the controls was a sister of a case. Demographic characteristics of the participants are presented in Table 1 and clinical characteristics of the cases in Table 2. The numbers of subjects included per subtest are presented in Figure 1.

Table 1 - Demographic characteristics

	Case group	Control group	P-value
Total group (N=20)	N=10	N=10	
Age (median (range))	15.5 (14.5 - 17.0)	15.1 (14.0 - 17.0)	0.60
Gender (male %)	80.0	60.0	0.63
Handedness (right handed %)	80.0	90.0	1.0
Ethnicity (western European %)	90.0	90.0	1.0

P-values were derived from Mann-Whitney U tests for continuous variables and Fisher's exact tests for categorical variables

Neuropsychological functioning

All but one 17-year-old case completed the six subtests of the NEPSY-II. The oldest control participated two weeks before she turned 17, and therefore conducted the NEPSY-II. Results did not significantly differ between cases and controls (Table 3 - uncorrected).

Chronic pain

Two cases (20%) reported to have pain for longer than three months, versus three controls (30%). The chronic pain was in the back, knee, or shoulder.

Table 2 - Clinical characteristics of the case group

		Case group N=10
General characteristics		
Gestational age in weeks (median, range)		38.3 (33.2 - 41.0)
Preterm born (n)		3
Birth weight (grams, median, range)		3178 (2200 - 4230)
Total score surgical stress * (median, range)		8.5 (6 - 15)
Age at ICU admission (days, median, range)		1.5 (0 - 29)
Age during surgery (days, median, range)		3.5 (1 - 30)
Surgical diagnosis (n)	Diaphragmatic hernia	3
	Malrotation	2
	Oesophageal atresia	1
	Malignancy (sacrococcygeal teratoma)	1
	Bladder exstrophy	1
	Perforation of the ductus choledochus	1
	Omphalocele	1
Mechanical ventilation postoperatively (% yes)		70
Pharmacological data		
Additional morphine administration first 24 hours (n (%) yes)		3 (30)
Cumulative morphine dose first 24 hours (μg kg^{-1} h^{-1}, median, range) **		10.0 (10.0 - 11.2)

** The surgical stress score measures the severity of surgical stress in neonates and has a range from 3-22, for more information see van Dijk et al. 2002 [1].*
*** Based on n=9 since one child was removed from the original RCT after 6 hours postoperatively due to incidental removal of the arterial line.*

Table 3 - Neuropsychological outcomes

NEPSY-II Subtests		Case group N=9	Control group N=10	P-value
Attention and executive functioning				
Auditory Attention (median (IQR))	Commission errors	0 (0 - 0)	0 (0 - 0)	0.34
	Omission errors	0 (0 - 0)	0 (0 - 0)	1.0
	Inhibitory errors	0 (0 - 0)	0 (0 - 0)	1.0
Response Set (median (IQR))	Commission errors	0 (0 - 1)	0 (0 - 2)	0.57
	Omission errors	0 (0 - 1)	1 (0 - 3)	0.06
	Inhibitory errors	0 (0 - 0)	0 (0 - 0)	0.56
Language				
Word Generation (total score, median (IQR))		36 (25 - 46)	48 (38 - 50)	0.09
Memory and learning				
Memory for Faces (total score, median (IQR))		11 (10 - 14)	12 (11 - 14)	0.48
Memory for Faces Delayed (total score, median (IQR))		13 (12 - 15)	14 (11 - 14)	1.0
Visuospatial processing				
Arrows (total score, median (IQR))		32 (32 - 35)	32 (29 - 33)	0.26
Geometric Puzzles (total score, median (IQR))		35 (33 - 38)	36 (32 - 37)	0.84

P-values were derived from Mann-Whitney U tests
Minimum and maximum scores of the subtests are: Auditory Attention commission errors: 0-180, omission errors: 0-30, inhibitory errors 0-35, Response Set commission errors: 0-180, omission errors: 0-36, inhibitory errors: 0-37, Word generation: 0-no maximum, Memory for faces: 0-16, Memory for faces delayed: 0-16, Arrows: 0-38, and Geometric puzzles: 0-40.

Figure 2 - Block design of both runs

Detection and pain thresholds

The mean MLE warmth detection threshold differed significantly between cases and controls, indicating that cases were less sensitive to warmth (Table 4 - uncorrected). The mean MLE cold detection threshold was not statistically different. Detection and pain thresholds obtained with the MLI technique were also not statistically different between both groups. NRS intensity scores for the painful stimulus of 46°C did not significantly

Table 4 - Detection- and pain thresholds

		Case group N=10	Control group N=10	P-value
Method of Limits (MLI)				
Cold detection threshold (°C)	mean (SD)	30.9 (0.9)	31.0 (0.4)	0.73
	median (IQR)	31.1 (30.8 - 31.4)	31.1 (30.7 - 31.3)	
Warm detection threshold (°C)	mean (SD)	33.6 (0.9)	33.4 (0.9)	0.54
	median (IQR)	33.3 (33.1 - 33.9)	33.0 (32.8 - 33.9)	
Cold pain threshold (°C)	mean (SD)	4.3 (7.4)	10.4 (6.7)	0.07
	median (IQR)	0.5 (0.0 - 6.6)	11.3 (5.0 - 16.4)	
Threshold not reached	(%)	50.0	20.0	0.35
Heat pain threshold (°C)	mean (SD)	48.7 (2.2)	46.6 (3.0)	0.09
	median (IQR)	49.9 (47.3 - 50.0)	47.3 (43.5 - 49.7)	
Threshold not reached	(%)	80.0	20.0	**0.02**
Method of Levels (MLE)				
Cold detection threshold (°C)	mean (SD)	30.8 (0.6)	31.3 (0.5)	0.08
	median (IQR)	30.8 (30.3 - 31.5)	31.4 (31.2 - 31.5)	
Number of stimuli	mean (SD)	11 (4)	11 (3)	0.95
Warm detection threshold (°C)	mean (SD)	34.2 (1.4)	33.1 (0.6)	**0.04**
	median (IQR)	33.9 (33.1 - 35.4)	32.9 (32.6 - 33.7)	
Number of stimuli	mean (SD)	10 (2)	11 (2)	0.35

P-values were derived from independent samples t-tests for continuous variables and Fisher's exact tests for categorical variables

differ between groups (median 3.0 (IQR 1.5 to 6.0) for cases versus 5.5 (IQR 2.8 to 7.0 for controls; p=0.16).

Structural imaging results

Brain volumes did not differ between cases and controls (Table 5 - uncorrected). Furthermore, cortical thickness was not significantly different between cases and controls (data not shown).

Table 5 - Global brain volumes and volumes of pain related brain regions

		Case group N=10	Control group N=8	P-value
Global Brain Volumes				
Total Brain Volume (mean (SD), cm^3)		1219 (100)	1232 (139)	0.34
Cerebral White Matter (mean (SD), cm^3)		434 (44)	446 (62)	0.27
Total Grey Volume (mean (SD), cm^3)		737 (62)	738 (79)	0.44
Parietal lobe (mean (SD), cm^3)	left	70 (8)	70 (7)	0.63
	right	75 (9)	74 (8)	0.76
Cerebellum (White Matter) (mean (SD), cm^3)	left	15 (2)	16 (2)	0.42
	right	15 (2)	16 (2)	0.81
Cerebellum (Cortex) (mean (SD), cm^3)	left	57 (5)	60 (8)	0.12
	right	58 (5)	62 (9)	0.10
Pain Related Brain Regions				
Thalamus (mean (SD), mm^3)	left	7510 (808)	7699 (808)	0.22
	right	7538 (809)	7523 (1023)	0.74
Amygdala (mean (SD), mm^3)	left	1476 (318)	1698 (196)	0.12
	right	1800 (273)	1842 (328)	0.48
Anterior Cingulate Cortex (mean (SD), mm^3)	left	2288 (630)	2465 (798)	0.40
	right	2641 (668)	2311 (445)	0.41
Insula (mean (SD), mm^3)	left	7422 (807)	7627 (972)	0.22
	right	7247 (927)	7590 (1042)	0.08

P-values were derived from linear regression analysis (with correction for age and gender)
After additional correction for total brain volume, the results remained insignificant (linear regression with correction for age, gender and total brain volume)

Functional imaging results

The fMRI analyses included two runs of all ten cases, two runs of seven controls and one run of two controls (second runs excluded due to movement. The warm stimulus of 41°C did not induce significant brain activation, neither in the case group nor in the control group.

In the case group, the painful stimulus of 46°C induced statistically significant activation in several brain areas, including the frontal and temporal lobes (Figure 3a, Table 6 - cor-

rected). In the control group, it induced statistically significant brain activation in several brain regions such as the lateral occipital cortex and the temporal gyrus (Figure 3b, Table 6 - corrected). A direct comparison revealed significantly more brain activation in mainly the lateral occipital cortex in the control group compared to the case group (Figure 3c and Table 6 - corrected).

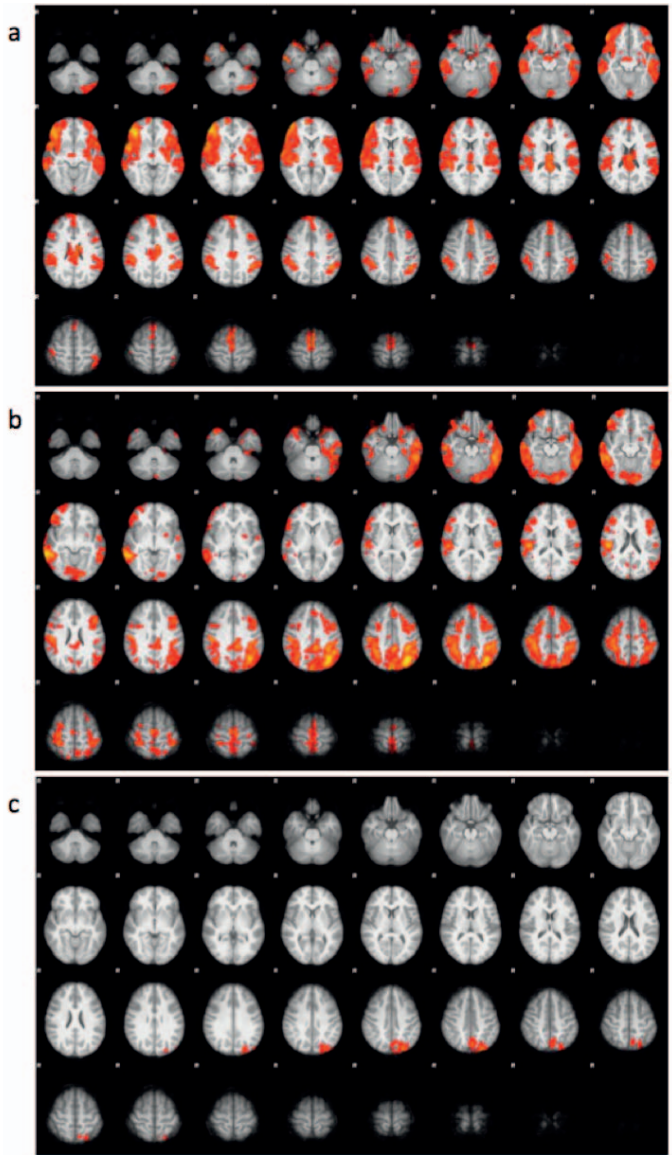

Figure 3 - The axial slices show areas of statistically significant activation during pain in the case group (a), the control group (b) and the direct comparison between both groups (control group > case group) (c) using a cluster significance threshold of $p<0.05$.

Table 6 - Areas of brain activation during pain

Cluster size (voxels)	P-value	MNI coordinates local maxima (mm)			Z-value	Anatomical area
		X	Y	Z		
Mean activation cases						
21434	<0.0001	−10	−8	24	3.92	Midline, Cingulate Gyrus
		−46	−52	38	3.89	Angular Gyrus (L)
		−46	−50	32	3.76	
		−38	−82	−44	3.74	Cerebellum (L)
		−50	−22	−14	3.70	Middle Temporal Gyrus (L)
		−52	12	−12	3.63	Temporal Pole (L)
20233	<0.0001	52	42	0	4.50	Frontal Pole (R)
		40	38	−4	4.44	
		52	46	−4	4.41	
		50	40	−10	4.22	
		48	52	8	4.12	
		66	8	−2	4.13	Superior Temporal Gyrus (R)
Mean activation controls						
42699	<0.0001	−36	−70	42	4.53	Lateral Occipital Cortex (L)
		−32	−76	38	4.25	
		66	−44	−6	4.28	Middle Temporal Gyrus (R)
		54	−46	−6	4.21	
		58	−48	−4	4.17	
		−48	−50	36	4.16	Supramarginal Gyrus (L)
Direct comparison (mean controls > mean cases)						
1747	0.03	−28	−80	42	3.67	Lateral Occipital Cortex (L)
		−22	−70	58	3.06	
		−46	−82	32	3.05	
		−36	−82	46	2.98	
		−26	−86	30	2.92	
		−2	−72	44	3.40	Precuneus Cortex (L)

Areas of activation during pain (46°C versus baseline) with cluster size, Z-values of the local maximum, Montreal Neurological Institute (MNI) coordinates, and the anatomical area of the local maximum (Harvard-Oxford Cortical Structural Atlas).
R: Right, L: Left

The median NRS intensity scores of the stimuli presented over the two fMRI runs were not significantly different between cases and controls (2.0 IQR 0.8 - 2.6 versus 2.5 IQR 1.3 - 6.0; p=0.15).

DISCUSSION

Since detrimental neurobiological effects after administration anaesthetic agents and opioids have been found in animals, we were interested if this was also true for humans. This is especially relevant in view of the ongoing debate on the necessity to shift from

general anaesthesia to loco-regional techniques and to postpone elective surgery in newborns.[3] Therefore, we conducted a neuropsychological assessment, determined detection- and pain thresholds, used structural MRI to measure brain morphology and tested brain functioning during pain using functional MRI in combination with thermal pain stimuli. We found that adolescents who had surgery in the first month of life and had been exposed to anaesthetics and opioids had less brain activation during pain and were less sensitive, although modest, for warm stimuli than controls without this history. Brain activation, however, was not significantly different in all visualized brain areas.

Different brain activation during pain between cases and controls mainly pertained to the occipital cortex. In a previous study, nine preterm born children showed significantly higher activations in the primary somatosensory cortex, anterior cingulate cortex and the insula compared to nine healthy controls during individualized thermal pain stimuli. Nine full term born children with a history of NICU admission did not show these differences in comparison to healthy controls.[28] In the current study, brain activation during pain in the occipital cortex in the case group was less intense than in the control group. The cases in the study by Hohmeister and colleagues had not undergone major surgery, however, and had therefore not been exposed to the combination severe pain, high doses of opioids and anaesthetics. This might explain the discrepancy in brain activation findings between these two studies. It is interesting that we found differences in brain activation in sensory regions such as the parietal and occipital lobe. Since primary cortical areas typically develop earlier than secondary or tertiary brain regions,[29] early stimuli such as anaesthetic exposure, surgical pain, and morphine exposure might have resulted in activity dependent neuronal changes in the primary and secondary sensorimotor cortical regions.

The cases in the present study were significantly less sensitive to detect a warm stimulus using the reaction time independent MLE method, although the significance disappears after correction for multiple testing. The cases also rated the painful stimulus of 46°C prior to scanning as less painful than did the controls, although this difference was not statistically significant. The mean NRS intensity scores of the stimuli presented during the fMRI scans were also lower in the case group. One other study reported global hyposensitivity, in nine children aged 9-12 years after cardiac surgery compared to nine healthy controls measured with both thermal and mechanical quantitative sensory testing.[30] Another previous study found alterations in pain sensitivity after neonatal intensive care treatment and thoracotomy, although this study was conducted in extremely preterm born children, and therefore hard to compare to our study.[31] We found no difference in the incidence of chronic pain, although chronic pain is a frequent symptom after surgical procedures.[32] However, a previous study suggested that the risk

for chronic pain was not higher if surgery was performed before the age of 3 months.[33] Another study likewise showed that the risk for chronic pain is lower if the surgery is performed at a younger age.[34] Cases in the present study were operated on in the first month of life, which thus would explain that the incidence of chronic pain comparable among cases and controls.

We found no differences in brain morphology between adolescents exposed to anaesthetics, opioids and surgery and controls without such a history. Since GABA, NMDA, and opioid receptors have a direct role in human neuronal development[35] and animal studies previously reported that anaesthetic agents induced neurotoxicity in rodents,[5,10,36] monkeys,[11,13,37,38] and piglets,[39] we expected an influence of anaesthetics and opioids on human brain morphology as well. On the other hand, experimental animals often receive much higher dosages of intravenous anaesthetic agents than humans receive and are much longer under anaesthesia.[3,35] Moreover, children are carefully monitored during anaesthesia in order to control for hypoxia and hypotension, while in animal studies physiologic derangement may often occur.[35] Furthermore, peak synaptogenesis may occur at different periods among species, and therefore the window of vulnerability between animals and humans may be different.[40] Additionally, post-mortem findings in animals cannot be compared to neuroimaging findings in humans.

Rodents showed learning and memory deficits after anaesthetic exposure,[8,9] and previous studies in humans found an increased rate of learning disabilities,[17] developmental and behavioral disorders,[18] and lower academic achievements[19,41] after early surgery and exposure to anaesthetics. The question arises whether it is the exposure to anaesthetics or rather the exposure to opioids or the surgery and pain that may lead to problems later in life. The increase in comorbidity and genetic vulnerability for learning disabilities related to the need for surgery in early life could also be the main cause. In our study, however, no major differences between cases and controls were found with respect to neuropsychological outcomes.

The strength of this study is that we assessed neuropsychological functioning, thermal sensitivity, brain morphology and brain functioning in a well-documented cohort of adolescents. All important information on anaesthetics and opioids consumption was available from the previous RCT, in contrast to other studies available in the literature. While large multi-center studies, such as the GAS study (ClinicalTrials.gov; NCT00756600; General Anaesthesia versus Spinal) which aims to compare the neurodevelopmental outcome between general anaesthesia and regional anaesthesia, are still ongoing, our study already provides insight in the long-term neurobiological effects of exposure to general anaesthetics. Moreover, the GAS study concerns inguinal hernia repair, which

is less painful than the major abdominal or thoracic surgery in our cohort, for which the children received opioids in our study.

Several limitations need to be addressed. First, since this was an exploratory study, the sample size was small. Not only the parents needed to give informed consent, the adolescents themselves had to assent as well. Probably due to their age and related puberty fewer than expected were willing to participate. However, even with only ten children in each group, we were able to detect statistically significant differences between both groups regarding brain activation during pain Moreover, a structural MRI study in comparable numbers of children found morphometric differences between groups.[42] The small sample size also did not permit to correct for the possibly confounding factors, i.e. the surgical procedure itself, morphine exposure, hospital admission, and comorbidity. But due to ethical reasons it is not possible to study the long-term effects of anaesthesia, surgery and subsequent analgesia separately. Future follow-up studies with different designs are needed.[43] Furthermore, by excluding children with contra-indications for the study procedures, we excluded children with a poor outcome. However, the majority of contra-indications were not attributable to neonatal exposure to anaesthesia and surgery, but rather to congenital anomalies such as Down's syndrome.

CONCLUSION

So should we be concerned about the long-term effects of exposure to anaesthetics and opioids in neonates? We did not find major or global neuropsychological or neurobiological long-term effects in adolescents who as neonates had been exposed to anaesthetics that warrant major concern. It is likely that the alarming findings regarding neurotoxicity in animals do not readily extrapolate to humans.

SUPPLEMENTARY DATA

Image acquisition

MR images were acquired on a 3 Tesla scanner (Discovery MR750, General Electric, Milwaukee, MI, USA) using an 8-channel head coil for signal reception. Cushions were used to comfortably support the participants' head and to minimize head motion. During the high-resolution structural MRI scan the adolescents were able to watch a movie or listen to music of their choice. The movie/music was stopped during the functional MRI scans. Participants wore an MRI-compatible headphone to reduce the scanner noise and to allow them to listen to the movie's audio track. The headphone also enabled communication with the MR operator between the scans.

We obtained a high-resolution structural T_1-weighted image using an inversion recovery fast spoiled gradient recalled (IR-FSPGR) sequence with the following parameters: TR = 10.3 ms, TE = 4.2 ms, TI = 350 ms, NEX = 1, flip angle = 16°, readout bandwidth= 20.8 kHz, matrix 256 x 256, imaging acceleration factor of 2, and an isotropic resolution of 0.9x0.9x0.9 mm^3. The scan time for the structural T_1 MRI scan was 5 minutes and 40 seconds. We conducted two runs of a functional MRI paradigm using single-shot echo-planar imaging (EPI) T_2^*-weighted sequences in transverse orientation sensitive to blood oxygen level dependent (BOLD) contrast (parameters: TR/TE 2000/30 ms, flip angle 85°, 64 x 64 matrix with a field-of-view of 260 x 260 mm^2; 39 slices and voxel sizes of 3.6 x 3.6 x 4.0 mm^3). Scan time was 6 minutes and 4 seconds (182 TRs) per run.

Functional MRI Block paradigm

The functional MRI (fMRI) component consisted of two runs and utilized a block paradigm. During each of these two runs the TSA-II thermode was applied to the thenar eminence of the non-dominant hand. During scanning the TSA-II thermode induced warm (41°C) and painful stimuli (46°C) (Figure 2). These temperatures were derived from a previous study from our research group.[44] Within each run, the temperature increased four times at a rate of 1.5°C per second from the baseline temperature of 32°C to a warm temperature of 41°C and four times to a potentially painfully hot temperature of 46°C. After each stimulus, the temperature decreased by 4.5°C per second back to baseline and stayed at the baseline temperature for 15 seconds before the increasing to the next warm or pain stimulus. The order and duration (8 - 16 seconds) of the stimuli was randomly determined at the beginning of the study and were different in both runs. In order to prevent anticipation to the stimuli, the order of warm and heat stimuli differed between the two runs. Figure 2 shows the block paradigm of the thermal stimuli for run 1 and run 2. Pain intensity of the thermal stimuli applied during the fMRI scans was measured again using the NRS scale.

Structural imaging analysis

We used the Freesurfer image analysis suite version 5.1.0 (http://surfer.nmr.mgh.harvard.edu/) for cortical reconstruction and volumetric segmentation. Freesurfer computes these measures in an automated approach, and technical procedures have been described extensively.[45] Each image was visually inspected and subjects with poor quality data were excluded. In subjects with small errors in the gray/white segmentation, control points, and white matter edits were added to identify and correct misclassified white matter regions. When the segmentation improved, the corrected images were used. Evaluation of surface-based cortical thickness FreeSurfer was performed using the built-in program QDEC with a smoothing filter of 10 millimeter. For the group analysis a general linear model (GLM) was fitted at each surface vertex. We corrected for age and gender and used a Monte Carlo correction ($p<0.05$) for multiple testing. Total brain volumes, volumes of the parietal lobe (associated with somatosensation) and volumes of pain related brain regions, such as the thalamus, anterior cingulate cortex and insula,[46] were compared between cases and controls using linear regression analysis with correction for age, gender, and total brain volume.

Functional imaging analysis

For our functional MRI analyses, we used AFNI (http://afni.nimh.nih.gov/) for slice timing and motion correction. Runs with more than 6 mm of motion (maximum displacement) were excluded from the analyses. Functional images were co-registered to the structural image of the subject and both the functional and structural images were normalized using the Montreal Neurological Institute (MNI) 152 atlas using FSL's non-linear registration tool FNIRT. Finally, data were spatially smoothed using AFNI with an 8-mm full width at half-maximum Gaussian kernel. Following the preprocessing steps, single-subject analyses were performed using FMRIB's fMRI Expert Analysis Tool FEAT (http://www.fmrib.ox.ac.uk/fsl/feat5/index.html), comparable to a previous report of our study group.[47] The time series for the pain runs were modeled using a block design. Design matrices were created for both runs using the data from each subject's stimulus log file from the TSA. These matrices were created independently for each individual using an automated MATLAB program (MATLAB 7.1, The MathWorks Inc., Natick, MA, 2000). This modeled time series was convolved with the hemodynamic response function. Next, a general linear model was implemented using FMRIB's Improved Linear Model. The two within-subject runs were combined using a fixed effects model. The higher-level group analyses, which compared patients and controls for each of the contrasts; 46°C versus baseline, and 41°C versus baseline, were performed using FMRIB's Local Analysis of Mixed Effects. Furthermore, we corrected for multiple comparisons using random Gaussian fields and significance was set at $p<0.05$ (two-tailed).

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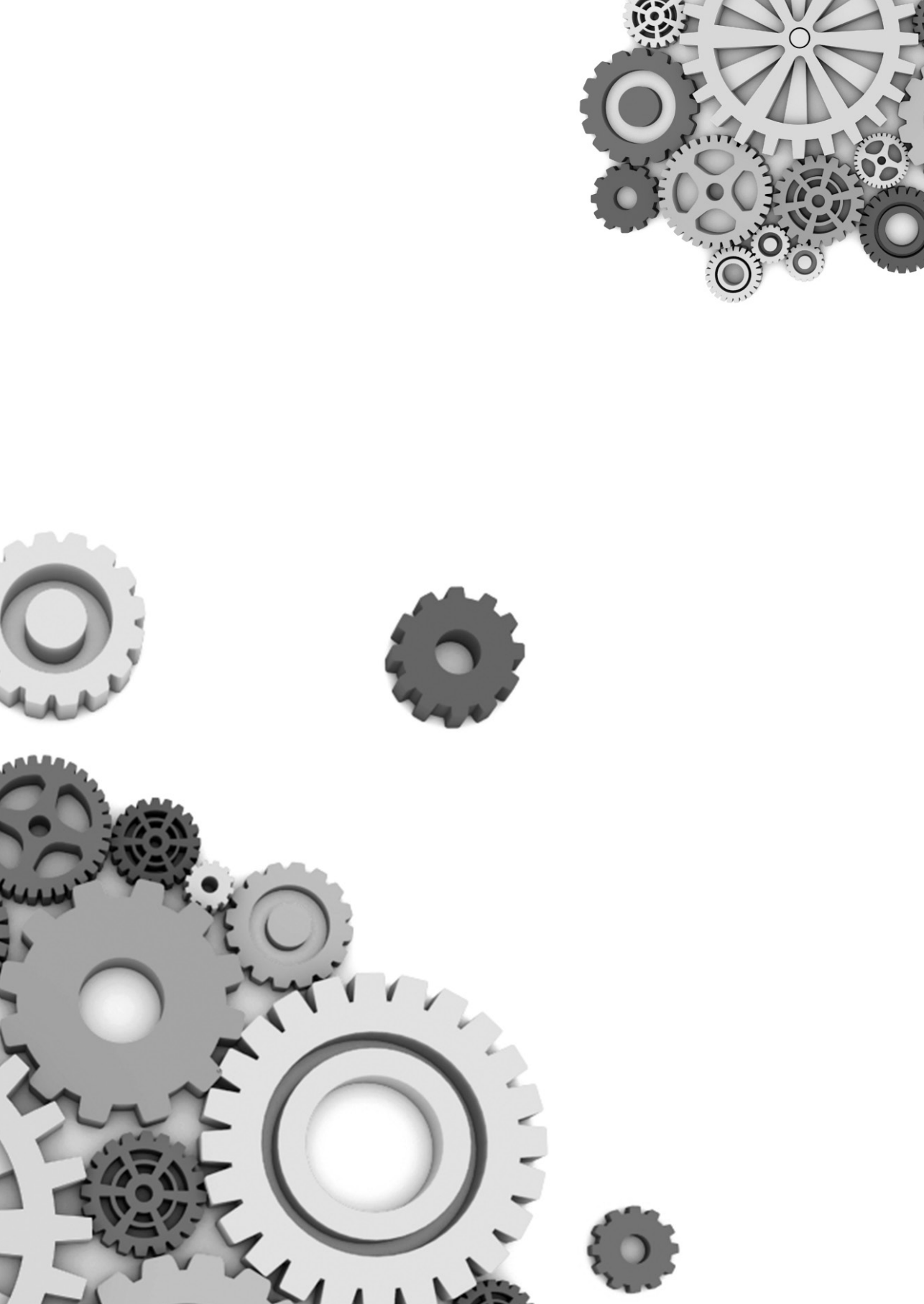

Chapter 8

Long-term effects of neonatal opioid and sedative exposure in ECMO patients

A neuroimaging study

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Submitted for publication

ABSTRACT

Objective Animal studies found negative long-term effects of exposure to sedatives and opioids in early life, especially when administered in the absence of pain. Around the world, children who require extracorporeal membrane oxygenation (ECMO) receive opioids and sedatives for extended periods, generally in the absence of major pain as ECMO cannulation is considered minor surgery. Therefore our objective was to determine the long-term effects of prolonged exposure to opioids and sedatives in the absence of severe pain with respect to pain sensitivity, brain functioning during pain, brain morphology, and neuropsychological functioning in humans.

Design Prospective follow-up study.

Setting Level III university hospital.

Subjects Thirty-six ECMO survivors (8.1-15.5 years) and 64 healthy controls (8.2-15.3 years).

Measurements and main results We measured detection- and pain thresholds, brain activity during pain (functional MRI), brain morphology (high resolution structural MRI), neuropsychological functioning, and collected information regarding the subject's experience of chronic pain. We found a significant difference in the detection threshold for cold measured in a reaction time dependent fashion (ECMO group 29.9°C (SD 1.4), control group 30.6°C (SD 0.8); $p<0.01$), but no differences in other modalities or in pain sensitivity between groups. Furthermore, no differences in brain activation during pain, brain morphology or in the occurrence of chronic pain were observed. However, ECMO survivors performed significantly worse on a verbal memory test compared to controls ($p=0.001$).

Conclusions While the most critically ill newborns receive ECMO and, relatedly, large doses of opioids and sedatives for extended periods, global measures of pain sensitivity, neurobiological and neuropsychological development appear to have minor long term consequences. Possible memory deficits in ECMO survivors require additional study, but neonatal exposure to opioids and sedatives seems less harmful to humans than animal studies suggest.

INTRODUCTION

Severe, but potentially reversible cardiac or respiratory failure in newborns can be treated with extracorporeal membrane oxygenation (ECMO), which is a complicated life support intervention with known survival benefits.[1] While ECMO therapy has immediate risks, including haemorrhaging and ischemic brain lesions,[2] the long-term survival of children has significantly improved with the advent of ECMO. To avoid accidental ECMO decannulation, children on ECMO generally receive continuous and prolonged amounts of opioids and sedatives. These are typically given in the absence of significant tissue damage, except when children require surgery for congenital diaphragmatic hernia (CDH) on ECMO. From rodent studies we know that sedatives such as midazolam can trigger neuroapoptosis in the developing brain[3] and that early opioid exposure in the absence of pain can have adverse long-term neurobiological, somatosensory, and cognitive effects.[4-7] On the other hand, opioids administered in the presence of pain exerted neuroprotective effects in animals.[8,9] Human in vitro studies have shown that midazolam induces apoptosis in cells of hematogenic, ectodermal and mesenchymal origin.[10] Moreover, we have shown that morphine administration to prematurely born neonates in the absence of severe pain does not affect neurological and cognitive outcome at school age.[11,12]

Follow-up studies of the UK collaborative randomised trial compared outcomes of neonatal ECMO-treated survivors with those of conventionally treated survivors. At age four, outcome of ECMO-treated children in terms of survival and severe disability was more favourable.[13] At age seven, both groups had similar learning problems with respect to spatial and processing tasks.[14] In our own prospective follow-up program in neonatal ECMO-treated children we found impaired health-related quality of life at age five[15] and intelligence within normal ranges with (subtle) concentration and behaviour problems.[16] In the present study we are the first who determined thermal detection and pain sensitivity, and brain functioning during a pain stimulus in school-age neonatal ECMO survivors and healthy controls. To obtain a comprehensive view, we also studied brain morphology and neuropsychological functioning. Based on animal studies we hypothesized that prolonged exposure to opioids and sedatives in the absence of severe pain would show long-term negative consequences with respect to pain sensitivity, neuropsychological functioning, and structural and functional brain development. ECMO patients can be considered the human equivalent to evaluate a proof-of-principle concept with respect to the long-term effects of prolonged neonatal opioid exposure.

PATIENTS AND METHODS

Study population

Cases

We studied children who as neonates had received venoarterial ECMO treatment in the Erasmus Medical Center in Rotterdam, the Netherlands from January 1997 to December 2003. Of these 165 children, 44 (27%) had died. Excluded were 15 children who did not join our follow-up program, and 46 children with contra-indications for participation in a MRI study or neuropsychological assessment e.g. genetic syndromes, or severe brain abnormalities found on previous cranial ultrasound images or MRI scans obtained during the neonatal period. These children would not properly understand the study procedures and brain abnormalities would influence our MRI outcomes. The perinatal and medical history of all patients was retrieved from medical records. The remaining eligible 60 children received an information letter and were invited to participate. Six families were not traceable and 17 declined participation. One child turned out to have permanent braces and was given the opportunity to participate in the non-MRI tests, but the family declined (Figure 1). Background characteristics of the remaining 36 cases were retrieved from the medical records. Information with respect top analgesic and sedative regimen is described in the supplementary information section.

Controls

Healthy controls were recruited in two ways. First, we asked all participating families whether they could recommend someone in the age range of 8-18 years. Second, we mailed invitation letters to parents of children attending a primary school in Rotterdam. Exclusion criteria were surgery in the neonatal period, ECMO treatment, prematurity, or severe mental or medical conditions. Candidates were screened on exclusion criteria and contra-indications for participation in an MRI study by phone prior to participation. Controls were matched within an age range of six months younger and older than the cases. Eventually, 64 children served as healthy controls.

The study was performed at the Erasmus Medical Center in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board at the Erasmus MC (MEC-2010-299). Informed consent was obtained from the parents of each child prior to participation. Informed assent was obtained from children 12 years of age and older prior to participation. Recruitment took place between March 2011 and March 2013.

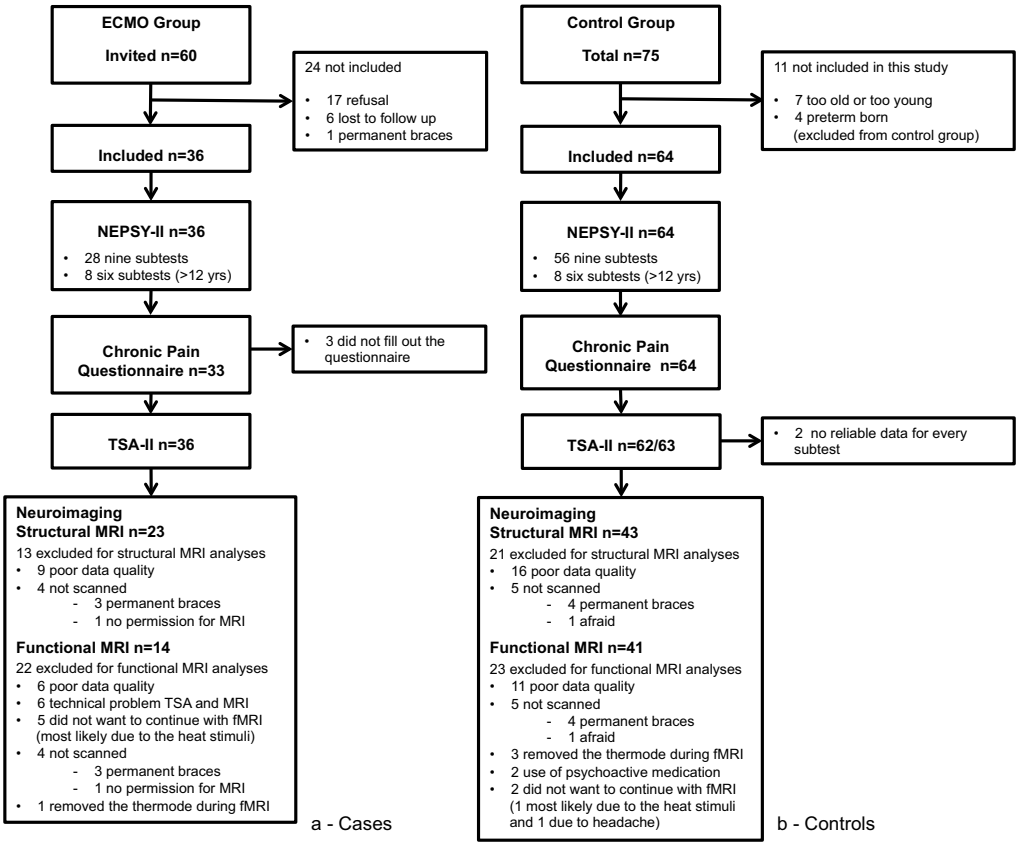

Figure 1a,b – Inclusion flowcharts
Inclusion flowchart of the ECMO group (a) and the control group (b).

Procedure

Cases and controls first underwent a neuropsychological assessment and were administered the Dutch Chronic Pain Questionnaire.[17] Next, they were placed in a mock scanner, allowing them to adjust to the environment of an MRI scanner. Subsequently, thermal detection- and pain thresholds were determined. Finally, a structural MRI scan and two task-based functional MRI scans with thermal pain stimuli were obtained.

Neuropsychological assessment

All subjects were administered subtests of the NEPSY-II-NL neuropsychological test (Pearson, Amsterdam), which is a Dutch translation of the North American NEPSY-II.[18] Children between 8 and 12 years of age performed nine subtests including domains of attention and executive functioning, language, memory and learning, sensorimotor functioning, and visuospatial processing. Older participants performed only 6 of these subtests due to the age limit of the 3 other tests.

Chronic pain questionnaire

Subjects were administered the Dutch chronic pain questionnaire,[17] which addresses whether subjects are currently having pain and whether this is chronic pain with a duration of more than three months.

Examination of the pain thresholds

Individual detection- and pain thresholds were obtained using the computer-controlled Thermal Sensory Analyzer (TSA type II, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel) with a Peltier-based contact thermode (30 x 30 mm). Skin temperature and room temperature were measured to check for a consistent test environment. As one of the TSA subtests is reaction time dependent, reaction time was tested with a subtest of the Amsterdam Neuropsychological Tasks (ANT).[19] Detection- and pain thresholds were obtained using a standardized protocol, as described previously.[20] In brief, detection thresholds for cold and warmth were measured using both the reaction time dependent Method of Limits (MLI) and the reaction time independent Method of Levels (MLE). Pain thresholds for cold and heat were measured using the MLI. Finally, children assigned a pain intensity score for a potentially painful stimulus of 46°C on a numeric rating scale (NRS).

Image acquisition

MR images were acquired on a 3 Tesla scanner (General Electric Discovery MR750, Milwaukee, MI, USA) using an 8-channel head coil. Cushions were used to comfortably support the participants' head and to minimize head motion. During the high-resolution structural MRI scan the participants were able to watch a movie or listen to music of their choice. The movie/music was stopped during the functional MRI scans. Participants wore an MRI-compatible headphone to reduce the scanner noise and allow them to listen to the movie's audio track. The headphone also enabled communication with the MR operator between the scans. We obtained high-resolution structural T_1-weighted images using an inversion recovery fast spoiled gradient recalled (IR-FSPGR) sequence with the following parameters: TR = 10.3 ms, TE = 4.2 ms, TI = 350 ms, NEX = 1, flip angle = 16°, readout bandwidth= 20.8 kHz, matrix 256 x 256, imaging acceleration factor of 2, and an isotropic resolution of 0.9x0.9x0.9 mm^3. The scan time for the structural T_1 MRI scan was 5 minutes and 40 seconds. We conducted two runs of a functional MRI paradigm using single-shot echo-planar imaging (EPI) T_2*-weighted sequences in transverse orientation sensitive to blood oxygen level dependent (BOLD) contrast (parameters: TR/TE 2000/30 ms, flip angle 85°, 64 x 64 matrix with a field-of-view of 260 x 260 mm^2; 39 slices and voxel sizes of 3.6 x 3.6 x 4.0 mm^3). Scan time was 182 TRs (6 minutes 4 seconds) per run.

Functional MRI Block paradigm

The functional MRI (fMRI) component consisted of two runs and utilized a block paradigm. During each of these two runs the TSA-II thermode was applied to the thenar eminence of the non-dominant hand. During scanning the TSA-II thermode induced warm (41°C) and painful stimuli (46°C). These temperatures were derived from a previous study from our research group.[21] Within each run, the temperature increased four times at a rate of 1.5°C/sec from the baseline temperature of 32°C to a warm temperature of 41°C and four times to a potentially painfully hot temperature of 46°C. After each stimulus, the temperature decreased with 4.5°C/sec back to baseline and stayed at the baseline temperature for 15 seconds before the increasing to the next warm or pain stimulus. The order and duration (8-16 seconds) of the stimuli was randomly determined at the beginning of the study and were different in both runs. In order to prevent anticipation to the stimuli, the order of warm and heat stimuli differed between the two runs. Figure 1 shows the block paradigm of the thermal stimuli for run 1 and run 2.

Structural imaging analysis

We used the Freesurfer image analysis suite version 5.1.0 (http://surfer.nmr.mgh.harvard.edu/) for cortical reconstruction and volumetric segmentation. Freesurfer computes structural morphometric measures in an automated approach. Technical procedures have been described extensively.[22] Each image was visually inspected and subjects with poor quality data were excluded. In subjects with small errors in the gray/white segmentation, control points, and white matter edits were added to identify and correct misclassified white matter regions. When the segmentation improved, the corrected images were used. Total brain volume and the volume of a priori selected pain related brain regions, including the thalamus, anterior cingulate cortex and insula,[23] were compared between cases and controls using ANCOVAs correcting for age, gender, and total brain volume. Statistical analyses were performed using SPSS version 20.0. Evaluation of surface-based cortical thickness FreeSurfer was performed using the built-in program QDEC[22] with a smoothing filter of 10 millimeter. For the group analysis a general linear model (GLM) was fitted at each surface vertex. We corrected for age and gender and used a Monte Carlo correction ($p<0.05$) for multiple testing.

Functional imaging analysis

For functional MRI analyses (fMRI), we used a combination of Analysis of Functional Neuroimages (AFNI, http://afni.nimh.nih.gov/)[24] and FSL's FMRIB's Software Library (FSL 5.0, FMRIB Software Library; FMRIB, Functional Magnetic Resonance Imaging of the Brain; http://www.fmrib.ox.ac.uk/fsl/).[25] AFNI was used for slice timing and motion correction. Runs with more than 6 mm of motion (maximum displacement) were excluded from the analyses. Functional images for each individual were co-registered to their high-

resolution T_1 image and both functional and structural images were registered to the Montreal Neurological Institute (MNI) 152 atlas using FSL's non-linear registration tool FNIRT. Finally, data were spatially smoothed using AFNI with an 8-mm full width at half-maximum Gaussian kernel.[26] Following the preprocessing steps, single-subject analyses were performed using FMRIB's fMRI Expert Analysis Tool FEAT (http://www.fmrib.ox.ac.uk/fsl/feat5/index.html), comparable to a previous report of our study group.[27] The time series for the pain runs were modeled using a block design. Design matrices were created for both runs using the data from each subject's stimulus log file from the TSA. These matrices were created independently for each individual using an automated MATLAB program (MATLAB 7.1, The MathWorks Inc., Natick, MA, 2000). This modeled time series was convolved with the hemodynamic response function. Next, a general linear model was implemented using FMRIB's Improved Linear Model. The two within-subject runs were combined using a fixed effects model. The higher-level group analysis, which compared patients and controls for the contrast; 46°C versus baseline, was performed using FMRIB's Local Analysis of Mixed Effects with correction for age and gender. We conducted the analyses with and without left-handed subjects. Furthermore, we corrected for multiple comparisons using random Gaussian fields and significance was set at $p<0.05$ (two-tailed).

Data analysis

Normally distributed variables are presented as mean (standard deviation) and non-normally distributed variables as median (range or interquartile range (IQR)). We used independent samples t-tests and Mann-Whitney U tests for continuous data and χ^2-tests for categorical data. We corrected for multiple testing using Bonferroni correction. All analyses were conducted with and without exclusion of data of children who had undergone repair of congenital diaphragmatic hernia, since those children had received analgesics and sedatives in the presence of severe pain. Correlations between ECMO duration and detection- and pain thresholds, neuropsychological outcome, and brain volumes were determined using Spearmans' rank order correlation coefficient. A p-value of 0.05 or less was considered statistically significant. Analyses were conducted with IBM SPSS 20.0.

RESULTS

Study population

The participants included seventeen boys and 19 girls with mean age 11.1 years (SD 2.4) and 64 controls (28 boys and 36 girls with a mean age of 11.1 years (SD 1.7)). Age and gender did not significantly differ between groups ($p=0.98$ and $p=0.74$, respectively).

The numbers of children included per sub-study are presented in Figure 1. Six cases underwent repair of congenital diaphragmatic hernia (CDH). Other clinical characteristics of the cases are presented in Table 1. One case that showed a minor subependymal haemorrhage on the neonatal ultrasound[2] was not excluded. There was no difference between the 36 included children and the 85 excluded children with regards to the following characteristics; age (p=0.76), gender (p=0.51), diagnosis (p=0.36), birth weight (p=0.18), duration of ECMO treatment (p=0.81) or duration of mechanical ventilation (p=0.80). Gestational age did differ, although the difference was minor (included children 40.3 weeks, excluded children 39.5 weeks; p=0.02).

Table 1 - Clinical characteristics ECMO group

			ECMO group N=36
General characteristics			
Gestational age in weeks, median (range)			40 (37 to 43)
Birth weight in grams, median (range)			3535 (2300 to 4985)
Age at ICU admission in days, median (range)			0 (0 to 16)
Oxygenation Index prior to ECMO treatment, median (range)			42 (21 to 106)
Age at start ECMO treatment in hours, median (range)			24 (5 to 398
ECMO duration in hours, median (range)			125 (53 to 369)
Duration of mechanical ventilation in days, median (range)			11 (2 to 70)
Surgery in the first months of life (% yes)			17
Diagnosis (%)	Meconium aspiration syndrome		64
	Congenital diaphragmatic hernia		17
	Sepsis		6
	Persistent pulmonary hypertension of the newborn (PPHN)		8
	Pneumonia		3
	Other		3
Pharmacological data			
Duration of opioid exposure (%)**		Less than one week	17
		One week - one month	71
		More than one month	11
Duration of sedative exposure (%)**		Less than one week	20
		One week - one month	66
		More than one month	14
Methadone treatment in the first year of life for weaning from opioids (% yes)			14

** Oxygenation index is a calculation to measure the fraction of inspired oxygen (FiO2) and its usage within the body.*
Based on n=34 due to missing data
*** Based on n=35 due to missing data*

Detection and pain thresholds

Reliable data was available from all 36 cases and 62/63 controls, depending on the subtest (Table 2). Cases were less sensitive to detect a cold stimulus compared to controls measured with the MLI method. The difference remained significant after correction for multiple testing. The cold detection threshold measured with the reaction time independent MLE method did not differ between both groups even as the other modalities (Table 2). The mean NRS score assigned for the painful stimulus did not statistically significantly differ between groups (cases 7.0 (IQR 3.0 to 9.0), controls 5.5 (IQR 1.0 to 9.0); p=0.12). No statistically significant correlations between ECMO duration (n=36) and detection and pain thresholds and NRS scores were found in the ECMO group (range of correlation coefficients (+/-) 0.02 to 0.17). Room temperature and mean reaction time did not differ between groups during testing (data not shown; p=0.47 and p=0.17, respectively). The skin temperature was significantly lower in cases than in controls, but as the difference was minor, skin temperature was not used as a covariate (36.4°C versus 36.7°C; p=0.01).

Table 2 - Detection- and pain thresholds

	ECMO group	Control group	P value
Method of Limits (MLI)	N=36	N=63	
Cold detection threshold in °C, mean (SD)	29.9 (1.4)	30.6 (0.8)*	**<0.01**
Warm detection threshold in °C, mean (SD)	34.4 (1.4)	34.0 (1.2)*	0.17
Cold pain threshold in °C, mean (SD)	11.7 (9.9)	9.9 (9.4)	0.35
Heat pain threshold in °C, mean (SD)	44.5 (4.7)	46.0 (4.4)	0.11
Method of Levels (MLE)	N=36	N=63	
Cold detection threshold in °C, mean (SD)	30.7 (0.9)	30.7 (1.2)	0.91
Warm detection threshold in °C, mean (SD)	33.9 (1.4)	33.7 (1.0)	0.31

Note: P-values were derived from independent samples T-tests
** n=62*

Functional imaging results

We compared 14 cases with 41 controls in the fMRI analyses. Eleven cases were included with two runs, and three with one run. Twenty-nine controls were included with both runs and 12 with only one.

After correction for age and gender, we found statistically significant brain activation in both the ECMO group and the control during administration of the painful stimulus i.e. in the frontal pole and temporal gyrus (Figure 2, Table 3). A direct comparison revealed no statistically significant differences between groups (Figure 2, Table 3). After exclusion of two left-handed subjects (a case and a control with one run each) the results remained comparable. Mean NRS score of the pain stimuli presented during the fMRI scans were significantly higher in all the scanned cases compared to all the scanned controls (median 6.0 (IQR 3.0 to 7.0) versus median 2.8 (IQR 0.0 to 6.0); p=0.02, respectively. When only

comparing the NRS pain scores of the children which were included in the fMRI analyses, the median NRS score in cases 5.3 (IQR 3.0 to 7.8) did not significantly differ from the median score in controls 3.5 (IQR 0.3 to 6.0); p=0.07).

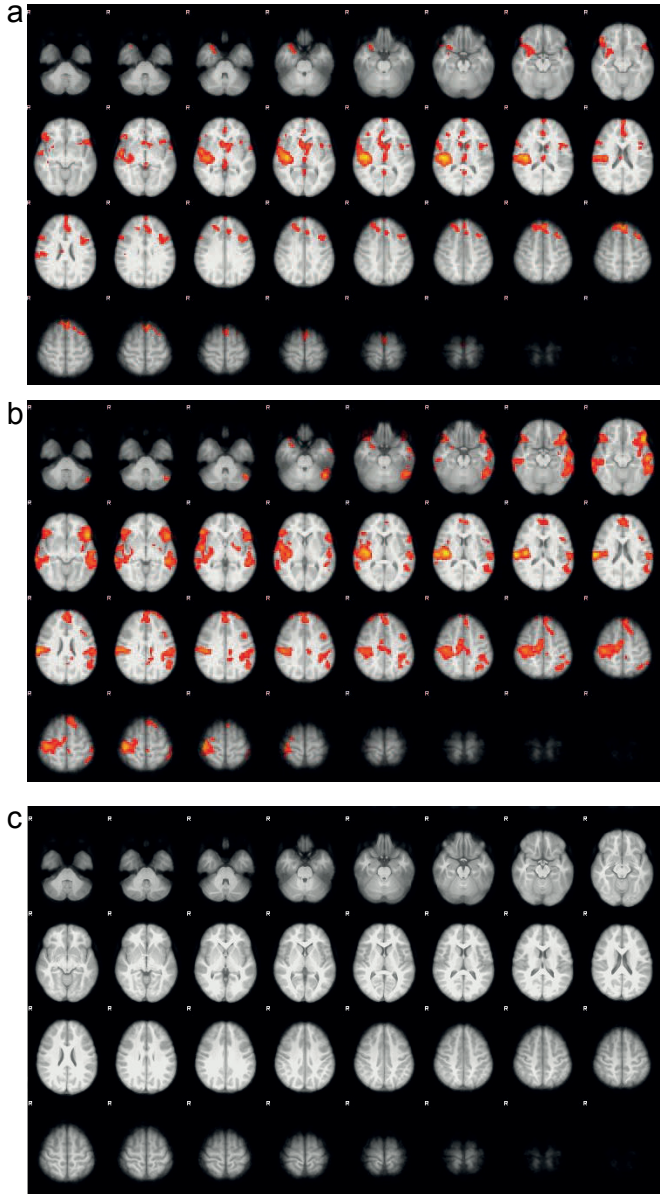

Figure 2 - Brain activation during pain
The axial slices show areas of statistically significant activation during pain in the ECMO group (a), the control group (b) and the direct comparison between both groups (c) using a cluster significance threshold of p<0.05 and corrected for age and gender.

Table 3 - Areas of brain activation during pain

Cluster size (voxels)	P-value	MNI coordinates local maxima (mm)			Z-value	Anatomical area
		X	Y	Z		
Mean activation ECMO group (n=14)						
5929	<0.001	2	44	54	4.07	Frontal Pole (R)
		−4	36	60	3.79	Superior Frontal Gyrus (L)
		0	8	72	3.68	Supplementary Motor Cortex (L)
		16	44	54	3.67	Frontal Pole (R)
		−8	8	2	3.54	Caudate (L)
		−58	16	−10	3.50	Temporal Pole (L)
5048	<0.001	42	−16	6	4.47	Heschl's Gyrus / Insula (R)
		42	−18	14	4.44	Central Opercular Cortex (R)
		48	−16	10	4.44	Heschl's Gyrus (R)
		62	26	8	3.96	Inferior Frontal Gyrus (R)
		54	−8	−6	3.72	Superior Temporal Gyrus (R)
		62	22	16	3.65	Inferior Frontal Gyrus (R)
Mean activation control group (n=41)						
12390	<0.001	42	−18	12	4.85	Heschl's Gyrus / Insula (R)
		62	−22	16	4.49	Parietal Operculum Cortex (R)
		72	−34	−8	4.26	Middle Temporal Gyrus (R)
		48	−14	64	4.25	Postcentral Gyrus (R)
		46	−18	66	4.20	Postcentral Gyrus (R)
		46	−22	66	4.07	Postcentral Gyrus (R)
10192	<0.001	−48	26	−10	4.39	Frontal Orbital Cortex (L)
		−56	−18	−12	4.20	Middle Temporal Gyrus, post.division (L)
		−54	32	−16	4.18	Frontal Orbital Cortex (L)
		−60	22	−4	4.00	Inferior Frontal Gyrus (L)
		−58	22	−8	3.91	Frontal Orbital Cortex (L)
2631	0.024	−30	58	32	3.87	Frontal Pole (L)
		−20	66	28	3.65	Frontal Pole (L)
		−24	64	28	3.64	Frontal Pole (L)
		−4	60	40	3.50	Frontal Pole (L)
		−4	70	28	3.40	Frontal Pole (L)
		4	56	24	3.32	Superior Frontal Gyrus (R)

Note: Areas of activation during pain corrected for age and gender with cluster size, Z-values of the local maximum, Montreal Neurological Institute (MNI) coordinates, and the anatomical area of the local maximum (Harvard-Oxford Cortical and Subcortical Structural Atlas).
R: Right, L: Left

Structural imaging results

Cortical thickness and global brain volumes did not differ between the 23 cases and 43 controls (Table 4). Regarding specific pain-related brain areas; only the left thalamus

was statistically significantly smaller in cases compared to controls. This difference did not remain significant after correction for multiple testing. The duration of ECMO treatment (n=23) was significantly negatively correlated with the volume of the left thalamus (Spearman's coefficient −0.42, p=0.05), as well as the volume of the right amygdala (Spearman's coefficient 0.44, p=0.04), although in the opposite direction. However, these findings did not survive Bonferroni correction for multiple testing.

Table 4 - Global brain volumes and volumes of pain related brain regions

		ECMO group	Control group	P value*	P value**
Global Brain Volumes		N=23	N=43		
Total Brain Volume Mean (SD), cm^3		1162 (102)	1155 (111)	0.99	NA
Cerebral White Matter Mean (SD), cm^3		390 (45)	394 (48)	0.59	0.18
Total Gray Volume Mean (SD), cm^3		728 (63)	717 (66)	0.68	0.18
Parietal lobe Mean (SD), mm^3	Left	73230 (7628)	72877 (8284)	0.99	1.0
	Right	75789 (8091)	75009 (8332)	0.86	0.75
Cerebellum (White Matter) Mean (SD), mm^3	Left	14202 (2141)	14959 (2150)	0.11	0.07
	Right	14493 (2625)	14867 (2195)	0.46	0.40
Cerebellum (Cortex) Mean (SD), mm^3	Left	56771 (5071)	55377 (4890)	0.38	0.30
	Right	57078 (5124)	55686 (4962)	0.39	0.32
Pain Related Brain Regions		N=23	N=43		
Thalamus Mean (SD), mm^3	Left	6796 (558)	7147 (821)	**0.04**	**0.01**
	Right	7004 (713)	7155 (695)	0.33	0.24
Amygdala Mean (SD), mm^3	Left	1590 (217)	1620 (288)	0.55	0.49
	Right	1712 (306)	1720 (280)	0.67	0.63
Anterior Cingulate Cortex Mean (SD), mm^3	Left	2434 (805)	2297 (608)	0.45	0.42
	Right	2588 (527)	2569 (631)	0.96	0.95
Insula Mean (SD), mm^3	Left	7525 (1169)	7470 (860)	0.97	0.95
	Right	7390 (969)	7423 (879)	0.68	0.59

** P-values were derived from ANCOVA test (correction for age and gender)*
*** P-values were derived from ANCOVA test (correction for total brain volume, age and gender)*
NA: Not applicable

Neuropsychological functioning

On the subtest Narrative memory, cases scored significantly worse than controls group (p=0.001; this difference remained significant after correction for multiple testing) (Table 5). Cases scored significantly better than the controls on the subtest Visuomotor Precision (p=0.05), but this difference was not significant after correction for multiple testing. The scores on all the other subtests were comparable between both groups (Table 5). Duration of ECMO treatment (n=28/36 depending on the subtest) was only significantly associated with total score for the subtest Word Generation (Spearman's coefficient 0.39, p=0.02). However, this did not survive correction for multiple testing.

Table 5 - Neuropsychological outcome

NEPSY-II Subtests		ECMO group N=36	Control group N=64	P-value
Attention and executive functioning				
Auditory Attention median (IQR)	Commission *errors*	0 (0 to 0)	0 (0 to 0)	0.71
	Omission *errors*	0 (0 to 1)	0 (0 to 1)	0.45
	Inhibitory *errors*	0 (0 to 0)	0 (0 to 0)	0.09
Response set median (IQR)	Commission *errors*	1 (1 to 3)	2 (0 to 4)	0.82
	Omission *errors*	3 (1 to 6)	3 (2 to 5)	0.79
	Inhibitory *errors*	0 (0 to 1)	0 (0 to 1)	0.92
Language				
Word Generation total score, median (IQR)		32 (25 to 40)	35 (27 to 40)	0.22
Memory and learning				
Memory for Faces total score, median (IQR)		12 (11 to 13) *	12 (10 to 13)	0.54
Memory for Faces Delayed total score, median (IQR)		12 (10 to 14)	12 (10 to 14)	0.99
Narrative Memory ** total score, median (IQR)	Free recall	18 (14 to 24)	24 (20 to 26)	**0.001**
	Free and cued recall	22 (19 to 25)	26 (22 to 29)	**0.001**
	Recognition	14 (14 to 15)	15 (15 to 16)	**0.001**
Sensorimotor functioning				
Visuomotor Precision *total errors*, median (IQR) **		7 (1 to 13)	10 (4 to 22)	**0.05**
Visuospatial processing				
Arrows total score, median (IQR)		28 (26 to 32)	28 (26 to 30)	0.53
Geometric Puzzles total score, median (IQR)		30 (27 to 33)	30 (27 to 34)	0.58
Route Finding total score, median (IQR) **		9 (8 to 10)	9 (8 to 10)	0.81

Note: P-values were derived from Mann-Whitney U test.
** n=35 due to missing data in one subject*
*** n=28 versus n=56 since 8 subjects in both groups conducted six subtests of the NEPSY-II (since they were older than 12 years of age)*
The minimum and maximum scores of these nine subtest are: Auditory Attention commission errors: 0-180, omission errors: 0-30, inhibitory errors 0-35, Response set commission errors: 0-180, omission errors: 0-36, inhibitory errors: 0-37, Word generation: 0-no maximum, Memory for faces: 0-16, Memory for faces delayed: 0-16, Narrative memory free and cued recall: 0-34, recognition: 0-16, Visuomotor precision: 0-382, Arrows: 0-38, Geometric puzzles: 0-40, and Route finding: 0-10 points.

Chronic pain

Seventeen of 33 cases (51.5%) had experienced pain in the three months before the visit versus 43 of 64 children in the control group (67.2%; p=0.13). Five cases (15.2%) and nine controls (14.1%) reported chronic pain, having lasted longer than three months (p=0.89).

Analyses after exclusion of subjects with congenital diaphragmatic hernia

After exclusion of the cases who underwent repair of CDH, findings on thermal and pain sensitivity, brain activation during pain, neuropsychological functioning and chronic

pain were sustained. Only with respect to brain morphology we found a difference when excluding the CDH patients, since only the difference between the left thalamus after correction for age, gender, and total brain volume remained significant (p=0.02).

DISCUSSION

Children who had received ECMO-treatment as a neonate were less sensitive than controls to detect a cold stimulus, but only when measured in a reaction time dependent fashion. No differences in pain sensitivity, brain activation during pain, brain morphology, or in the occurrence of chronic pain were found. Neuropsychological testing found that the ECMO survivors performed significantly worse on a narrative memory subtest.

The difference in the temperature perceived to be cold between ECMO survivors and controls was no more than 0.7°C and there was no difference when applying the reaction time independent Method of Levels. The latter also held true for the warm detection and pain thresholds. Likewise, in the functional MRI study, no differences in brain activation were observed during pain. However, the NRS pain scores assigned to the painful stimulus were significantly higher in the ECMO group suggesting hypersensitivity to thermal heat pain compared to controls. Note that this only was found when comparing all the scanned children and not when only comparing the children included in the fMRI analyses. Five of the 32 ECMO children did not want to continue with fMRI scanning after the thermode was perceived as too hot. The corresponding proportion of control children was smaller (Flowchart Figure 1). Furthermore, the proportion of children with poor data quality due to movement in the ECMO group was higher than that in the control group (Flowchart Figure 1). Possibly, since the stimulus was too painful for the ECMO children, although we found no significant differences in pain thresholds between groups. The absence of differences in brain activation during pain in this study is in line with the only previous fMRI study in children, which nevertheless found differences in brain activation during pain between former preterm born children (not treated with ECMO) and healthy controls, but not between full term born NICU children (not treated with ECMO either) and healthy controls.[28]

The ECMO children's thalamus had significant smaller volume (0.3 $cm^{3)}$ than that of controls. However, this finding did not remain significant after correction for multiple testing. Therefore, the clinical relevance remains unclear. Duration of ECMO was negatively correlated with left thalamus volume, although not significant after correction for multiple testing. Interestingly, a previous study using cranial ultrasound images of neonates on ECMO also found that lesions mainly occurred in the left hemisphere,[2] while the right carotid artery was and right internal jugular vein were cannulated in general.[2]

On the basis of an animal study that found impaired adult cognitive functioning after early opioid exposure,[7] and a study in ECMO survivors that found concentration and behaviour problems,[16] we expected to find neuropsychological problems in our cohort of neonatal ECMO survivors as well. However, ECMO children performed comparably with healthy controls on the NEPSY-II subtests, except for memory performance. Possible memory deficits in ECMO survivors deserve further study since parents and children themselves also often mention this problem when they visit our outpatient clinic. Our finding that continuous and prolonged opioid exposure in the absence of severe pain induces no global neuropsychological problems seems to confirm the normal IQ scores later in childhood found in previous follow-up studies in preterm born children exposed to opioids.[11,12] Moreover, a follow-up study among neonatal ECMO survivors showed a normal range of intelligence.[16]

While rodent studies found major negative long-term effects of both early opioid and midazolam exposure in the absence of pain,[3-7] our findings only show minor effects on somatosensory processing, brain morphology, and neuropsychological functioning. Apart from the fact that animal data cannot be readily extrapolated to humans, differences in age of exposure, supratherapeutical dosages, duration of exposure, plasticity of the brain, and experimental methodology could account for the discrepancies with animal studies.[29]

The strength of this study is the multifaceted exploration of a unique cohort of children who had been exposed to opioids, sedatives, and some to methadone[30] from several days to months in the absence of severe pain, except for the ECMO group who received surgery for diaphragmatic hernia. Therefore, we conducted the analyses with and without those children. Findings from with and without the CDH children did not differ. There is a potential weakness of our study. Selection bias may represent a limitation to the generalizability of our findings. Children with the most severe neurological and cognitive outcomes were not invited for this study, as they were unable to participate in the neuropsychological and MRI assessments. However, no significant differences with respect to diagnosis, duration of ECMO treatment or duration of mechanical ventilation were observed between the included and excluded ECMO survivors. Moreover, the included children all had received ECMO therapy with high amounts of opioids and sedatives and had all been critically ill as neonates.

CONCLUSION

We found only subtle differences in thermal sensitivity and neuropsychological functioning between ECMO survivors and healthy controls. The ECMO survivors' significantly poorer outcome in the memory task warrants further investigation since it may explain why they generally need extra support in regular education or even special education at school age.[16] In conclusion, prolonged continuous administration of opioids and sedatives in the absence of pain does not negatively affect pain sensitivity, brain morphology, cortical thickness and brain functioning during pain in ECMO survivors, suggesting that the inherent plasticity of the human brain can overcome early negative stimuli such as drug exposure and ECMO therapy.

SUPPLEMENTARY DATA

Analgesic and sedative regimen on ECMO

During the study period the regimen for providing analgesia and/or sedation did not change and consisted of;

1. Cannulation under muscle relaxation and dosages of fentanyl (1-5 mcg/kg)
2. A continuous infusion of morphine in a starting dosage of 10 mcg/kg/hour
3. A continuous infusion of midazolam of 0.1-0.2 mg/kg/hour
4. In case of documented pain, boluses of morphine (10 mcg/kg) were given. Hereafter, the amount of pain was re-evaluated (Comfort scale). After three boluses with an inadequate response the continuous infusion was increased to 20 mcg/kg/hour
5. In cases of documented agitation in the absence of pain, midazolam infusions were increased to 0.2-0.3 mg/kg/hour following the same guideline of behavioral signs of agitation (see point 4)

Dose adjustment in the oldest group (1997-2000) was based on clinical observations by the care-taking nurses. From 2000 on, following the validation of the Comfort score for postoperative newborns and infants, standardized algorithms were used, which we also published (see literature[31-33]). We also published on the longitudinal changes in morphine and its degradation products M3-and M6 glucuronide.[34] The implementation of pain algorithms took place in 2000 and all patients from that point on, both ECMO and non-ECMO patients, have been treated according to our published algorithms.

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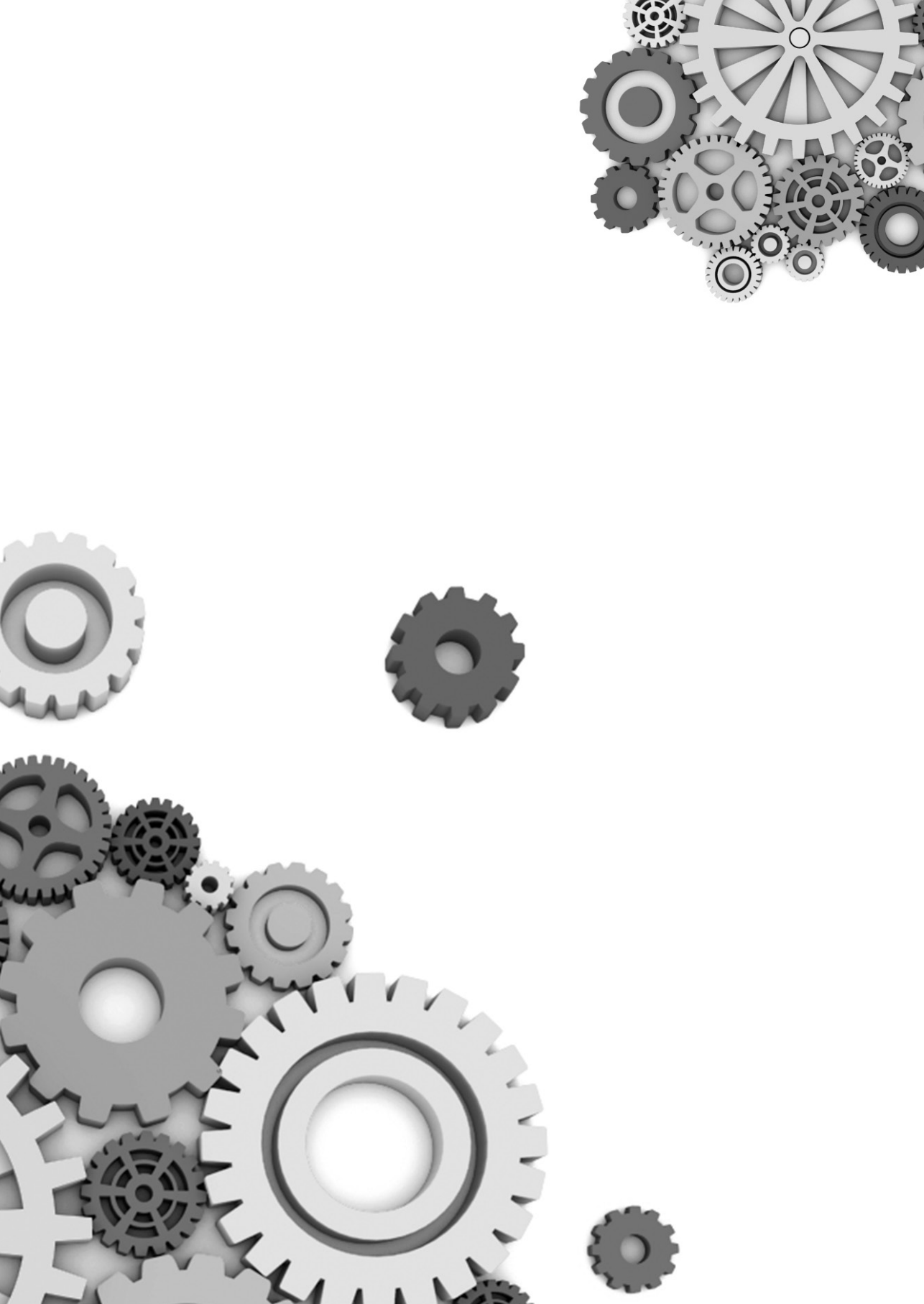

Chapter 9

Prematurity, opioid exposure and neonatal pain: Does it affect the developing brain?

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ABSTRACT

Background Ten years ago, preterm born children often routinely received morphine, especially during mechanical ventilation. Studies in neonatal rat pups, whose stage of brain development roughly corresponds to that of preterm born children, found negative long-term effects after exposure to pain and opioids.

Objectives We studied possible effects of prematurity, procedural pain and opioid exposure in humans some ten years later. Our hypothesis was that these factors would negatively influence neurobiological, neuropsychological and thermal sensory development later in life.

Methods We evaluated 19 preterm born children who as neonates participated in a RCT on the short-term effects of morphine administration and who previously participated in our follow-up studies on cognitive functioning, thermal sensitivity, and stress reactivity at ages 5 and 8 years. We assessed associations between brain morphology, neuropsychological functioning, thermal sensitivity and prematurity, opioid exposure and neonatal pain.

Results Significant correlations (coefficients 0.60-0.83) between gestational age, number of painful procedures, morphine exposure and brain volumes were observed. Significant correlations between these factors and thermal sensitivity were not established. Neuropsychological outcome was significantly moderately correlated with morphine exposure in only two subtests, and children performed in general 'Average' by Dutch norms.

Conclusions Although prematurity, opioid exposure and neonatal pain were significantly associated with brain volume, no major associations with respect to cognitive functioning or thermal sensitivity were detected. Administration of morphine in international used doses in neonatal life does not appear to affect neurocognitive performance or thermal sensitivity during childhood in preterm born children without brain damage during early life.

INTRODUCTION

The last trimester of gestation is very important for the maturation of the nervous system. Preterm born children, however, spend part of this trimester outside the protective environment of the uterus when the brain is still vulnerable to external perturbations.[1] Moreover, admitted to the neonatal intensive care unit (NICU) they undergo many potentially painful procedures, estimated even today at approximately 10 daily.[2,3] These may cause pain-related stress and alterations in the intracranial blood volume and blood pressure, with risk of intraventricular haemorrhage (IVH) and periventricular leukomalacia.[4,5] Pain management traditionally consisted of opioids, but many NICUs nowadays are reluctant to use these. For one thing, there is uncertainty about the effects that procedural pain and opioid exposure in preterm born children may have on the long term. Furthermore, previous RCTs have not found beneficial effects of the routine use of morphine infusions in ventilated preterm newborns.[6,7]

Studies in neonatal rat pups, whose stage of brain development roughly corresponds to that of preterm born children,[8] have found increased neuroapoptosis[9] and impaired cognitive functioning after exposure to pain and opioids.[10] However, these effects mainly occurred in response to an induced chronic inflammatory response not necessarily mimicking the situation in humans. In humans, neurological and developmental disabilities were found in almost half of a cohort of extremely preterm born children at the median age of 30 months.[11] Furthermore, a significant association between more skin-breaking procedures and poorer cognition,[12] smaller brain volumes,[13] and alterations in pain sensitivity has been described in former preterms.[14]

As previous studies found short- and long-term effects of pain and pain treatment in several separate domains, including brain development, cognition and pain sensitivity, our goal was to study all these long-term consequences in a single, well-defined cohort of preterm born children who participated in an RCT as a neonate[6] and who we have followed for about ten years.[15,16] The use of morphine was significantly negatively correlated with one IQ subtest at the age of 5 years,[15] and positively correlated to executive functioning at 8/9 years of age.[16] The obtain more insight in their long-term neurobiological outcome, we conducted structural magnetic resonance imaging (MRI) to study brain morphology and assessed neuropsychological functioning and thermal sensitivity.

PATIENTS AND METHODS

Study population

Preterm born children were recruited from a cohort of that had participated in an RCT as neonate between 2000 and 2002 comparing continuous infusion of morphine with placebo. Details have been published previously.[6,17] Some of these children also participated in two follow-up studies (Figure 1).[15,16] Since formal power analyses are hard to conduct in fMRI studies, we aimed to include at least as many children as in the only previous fMRI pain study determining the long-term effects of neonatal pain including nine children per subgroup.[18] For feasibility reasons we chose to only include children of the original RCT which were recruited in Rotterdam and included in the local follow-up program (n=44).[16] Participants were recruited from both arms of the original RCT, as short-term survival and long-term cognition did not essentially differ between the groups.[6,15,16] Reasons for exclusion were the following: twins or triplets (n=5), contra-indications for participation in an MRI study or neuropsychological assessment (n=11), such as documented intellectual disabilities (IQ 80 or less), brain abnormalities such as a delay in myelinisation or IVH, or hearing loss since these children could not properly understand the procedure and brain abnormalities could possibly influence brain functioning during pain or brain morphology. Furthermore, six term born children were excluded. Invitation letters eventually went out to 22 families.

Children with a specific contraindication for participation in an MRI study (i.e., permanent braces or claustrophobia) were invited to participate in the other components of the study. The study was performed at the Erasmus University Medical Center (Erasmus MC) in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Erasmus MC Institutional Review Board (MEC-2010-299). Informed consent was obtained from the parents of each child prior to participation. Children were recruited from July 2011 to February 2012.

Neuropsychological testing

Neuropsychological functioning was tested with the NEPSY-II-NL neuropsychological test (Pearson).[19] Norm scores and percentile scores are available for Dutch children aged between 5 and 12 years old. Participants completed nine subtests addressing areas of cognitive functioning such as attention and executive functioning, language, memory and learning, sensorimotor functioning, and visuospatial processing.

Chronic pain questionnaire

All participants filled out the Dutch chronic pain questionnaire, which addresses the presence of current pain and chronic pain.[20]

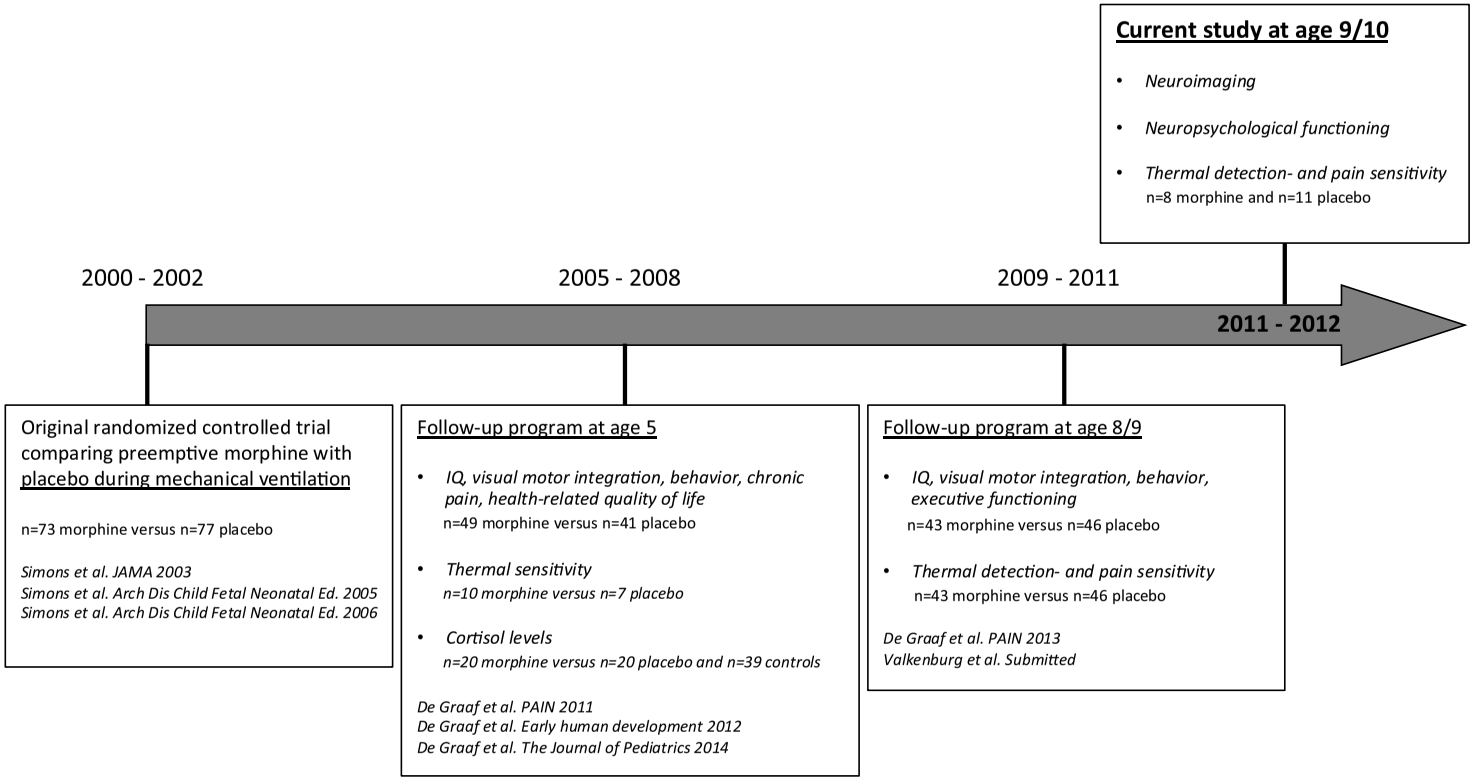

Figure 1 - Follow-up program

Examination of the detection and pain thresholds

Detection- and pain thresholds were obtained using the computer-controlled Thermal Sensory Analyzer (TSA type II, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel) with a Peltier-based contact thermode (30 x 30 mm). After explaining the TSA test, we determined detection- and pain thresholds using a standardized protocol. Detection thresholds were measured using both the reaction time dependent Method of Limits (MLI) and the reaction time independent Method of Levels (MLE). For more details see van den Bosch et al. 2014.[21]

Image acquisition

MR images were acquired on a 3 Tesla scanner (General Electric Discovery MR750, Milwaukee, MI, USA) using an 8-channel head coil for signal reception. Cushions supported the child's head and minimized head motion. We obtained a high-resolution structural T_1-weighted image using an inversion recovery fast spoiled gradient recalled (IR-FSPGR) sequence with the following parameters: TR = 10.3 ms, TE = 4.2 ms, TI = 350 ms, NEX = 1, flip angle = 16°, readout bandwidth= 20.8 kHz, matrix 256 x 256, imaging acceleration factor of 2, and an isotropic resolution of 0.9x0.9x0.9 mm^3. The scan time was 5 minutes 40 seconds.

Structural imaging analysis

Structural imaging analyses was performed using the Freesurfer image analysis suite version 5.1.0 (http://surfer.nmr.mgh.harvard.edu/).[22] Each image was first visually inspected and subjects with poor quality data were excluded. In subjects with small errors in the grey/white segmentation, control points, and white matter, edits were added to identify and correct misclassified white matter regions. When the segmentation improved, the corrected images were used.

Statistical analysis

Normally distributed variables are presented as mean (standard deviation) and non-normally distributed variables as median (range or interquartile range (IQR)). Spearman rank order correlation coefficient (with 95% confidence intervals) was applied to calculate correlations between the non-normally distributed variables gestational age, number of painful procedures in the first 14 days of life and total morphine exposure in the first 28 days of life with brain volumes, NEPSY-II outcomes, and detection- and pain thresholds. A p-value of 0.05 or less was considered statistically significant. Analyses were conducted with IBM SPSS 20.0.

RESULTS

Study Population

Twenty-two families received an information letter. As one child was lost to follow-up and two families declined participation, 19 children participated; 13 boys and six girls with mean age 10.2 (SD 0.4) years. Numbers of children included in the different analyses are presented in Figure 2. Of the 19 children, 11 received placebo in the original RCT and 8 received pre-emptive morphine. Of the children in the placebo arm of the original RCT, only 4 did not receive additional open-label morphine. One child had undergone surgery in the neonatal period (clipping of patent ductus arteriosus and ileostomy) and was not scanned due to contra-indications for MRI. Other characteristics of these 19 children are presented in Table 1. The 19 included children did not differ from the 25 excluded children with regards to gender (p=0.40), gestational age (p=0.69), number of painful procedures in the first 14 days of life (p=0.55), or morphine exposure in the first 28 days of life (p=0.65).

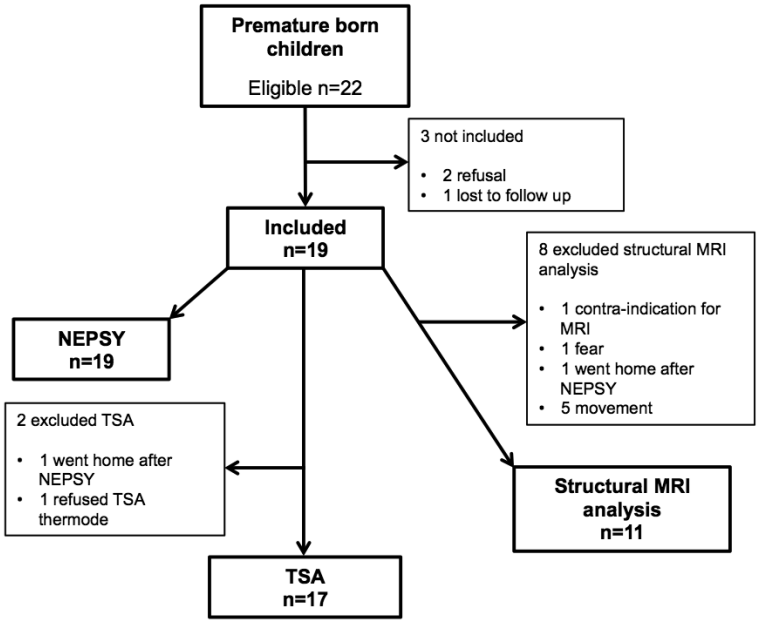

Figure 2 - Inclusion flowchart

Correlation coefficients

The variables gestational age, number of painful procedures in the first 14 days of life (mean per day), and morphine exposure in the first 28 days of life, were not significantly correlated with each other, although the direction of the correlation was as expected

Table 1 - Demographic and clinical characteristics

	Preterm born children N=19
General characteristics	
Age (Mean (SD))	10.2 (0.4)
Gender (male %)	68.4
Ethnicity (Western European %)	68.4
Gestational age in weeks (median, range)	31.1 (26.1 - 36.3)
Birth weight (grams, median, range)	1415 (675 - 2895)
Number of painful procedures per day* (median, range)	12 (4 to 18)
CRIB score (median, range)	4 (0 - 8)
Age at ICU admission in days (days, median, range)	0 (0 - 0)
Duration of ICU stay in days (days, median, range)	15 (4 - 63)
Duration of mechanical ventilation (days, median, range)	4 (2 - 26)
Pharmacological data	
Morphine administration (% yes)	78.9
Cumulative use of IV morphine in the first 28 days in mcg/kg (median, range)	393.6 (0 - 4873)

Note: CRIB: Clinical Risk Index for Babies, IV: intravenous.

* *Measured in the first 14 days, presented as mean per day. Based on n=14 due to missing data*

Table 2 - Global brain volumes and volumes of pain related brain regions

		Preterm born children N=11
Global Brain Volumes		
Total Brain Volume (Mean (SD), cm^3)		1129 (111)
Cerebral White Matter (Mean (SD), cm^3)		372 (41)
Total Grey Volume (Mean (SD), cm^3)		713 (64)
Parietal lobe (Mean (SD), cm^3)	Left	72 (8)
	Right	74 (8)
Cerebellum (White Matter) (Mean (SD), cm^3)	Left	13 (2)
	Right	13 (2)
Cerebellum (Cortex) (Mean (SD), cm^3)	Left	56 (5)
	Right	57 (6)
Pain Related Brain Regions		
Thalamus (Mean (SD), cm^3)	Left	6.6 (0.8)
	Right	6.6 (0.9)
Amygdala (Mean (SD), cm^3)	Left	1.6 (0.2)
	Right	1.6 (0.2)
Anterior Cingulate Cortex (Mean (SD), cm^3)	Left	2.0 (0.3)
	Right	2.7 (0.5)
Insula (Mean (SD), cm^3)	Left	6.9 (0.7)
	Right	6.8 (0.8)

(gestational age and painful procedures; −0.40 (p=0.29, 95% confidence interval −0.84 to 0.36), gestational age and morphine exposure; −0.50 (p=0.12, 95% confidence interval −0.85 to 0.14), and painful procedures and morphine exposure; 0.30 (p=0.43, 95% confidence interval −0.45 to 0.80).

Structural imaging results

No incidental brain anomalies were detected on the MRI scans. Brain volumes of the 11 scanned children with good data quality are presented in Table 2. We found statistically significant strong to very strong correlations between gestational age (range of the correlation coefficients 0.62 to 0.76), number of painful procedures (−0.73 to −0.83) and morphine exposure (−0.60 to −0.74), and volumes of brain regions (Table 3).

Neuropsychological functioning

No statistical significant correlations between gestational age and any of the NEPSY outcomes were found. Furthermore, the correlation coefficients indicated a very weak to moderate correlation (range of the correlation coefficients; −0.20 to −0.07 and 0.03 to 0.37). The number of painful procedures was also not significantly correlated to NEPSY outcomes and the correlation coefficients were very weak to moderate as well (range of the correlation coefficients; −0.41 to −0.10 and 0.03 to 0.47). A significant correlation was found between morphine exposure in the first 28 days and the total amount of commission errors in the subtest Response Set (coefficient −0.46, p=0.05). Furthermore, there was a significant correlation between morphine exposure and the total score for Recognition in the subtest Narrative Memory (coefficient −0.46, p=0.05). Children in general scored 'average' by Dutch norms (Pearson NEPSY-II-NL manual) (Table 4). Only the number of Response Set Omission errors and Visuomotor Precision errors corresponded to a 'low average' score.

Detection and pain thresholds

Reliable data on detection and pain- thresholds were obtained from 16/17 children, depending on the subtest (Table 5). We found no statistically significant correlations between gestational age, number of painful procedures and morphine exposure with detection thresholds (MLI and MLE) and pain thresholds. Moreover, the correlation coefficients indicated a very weak to moderate correlation (range correlation coefficients; −0.44 to −0.07 and 0.01 to 0.40).

Chronic pain

Thirteen of the 19 children (68.4%) had experienced pain in the three months before the visit. Three children (15.8%) had chronic pain, i.e. lasting longer than three months.

Table 3 - Correlations between brain volumes and gestational age, morphine exposure and number of painful procedures in preterm born children

		Gestational age N=11	Morphine exposure N=11	Painful procedures N=9*
Global Brain Volumes		Correlation coefficient (95% confidence interval)	Correlation coefficient (95% confidence interval)	Correlation coefficient (95% confidence interval)
Total Brain Volume		**0.76 (0.30 to 0.93)**	**−0.67 (−0.91 to −0.12)**	−0.47 (−0.86 to 0.28)
Cerebral White Matter		**0.62 (0.03 to 0.89)**	**−0.74 (−0.93 to −0.25)**	−0.45 (−0.86 to 0.31)
Total Grey Volume		**0.73 (0.23 to 0.92)**	**−0.60 (−0.88 to −0.001)**	−0.43 (−0.85 to 0.33)
Parietal lobe	Left	**0.67 (0.12 to 0.91)**	**−0.68 (−0.91 to −0.14)**	−0.37 (−0.83 to 0.39)
	Right	**0.76 (0.30 to 0.93)**	−0.47 (−0.83 to 0.18)	−0.42 (−0.85 to 0.34)
Cerebellum (White Matter)	Left	**0.67 (0.12 to 0.91)**	**−0.65 (−0.90 to −0.08)**	**−0.83 (−0.96 to −0.37)**
	Right	0.49 (−0.16 to 0.84)	−0.52 (−0.85 to 0.12)	**−0.80 (−0.96 to −0.29)**
Cerebellum (Cortex)	Left	0.53 (−0.10 to 0.86)	−0.47 (−0.83 to 0.18)	−0.65 (−0.92 to 0.02)
	Right	0.36 (−0.31 to 0.79)	−0.18 (−0.70 to 0.47)	−0.35 (−0.82 to 0.41)
Pain Related Brain Regions				
Thalamus	Left	0.40 (−0.26 to 0.81)	−0.46 (−0.83 to 0.19)	**−0.73 (−0.94 to −0.13)**
	Right	0.52 (−0.12 to 0.85)	−0.53 (−0.86 to 0.10)	−0.52 (−0.88 to 0.22)
Amygdala	Left	0.27 (−0.39 to 0.75)	−0.35 (−0.79 to 0.32)	0.28 (−0.47 to 0.80)
	Right	0.35 (−0.32 to 0.79)	**−0.67 (−0.91 to −0.12)**	0.00 (−0.66 to 0.66)
Anterior Cingulate Cortex	Left	0.08 (−0.55 to 0.65)	0.39 (−0.27 to 0.80)	0.35 (−0.41 to 0.82)
	Right	**0.66 (0.10 to 0.90)**	−0.45 (−0.83 to 0.21)	−0.22 (−0.77 to 0.52)
Insula	Left	−0.17 (−0.70 to 0.48)	−0.37 (−0.79 to 0.30)	−0.10 (−0.72 to 0.60)
	Right	0.11 (−0.52 to 0.67)	−0.57 (−0.87 to 0.05)	−0.27 (−0.79 to 0.48)

Note: Correlation coefficients were derived from Spearman's correlation test

** Based on n=9 due to missing data*

Table 4 - Neuropsychological outcome

NEPSY-II Subtests		Preterm born children N=19
Attention and executive functioning		
Auditory Attention (median (IQR))	Commission errors Omission errors Inhibitory errors	0 (0-0) 1 (0-2) 0 (0-0)
Response set (median (IQR))	Commission errors Omission errors Inhibitory errors	2 (1-4) 5 (2-8) 1 (0-2)
Language		
Word Generation (total score, median (IQR))		28 (24-36)
Memory and learning		
Memory for Faces (total score, median (IQR))		10 (7-12)
Memory for Faces Delayed (total score, median (IQR))		11 (9-13)
Narrative Memory (total score, median (IQR))	Free and cued recall Recognition	25 (23-28) 15 (14-16)
Sensorimotor functioning		
Visuomotor Precision (total errors, median (IQR))		12 (5-18)
Visuospatial processing		
Arrows (total score, median (IQR)) Geometric Puzzles (total score, median (IQR)) Route Finding (total score, median (IQR))		27 (24-31) 30 (28-32) 9 (8-10)

Note: The minimum and maximum are: Auditory Attention commission errors: 0-180, omission errors: 0-30, inhibitory errors 0-35, Response set commission errors: 0-180, omission errors: 0-36, inhibitory errors: 0-37, Word generation: 0-no maximum, Memory for faces: 0-16, Memory for faces delayed: 0-16, Narrative memory free and cued recall: 0-34, recognition: 0-16, Visuomotor precision: 0-382, Arrows: 0-38, Geometric puzzles: 0-40, and Route finding: 0-10 points.

Table 5 - Detection- and pain thresholds

		Preterm born children N=17
Method of Limits (MLI)		
Cold detection threshold (°C) Warm detection threshold (°C)	Mean (SD) Mean (SD)	30.0 (1.9) 34.8 (2.4)
Cold pain threshold (°C)* Threshold not reached**	Mean (SD) (n, %)	13.5 (9.1) 6 (37.5)
Heat pain threshold (°C)* Threshold not reached**	Mean (SD) (n, %)	45.0 (4.4) 6 (37.5)
Method of Levels (MLE)		
Cold detection threshold (°C) Number of stimuli	Mean (SD) Mean (SD)	30.6 (1.3) 10 (3)
Warm detection threshold (°C) Number of stimuli	Mean (SD) Mean (SD)	33.6 (1.3) 10 (3)

*Note: * 16 children*
*** The child did not press the button before the minimum or maximum temperature of 0°C or 50°C at least once during the test.*

DISCUSSION

We found that gestational age, neonatal pain and morphine exposure were correlated with brain volume, but not with cognitive performance or thermal detection and pain thresholds. The associations with respect to brain volume indicated that a lower gestational age, higher number of painful procedures in the first 14 days of life, and higher exposure to morphine in the first 28 days of life was correlated with smaller brain volumes. Interestingly, we did find in general average scores on cognitive functioning, in contrast to our expectations based on animal studies, but in line with previous follow-up studies in preterm born children at our department.[15,16] While the factors gestational age, pain and morphine exposure are correlated to a smaller brain volume in preterm born children Thus, our findings do not support major differences in cognitive functioning later in life.

Previous studies found altered brain morphology and functioning during pain in preterm born children.[13,18,23] We also found that prematurity, opioid exposure and neonatal pain was associated with reduced cortical and white matter volumes. Comparing the MRI scans of the preterm born children with those of healthy controls, obtained for other follow-up studies of our department,[24] we found no differences in cortical thickness and no differences in brain volumes after correction for age, gender, total brain volume, and multiple testing (data not shown). A possible explanation is that any reductions in brain volume and size at term-equivalent age had disappeared over time due to the inherent plasticity of the human brain associated with development.

A possible explanation for the lack of significant correlations in the present study with respect to cognitive development and thermal sensitivity would be the relatively low dose of 10μg/kg/h morphine administered to the morphine group in the original RCT. In the only other comparable RCT in neonates born between 30-32 weeks of gestation the dose was 30μg/kg/h.[7] A likely explanation for our lack of results is the relatively small sample size, which however should have permitted to detect significant correlations as in the structural MRI results. Still it would seem that gestational age, morphine exposure and painful procedures exert an effect mainly on brain volume but not on brain function. The previous follow-up studies in this unique cohort likewise did not evidence major negative effects of neonatal morphine exposure on cognition.[15,16]
The neuropsychological test results of all children were generally comparable to Dutch norm scores – in line with what we found previously.[16] A previous study in rodents did find impaired cognitive functioning in adulthood after neonatal morphine administration.[10] Findings are hard to compare; for one thing because cognitive functioning obviously was measured in different ways. In human preterm born children, an association was found

between the number of skin-breaking procedures and poorer cognition measured at 18 months after birth.[12] Although the children in our cohort had experienced approximately 12 skin-breaking procedures per day as a neonate, we did not confirm this association. Comparing the neuropsychological test to those of a healthy age- and gender-matched control group we found no significant differences in neuropsychological functioning (data not shown). A possible explanation is that possible existing effects at a very young age may have disappeared during childhood due to great plasticity of the brain.

While previous studies found evidence for hypersensitivity for pain in preterm born children with a history of procedural pain and opioid exposure,[14,25] we did not find significant correlations between clinical characteristics and detection- or pain thresholds. When comparing these children to healthy controls,[24] no statistical differences were obtained (data not shown). Moreover, our obtained pain threshold for heat was roughly comparable to that of preterm born children described in the literature.[18]

The strength of this study is that relevant prospectively collected information regarding, pain exposure (number of skin breaking procedures) and morphine consumption was available from the prior RCT. A limitation is the relatively small sample size. However, this unique cohort participated in previous follow-up studies of our department at younger ages.[15,16] By adding neuroimaging to the previous follow-up programs, we present a comprehensive and unique view of the long-term effects of low-dose morphine administration and procedural pain in preterm born children.

CONCLUSION

We report strong to very strong correlations between prematurity, opioid exposure and neonatal pain with brain volumes. However, and in our view more important, we did not observe strong correlations with neurocognitive performance or thermal sensitivity. Furthermore, preterm born children scored average according to norm scores on cognitive tests indicating an effect mainly on brain volume but not brain function. We conclude that the administration of morphine in low doses in the neonatal period does not appear to affect neurocognitive performance or thermal sensitivity in the long run in preterm born children without brain damage during early life.

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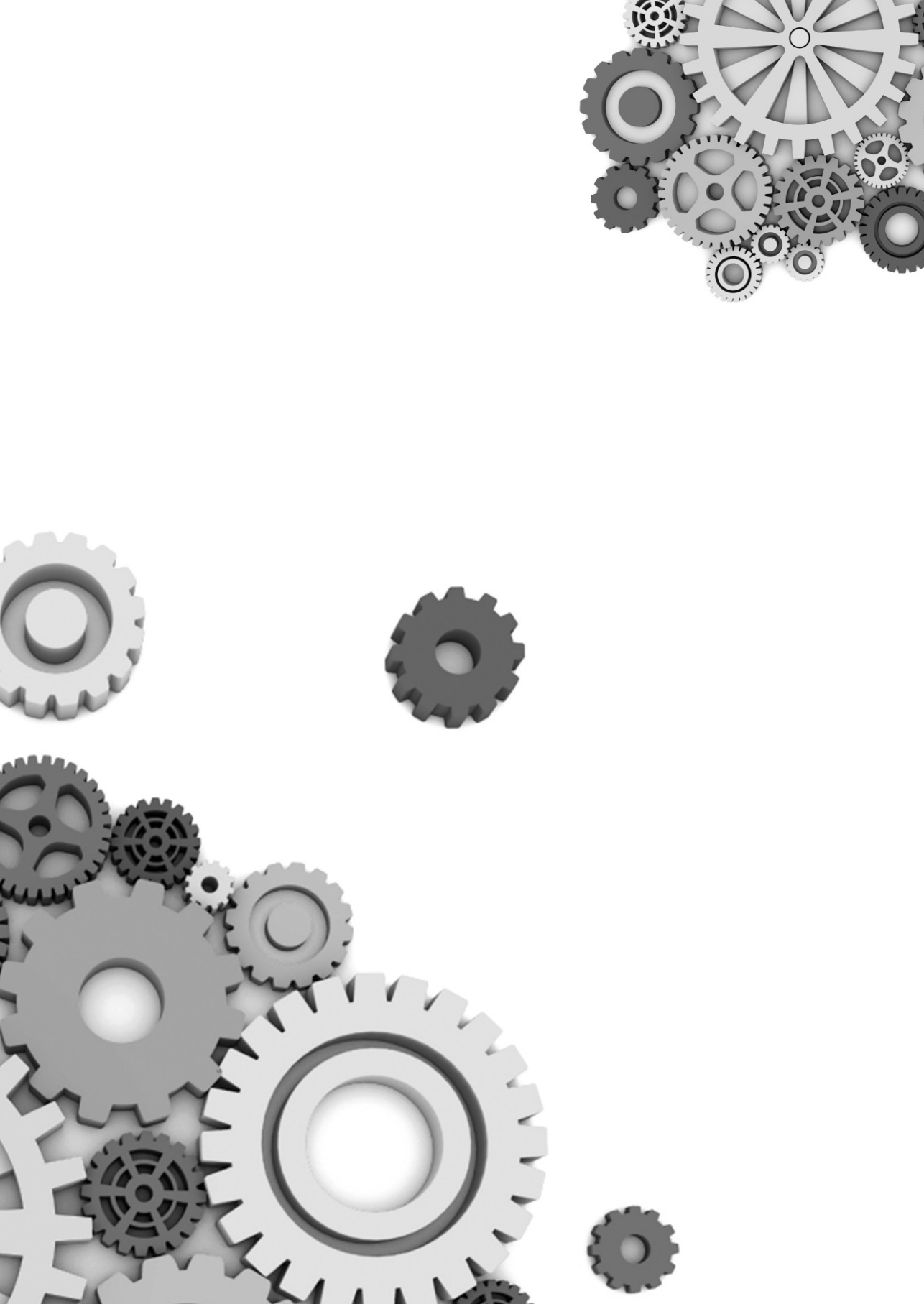

Chapter 10

Long-term effects of opioid exposure in utero

A neuropsychological and neuroimaging study

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ABSTRACT

Background The number of children exposed to heroin and prescription opioids in utero is growing, especially in the United States. These children do not suffer from pain and therefore serve as a unique human model to study the long-term effects of early opioid exposure in the absence of pain. This is useful since animal studies showed negative outcomes in terms of neurotoxicity and pain sensitivity when opioids were given without pain. We studied the long-term effects of early opioid exposure in the absence of pain and hypothesized alterations in pain sensitivity and brain activation during pain, worse neuropsychological functioning, and smaller brain volumes.

Methods Fifteen individuals prenatally exposed to opioids (9.4-19.4 years) were compared to 71 healthy controls (8.2-17.9 years). Primary outcomes were thermal sensitivity and brain functioning during pain (functional MRI). Secondary outcomes were brain morphology (high-resolution MRI) and neuropsychological functioning.

Results We observed no statistically significant differences in thermal and pain sensitivity or brain morphology. However, cases showed statistically significant less brain activation in the frontal lobe during pain. Additionally, cases performed significantly worse on four subtests of the neuropsychological test, involving visiospatial processing, language, attention and executive functioning ($p<0.01$).

Conclusions Early opioid exposure in the absence of pain is associated with less brain activation during pain in the frontal lobe, which is a brain region typically found to be associated with attention and executive functioning rather than pain, and poorer neuropsychological functioning. Interestingly, no differences in pain sensitivity or brain morphology were observed indicating primarily neuropsychological effects.

INTRODUCTION

Misuse of prescription opioids and abuse of illicit drugs is a growing problem among pregnant women, especially in the USA.[1,2] Newborn infants of these mothers are at risk of developing neonatal abstinence syndrome (NAS), which includes increased muscle tone, irritability, diarrhea, feeding difficulties and requires hospital admission.[1] In vitro studies have shown that prenatal opioid exposure increases apoptosis of fetal human microglial cells.[3] Prenatal opioid exposure has also been associated with neurodevelopmental impairments at several domains, hyperactivity in infancy, and smaller brain volumes as compared to controls.[4-9]

Animal studies have shown that the negative long-term effects of postnatal opioid exposure may differ depending on whether they were given in the absence or presence of pain, with protective effects in terms of pain sensitivity and neurotoxicity in animals in the latter case.[10-15] In humans it is impossible to study the long-term effects of neonatal opioid exposure in the absence of pain, since the clinical use of opioids is linked with the presence of pain and it is unethical to administer opioids to pediatric patients in the absence of pain. Children and adolescents with prenatal exposure to synthetic opioids such as methadone, however, could serve as a unique model in this respect. The present study is the first to study possible effects of prenatal opioid exposure on pain processing and brain functioning during pain in children and adolescents. We measured thermal and pain sensitivity and brain activity during a painful stimulus, and compared outcomes in prenatally exposed children and adolescents with those of healthy controls. To provide a complete picture, we also imaged brain morphology by MRI and tested neuropsychological functioning. Based on previous studies in animals and humans we hypothesized that children and adolescents prenatally exposed to opioids, would show alterations in thermal and pain sensitivity and in brain activation during pain, worse neuropsychological functioning, and smaller brain volumes.

PATIENTS AND METHODS

Study population

Children and adolescents who were prenatally exposed to opioids (Cases)

From October 1993 to May 2005, 80 newborn infants were admitted to the Erasmus MC-Sophia Children's Hospital in Rotterdam, the Netherlands, for treatment of NAS due to prenatal opioid exposure. The mothers of these children used heroin and methadone during pregnancy and had been intensively coached at a special outpatient clinic for drug abusing pregnant women throughout pregnancy. Information on type of drugs

used during pregnancy is therefore available. Urine samples for toxicology were randomly collected throughout pregnancy and postpartum urine samples of the child were collected within 12 hours after birth. The newborn infants were admitted to the neonatology ward and treated for symptoms of NAS if indicated by high Finnegan scores.[16] One of these 80 children died, 17 were lost to follow-up and 19 were excluded from this study for several reasons including medical problems, such as severe hearing loss, since these children could not properly participate in the different tests (See Figure 1). A letter with relevant information was sent to the remaining 43 cases and these cases were asked by phone two weeks later if they were willing to participate. Seventeen could not be reached by phone, and 10 cases declined participation. One case was excluded because of previously unknown intellectual disabilities (Flowchart Figure 1). Clinical background characteristics of the remaining 15 cases were retrieved from the medical records.

Control group

A control group of 8 to 18-year-olds without a history of or intra-uterine opioid exposure or neonatal pain necessitating opioid treatment was recruited in two ways. First, we asked participants for this and other studies in our department whether they could recommend a volunteer.[17,18] A letter with relevant information was sent to these potential volunteers and they were asked by phone two weeks later if they were willing to participate. Second, we mailed invitation letters to parents of children attending three primary schools in Rotterdam. Interested parents were asked to contact the researcher for further information or to set a date for the study procedure. A total of 75 controls were recruited. Four of them were excluded since they had been born prematurely. The other 71 were included in this study. Children who had a contraindication for participation in an MRI study (pacemaker or permanent braces) were invited to participate only in the behavioral component of the study. The use of psychoactive medication on the day of MRI scanning was a contraindication for the fMRI experiment.

Setting

The study was performed at Erasmus MC in Rotterdam in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board. Informed consent was obtained from the parents and assent from the participant from the age of 12 years. Controls were recruited from June 2011 to March 2013. The cases were recruited in November and December 2013.

Procedure

Testing started with a neuropsychological test administered to all cases and controls up to 16 years of age. Then, subjects of all ages filled out the Dutch Chronic Pain

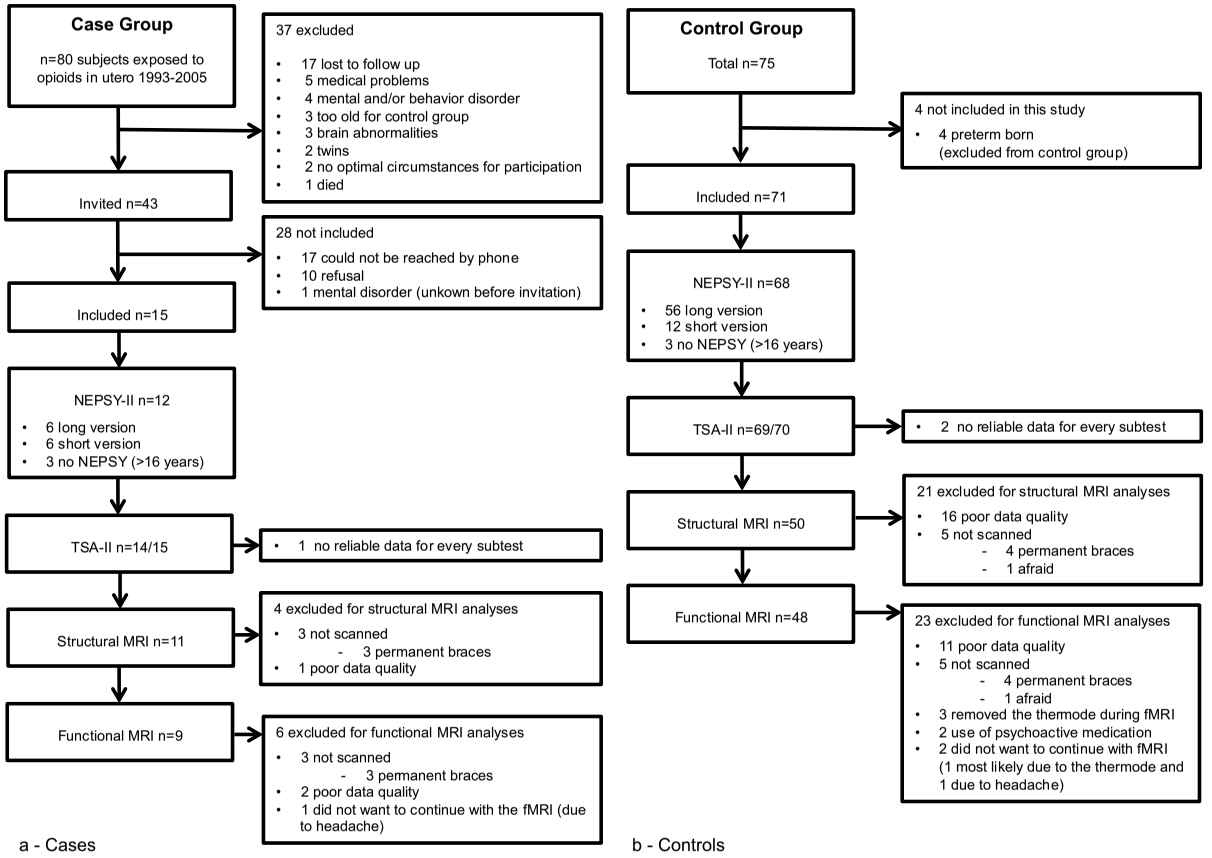

Figure 1a,b – Inclusion flowcharts
Inclusion flowchart of the case group (a) and the control group (b).

Questionnaire.[19] Next, subjects were instructed on the MRI experiment and underwent a mock scan. Subsequently, detection- and pain thresholds for cold and warmth were determined. The final part was a high-resolution structural T1 weighted MRI scan and two runs of a functional MRI scan during which subjects received thermal pain stimuli. Tests are further detailed below.

Neuropsychological testing

Children between 8 and 12 years of age were administered nine subtests of the NEPSY-II neuropsychological test (Pearson),[20] addressing five different domains of cognitive functioning, i.e. attention and executive functioning, language, memory and learning, sensorimotor functioning, and visiospatial processing. Children aged between 13 and 15 years were administered only six subtests, due to the age limit of the other three subtests. These three excluded subtests addressed memory and learning, sensorimotor functioning, and visiospatial processing.

Chronic pain questionnaire

The Dutch chronic pain questionnaire[19] obtains information on current pain and whether pain was present for more than three months, in which case it was defined as chronic.[19]

Thermal detection and pain threshold testing

Individual detection- and pain thresholds were obtained and pain stimuli were applied using the MRI-compatible, computer-controlled Thermal Sensory Analyzer (TSA type II, Medoc Ltd. Advanced Medical Systems, Ramat Yishai, Israel) with a Peltier-based contact thermode (30 x 30 mm). After explaining the thermal threshold test, we determined detection- and pain thresholds using a standardized protocol applying both the reaction time dependent Method of Limits (MLI) and the reaction time independent Method of Levels (MLE). Furthermore, subjects rated pain intensity of a standardized thermal stimulus of 46°C on a numerical rating scale (NRS). For more details see van den Bosch et al.[18]

Image acquisition and analyses

MR images were acquired on a 3 Tesla scanner (Discovery MR750, General Electric, Milwaukee, MI, USA), and analyses were conducted using the Freesurfer image analysis suite version 5.1.0 (http://surfer.nmr.mgh.harvard.edu/) for structural MRI analyses and FMRIB's fMRI Expert Analysis Tool FEAT (http://www.fmrib.ox.ac.uk/fsl/feat5/index.html) for the functional MRI analyses. For more details see the supplementary data and Figure 2.

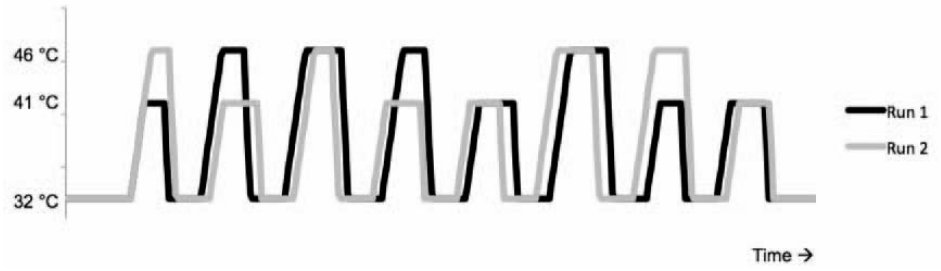

Figure 2 - Block design of both runs

Non-imaging statistical analysis

Normally distributed variables are presented as mean (with standard deviation) and non-normally distributed variables as median (with inter-quartile range or range). Independent samples t-tests and Mann-Whitney U tests were applied for continuous data; Chi squared tests or Fisher Exact tests for categorical data. Tests were conducted with a two-sided significance level and with (using ANCOVA test) and without correction for age and gender since the difference in age and gender could possibly influence the results. Bonferroni correction served to correct for multiple testing. A p-value of 0.05 or less was considered statistically significant. Analyses were conducted using IBM SPSS 20.0.

RESULTS

Study Population

Fifteen cases with a median age of 15.1 years (range 9.4 to 19.4) were compared to 71 controls with a median age of 11.1 years (range 8.2 to 17.9). Controls were significantly younger than the cases, which we controlled for in the analyses ($p<0.01$) (Table 1). Both groups showed a female predominance (cases 73% and controls 58%; $p=0.39$). From the medical records it appeared that eight cases (53%) had been exposed to opioid-related substances throughout pregnancy. For the other seven cases this could be confirmed for the last trimester of pregnancy only; information about maternal drug abuse in the first and second trimesters was less reliable due to late first prenatal check-ups. Other clinical background characteristics are presented in Table 2. Numbers of subjects included and excluded per subtest are presented in Figure 1.

Table 1 - Demographic characteristics

	Case group	Control group	P value
Total group (N=86)	N=15	N=71	
Age (Mean (SD))	14.2 (3.2)	11.7 (2.5)	**0.01**
Gender (male %)	26.7	42.3	0.39
Handedness (Right %)	86.7	95.8	0.21
Structural MRI analysis (n=61)	N=11	N=50	
Age (Mean (SD))	14.8 (3.3)	12.1 (2.5)	**<0.01**
Gender (male %)	36.4	42.0	1.0
Functional MRI analysis (n=57)	N=9	N=48	
Age (Mean (SD))	15.0 (3.5)	12.1 (2.7)	**<0.01**
Gender (male %)	33.3	47.9	0.49

P-values were derived from Independent samples T-test test for continuous variables and Fishers exact tests for categorical variables

Table 2- Background characteristics of the case group

Background Characteristics		Case group N=15
Birth characteristics		
Gestational age, weeks, median (IQR)		38 (36 to 41)
Prematurely born (less than 37 weeks of gestation), n (%)		4 (27%)
Birth weight, in grams, median (IQR)		2935 (2400 to 3215)
Apgar scores after 1 minute, median (IQR)		9 (7 to 9)
Apgar scores after 5 minutes, median (IQR)		10 (9 to 10)
Apgar scores after 10 minutes, median (IQR)		10 (10 to 10)
Born in our Hospital, n (%)		15 (100)
Intensive care admission, n (%)		3 (20)
Length of stay, in days, median (IQR)		17 (11 to 22)
Pharmacological characteristics		
Prenatal exposure to Methadone, n (%)		13 (87)
Prenatal exposure to Heroine, n (%)		12 (80)
Prenatal opioid exposure in combination with:	Cocaine, n (%)	13 (87)
	Benzodiazepines, n (%)	1 (7)
NAS		
NAS (Finnegan score ≥ 8)*, n (%)		14 (93)
Phenobarbital treatment, n (%)		14 (93)
Demographic characteristics		
West-European, n (%)		8 (53)
Caregiver	Adopted/foster parents, n (%)	13 (87)
	With relatives (grandmother), n (%)	3 (23)
	Biological parents, n (%)	2 (13)
Education	Special primary education, n (%)	2 (13)
	Primary education, n (%)	4 (27)
	Lower vocational education, n (%)	5 (33)
	Intermediate vocational education, n (%)	3 (20)
	Higher vocational education, n (%)	1 (7)

IQR - Interquartile range

** NAS: Neonatal Abstinence Syndrome*

Neuropsychological functioning

Cases scored poorer on two visiospatial processing subtests; *Geometric Puzzles* (p=0.02) and *Route Finding* (p=0.02) (Table 3). After correction for age and gender the subtest *Geometric Puzzles* remained statistically significantly different (p=0.002). Furthermore, cases scored significantly worse on the subtests *Response Set* (more omission errors) (p=0.002), *Word Generation* (p=0.002), and *Arrows* (p=0.002) (Table 3). These four subtests remained significantly different after correction for multiple testing.

Table 3 - Neuropsychological outcome

NEPSY-II Subtests		Case group N=12	Control group N=68	P-value*	P-value**
Attention and executive functioning					
Auditory Attention median (IQR)	Commission errors	0 (0 to 2)	0 (0 to 0)	0.17	0.43
	Omission errors	0 (0 to 4)	0 (0 to 1)	0.46	0.06
	Inhibitory errors	0 (0 to 0)	0 (0 to 0)	0.30	0.49
Response set median (IQR)	Commission errors	2 (0 to 5)	2 (0 to 4)	0.40	0.18
	Omission errors	4 (2 to 6)	3 (1 to 5)	0.18	**0.002**
	Inhibitory errors	0 (0 to 2)	0 (0 to 1)	0.74	0.24
Language					
Word Generation total score, median (IQR)		30 (25 to 35)	35 (27 to 41)	0.15	**0.002**
Memory and learning					
Memory for Faces total score, median (IQR)		12 (10 to 13)	12 (10 to 13)	0.84	0.94
Memory for Faces Delayed total score, median (IQR)		13 (9 to 13)	12 (10 to 14)	0.75	0.29
Narrative Memory *** total score, median (IQR)	Free and cued recall	25 (20 to 29)	26 (22 to 29)	0.74	0.54
	Recognition	15 (14 to 15)	15 (15 to 16)	0.26	0.31
Sensorimotor functioning					
Visuomotor Precision total errors, median (IQR)***		15 (5 to 46)	10 (4 to 22)	0.52	0.41
Visiospatial processing					
Arrows total score, median (IQR)		26 (20 to 32)	28 (26 to 31)	0.12	**0.002**
Geometric Puzzles total score, mean (IQR)		27 (25 to 31)	30 (28 to 34)	**0.02**	**0.002**
Route Finding total score, median (IQR)***		8 (7 to 8)	9 (8 to 10)	**0.02**	0.33

** P-values were derived from Mann-Whitney U test*

*** P-values were derived from ANCOVA tests adjusted for gender and age*

****n=6 versus n=56 since 6 cases and 12 controls conducted the short version of the NEPSY-II (13-16 years old)*

Chronic pain

Ten cases (67%) and 49 controls (69%) reported an episode of pain within the last three months before their study visit. Abdominal pain was the most frequently reported type of pain. The pain experienced could be defined as chronic pain for three cases versus 11 controls (p=0.70).

Thermal detection and pain thresholds

Detection and pain thresholds did not differ between cases and controls (corrected and uncorrected for age and gender) (Table 4) and nor did the pain intensity (NRS) score assigned upon the 46°C stimulus (cases 5.0 (IQR 1.0 to 8.0), controls 6.0 (IQR 1.0 to 9.0); p=0.38). Mean reaction time and skin temperature did not differ between groups during testing (p=0.84 and p=0.39, respectively). Cases were tested at a significantly higher room temperature although (cases 24.1 (SD 0.7) and controls 23.0 (SD 1.3); p<0.01). As the difference was only 1.1°C, room temperature was not a covariate in the analyses.

Table 4 - Thermal Quantitative Sensory Testing

	Case group N=15	Control group N=70	P-value*	P-value**
Method of Limits (MLI)				
Cold detection threshold °C, mean (SD)***	30.7 (0.7)	30.7 (0.7)	0.88	0.37
Warm detection threshold °C, mean (SD)***	33.9 (1.7)	33.9 (1.2)	1.00	0.16
Cold pain threshold °C, mean (SD)	11.9 (8.9)	9.9 (9.1)	0.45	0.81
Threshold not reached n (%)	3 (20%)	28 (40%)	0.24	NA
Heat pain threshold °C, mean (SD)	47.2 (3.4)	45.9 (4.2)	0.28	0.15
Threshold not reached n (%)	7 (47%)	29 (41%)	0.71	NA
Method of Levels (MLE)				
Cold detection threshold °C, mean (SD)	30.9 (0.8)	30.8 (1.2)	0.69	0.74
Number of stimuli Mean (SD)	10 (2)	11 (3)	0.21	0.15
Warm detection threshold °C, mean (SD)	33.2 (0.8)	33.6 (1.0)	0.15	0.51
Number of stimuli Mean (SD)	10 (2)	9 (3)	0.16	0.15

** P-values were derived from Independent Samples T-test for continuous data and chi squared tests or Fisher's exact tests for categorical data.*
*** P-values were derived from ANCOVA tests adjusted for gender and age*
****14 cases versus 69 controls*
NA: Not applicable

Structural imaging results

MRI-scanning was not performed in three cases and in five controls. Data from one case and 16 controls were excluded due to poor quality. As a consequence we compared imaging results of 11 cases and 50 controls. Cortical thickness and global brain volumes were not significantly different between groups (Table 5). With respect to specific pain-related brain areas, the only significant difference was a smaller volume of the right insula in case subjects after correction for age and gender (cases 6.6 cm^3 (0.6), controls 7.4 cm^3 (0.9); p=0.05). This difference remained significant after additional correction for total brain volume, but the significance disappeared after correction for multiple testing. No incidental brain abnormalities were observed.

Table 5 - Global brain volumes and volumes of pain related brain regions

		Case group	Control group	P value*	P value**
Global Brain Volumes		N=11	N=50		
Total Brain Volume cm^3, mean (SD)		1129 (121)	1159 (119)	0.53	NA
Cerebral White Matter cm^3, mean (SD)		401 (66)	399 (53)	0.69	0.57
Total Gray Volume cm^3, mean (SD)		683 (61)	716 (68)	0.41	0.44
Parietal lobe cm^3, mean (SD)	Left	69 (8)	72 (8)	0.69	0.13
	Right	70 (8)	74 (9)	0.94	0.42
Cerebellum (White Matter) cm^3, mean (SD)	Left	15 (2)	15 (2)	0.74	0.96
	Right	15 (2)	15 (2)	0.96	0.79
Cerebellum (Cortex) cm^3, mean (SD)	Left	54 (7)	56 (6)	0.22	0.29
	Right	54 (6)	56 (6)	0.12	0.14
Pain Related Brain Regions		N=11	N=50		
Thalamus cm^3, mean (SD)	Left	7.2 (0.7)	7.2 (0.9)	0.96	0.53
	Right	7.0 (0.8)	7.2 (0.7)	0.64	0.95
Amygdala cm^3, mean (SD)	Left	1.5 (0.3)	1.6 (0.3)	0.52	0.68
	Right	1.8 (0.2)	1.7 (0.3)	0.90	0.85
Anterior Cingulate Cortex cm^3, mean (SD)	Left	1.9 (0.4)	2.3 (0.6)	0.09	0.12
	Right	2.7 (0.7)	2.5 (0.6)	0.35	0.15
Insula cm^3, mean (SD)	Left	6.9 (0.5)	7.5 (0.9)	0.16	0.18
	Right	6.6 (0.6)	7.4 (0.9)	**0.05**	**0.03**

** P-values were derived from ANCOVA test (correction for age and gender)*
*** P-values were derived from ANCOVA test (correction for total brain volume, age and gender)*
NA: Not applicable

Functional imaging results

Nine cases (eight with two good quality runs and one with only one run) and 48 controls (36 with two good quality runs and 12 with only one run) were included in the fMRI analyses. Mean brain activation induced by the 41°C stimulus did not differ from that at the baseline 32°C temperature, neither in the case group nor in the control group. The 46°C stimulus induced statistically significant activation in one cluster in the case group, which included the right insula and in three clusters in the control group including multiple brain regions such as the frontal and temporal lobe (Figure 3, Table 6). A direct comparison revealed statistically significantly more brain activation in one cluster consisting mainly of the frontal pole in the control group compared to the cases (Figure 3, Table 6). In the direct comparison among groups, none of the brain regions showed statistically significantly more activation in cases compared to controls during pain After correction for age and gender the significantly higher brain activation during pain in the case group did not remain significant.

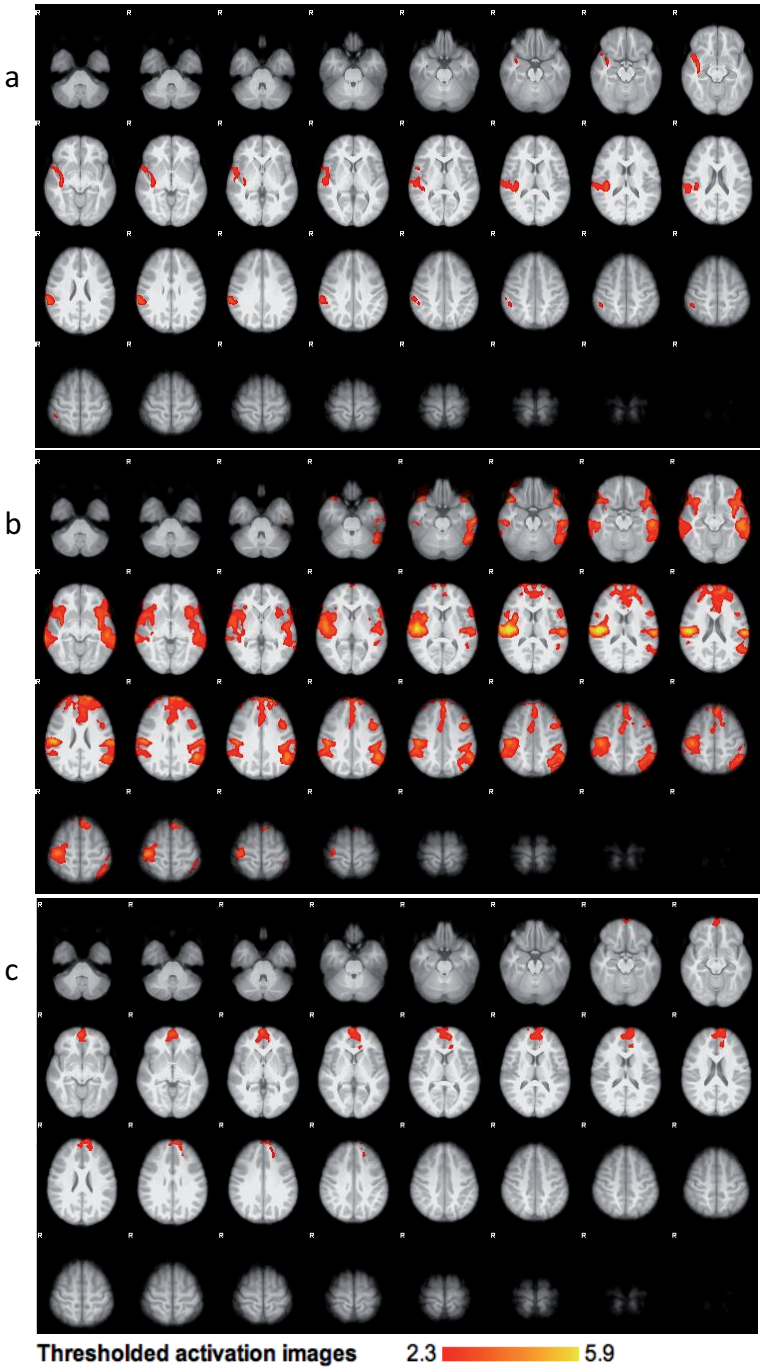

Figure 3 - The axial slices show areas of statistically significant activation during pain in the case group (a), the control group (b) and the direct comparison between both groups (control group > case group) (c) using a cluster significance threshold of $p<0.05$.

Table 6 - Areas of brain activation during pain

Cluster size (voxels)	P-value	MNI coordinates local maxima (mm)			Z-value	Anatomical area
		X	Y	Z		
Mean activation cases						
2767	0.01	66	−32	28	3.63	Supramarginal Gyrus (R)
		60	−38	26	3.48	
		38	−6	−12	3.42	Insula (R)
		38	−14	−6	3.16	
		40	−26	18	3.40	Parietal Operculum Cortex (R)
		38	−16	−10	3.18	Planum Polare (R)
Mean activation controls						
14473	<0.0001	−60	−24	18	5.12	Parietal Operculum Cortex (L)
		−52	−48	30	4.57	Supramarginal Gyrus (L)
		−52	30	−18	4.52	Frontal Pole (L)
		−56	−24	−14	4.49	Middle Temporal Gyrus (L)
		−50	26	−22	4.38	Temporal Pole (L)
		−60	−58	40	4.36	Lateral Occipital Cortex (L)
12820	<0.0001	46	−18	14	6.00	Central Opercular Cortex (R)
		66	−16	14	4.94	
		36	6	10	4.25	
		50	24	−20	4.76	Temporal Pole (R)
		54	22	−18	4.74	
		70	−34	−4	4.42	Middle Temporal Gyrus (R)
7226	<0.0001	−2	70	26	4.79	Frontal Pole (L)
		−20	66	22	4.67	
		−2	66	30	4.62	
		−2	62	38	4.08	
		20	74	16	4.20	Frontal Pole (R)
		2	74	14	4.06	Frontal Pole (R)
Direct comparison (mean controls > mean cases)						
2604	0.02	4	60	−4	3.80	Frontal Pole (R)
		6	66	2	3.42	
		2	68	30	3.24	
		−6	64	28	3.52	Frontal Pole (L)
		−8	68	22	3.37	
		−8	54	6	3.22	Paracingulate Gyrus (L)

Areas of activation during pain (46°C versus baseline) with cluster size, Z-values of the local maximum, Montreal Neurological Institute (MNI) coordinates, and the anatomical area of the local maximum (Harvard-Oxford Cortical Structural Atlas).
R: Right, L: Left

While significant differences in brain activation during pain were found with respect to the frontal pole, the median NRS scores of the pain stimuli presented over the two fMRI runs were not statistically significantly different between cases (2.5 (IQR 0.3 – 5.0)) and controls (3.8 (IQR 0.5-6.4); p=0.37).

DISCUSSION

The aim of this study was to determine the long-term consequences of exposure to opioid-related substances in utero as a unique model for early opioid exposure in the absence of pain. Case subjects showed significantly less brain activation in the frontal lobe during pain than did controls, but the significance disappeared after correction for age and gender. Differences in thermal and pain sensitivity or brain morphology were not detected. Performance of case subjects on the neuropsychological tests was statistically significantly worse than that of the controls.

While both groups showed statistically significant brain activation during pain, cases showed significantly less activation specifically in the frontal lobe, which is an area not associated with fMRI studies of pain, but rather a region associated with attention and executive functioning.[21] It is noteworthy that pain threshold test results and occurrence of chronic pain did not differ between both groups, indicating no long-term effects of opioids with respect to pain sensitivity later in life. It is possible that the differences in brain activation in the frontal pole represent differences in attention, rather than differences in pain perception. The comparable NRS pain intensity scores of the stimuli presented over the fMRI runs are in line with this hypothesis. Moreover, the fact that the case subjects performed worse on a subtest in the attention and executive functioning domain of the NEPSY-II support this hypothesis as well. The difference in brain activation during pain did not remain significant after correction for age and gender, probably due to the decrease in degrees of freedom related to the relatively low sample size.

With regard to brain morphology, brain volumes as well as cortical thickness were comparable between both groups. Probably due to great plasticity of the human brain, no major effects of early opioid exposure were detected with regards to brain morphology. Walhovd and colleagues included 14 children in their MRI study who had been prenatally exposed to poly-substances and found several brain regions were significantly smaller compared to 14 controls.[4] They found the same when comparing only eight children who were uniquely prenatally exposed to opioids with healthy controls. Therefore, we expected to find the same effect as well. Walhovd and colleagues did not correct for multiple testing in their cortical thickness analyses, which could explain this discrepant finding. Remarkably, all mothers of the exposed children smoked tobacco during pregnancy in the study of Walhovd.[4] In a study in 6 to 8-year-old children, prenatal tobacco exposure was associated with smaller brain volumes and cortical thinning.[22] In the present study maternal smoking habits were not recorded properly. However we know from the follow-up program that most of the cases had been exposed to tobacco.[4] The long-term outcome of children with NAS might well depend on genetic factors, since short-term outcome

such as length of hospital stay due to NAS was found to be associated with variations in specific genotypes.[23] The relatively positive long-term outcome for NAS children in our study could be caused by the fact that these children were intensively seen until approximately age 2 at our outpatient clinic. Early signs of medical or psychological problems were therefore detected and treated in an early stage. Moreover, good perinatal and general care is available for drug abusers in the Netherlands, offering intense programs for drug abusing pregnant women and mothers with the aim of enhancing the children's health and development. In addition, an excellent network for foster parents is available in the Netherlands. It is known that adoption is associated with a better developmental outcome in children with NAS.[6,7] In our cohort, the majority of case subjects were raised by foster parents, which could have influenced the positive outcome with respect to brain morphology. The small sample size did not permit comparison between children raised by biological parents and children raised by foster parents.

The cases scored statistically significantly lower than the controls on four subtests of the NEPSY-II neuropsychological test, in line with previous studies in children exposed to illicit drugs in utero.[5-7] One of these subtests addressed visiospatial processing. Regarding this domain, a study by De Graaf and colleagues also found a relation between postnatal opioid exposure and lower performance on the 'visual analysis' IQ subtest at age 5.[24]

The strength of this study is that we examined a unique group of subjects exposed prenatally to opioid-related illicit drugs using brain imaging, detection- and threshold testing, and neuropsychological assessments at later age. However, several limitations need to be addressed. First, the sample size is relatively small and therefore we were unable to detect minor differences between groups. Another limitation is the high risk for confounding, as we were unable to correct in the analyses for possible confounders such as maternal socioeconomic state or the additional use of cocaine, or other drugs of abuse. Furthermore, the case subjects were statistically significantly older than the healthy controls, but this was corrected for in the analyses. Finally, information regarding alcohol consumption and smoking habits of the mothers was not properly recorded.

CONCLUSION

In line with the animal studies in this area of research,[11,12,25] we indeed found minor negative effects of early opioid exposure in the absence of pain, mainly of a neuropsychological nature. However, and even more important, no effects with respect to pain sensitivity and brain morphology were found. The question remains whether the negative neuropsychological effects were induced by the prenatal opioid exposure or by other factors

related to maternal illicit drug abuse. Future studies and follow-up programs for children with NAS are needed to prevent or minimize cognitive delays, especially since it is a serious and growing problem.[1,2] Moreover, future studies with similar methodologies are needed to evaluate if comparable effects are seen in children exposed to opioids in the presence of pain, such as in the case of major neonatal surgery.

SUPPLEMENTARY DATA

Image acquisition

MR images were acquired on a 3 Tesla scanner (Discovery MR750, General Electric, Milwaukee, MI, USA) using an 8-channel head coil for signal reception. Cushions were used to comfortably support the participants' head and to minimize head motion. During the high-resolution structural MRI scan the adolescents were able to watch a movie or listen to music of their choice. The movie/music was stopped during the functional MRI scans. Participants wore an MRI-compatible headphone to reduce the scanner noise and to allow them to listen to the movie's audio track. The headphone also enabled communication with the MR operator between the scans.

We obtained a high-resolution structural T_1-weighted image using an inversion recovery fast spoiled gradient recalled (IR-FSPGR) sequence with the following parameters: TR = 10.3 ms, TE = 4.2 ms, TI = 350 ms, NEX = 1, flip angle = 16°, readout bandwidth= 20.8 kHz, matrix 256 x 256, imaging acceleration factor of 2, and an isotropic resolution of 0.9x0.9x0.9 mm^3. The scan time for the structural T_1 MRI scan was 5 minutes and 40 seconds. We conducted two runs of a functional MRI paradigm using single-shot echo-planar imaging (EPI) T_2*-weighted sequences in transverse orientation sensitive to blood oxygen level dependent (BOLD) contrast (parameters: TR/TE 2000/30 ms, flip angle 85°, 64 x 64 matrix with a field-of-view of 260 x 260 mm^2; 39 slices and voxel sizes of 3.6 x 3.6 x 4.0 mm^3). Scan time was 6 minutes and 4 seconds (182 TRs) per run.

Functional MRI Block paradigm

The functional MRI (fMRI) component consisted of two runs and utilized a block paradigm. During each of these two runs the TSA-II thermode was applied to the thenar eminence of the non-dominant hand. During scanning the TSA-II thermode induced warm (41°C) and painful stimuli (46°C) (Figure 2). These temperatures were derived from a previous study from our research group.[26] Within each run, the temperature increased four times at a rate of 1.5°C per second from the baseline temperature of 32°C to a warm temperature of 41°C and four times to a potentially painfully hot temperature of 46°C. After each stimulus, the temperature decreased by 4.5°C per second back to baseline and stayed at the baseline temperature for 15 seconds before the increasing to the next warm or pain stimulus. The order and duration (8 - 16 seconds) of the stimuli was randomly determined at the beginning of the study and were different in both runs. In order to prevent anticipation to the stimuli, the order of warm and heat stimuli differed between the two runs. Figure 2 shows the block paradigm of the thermal stimuli for run 1 and run 2. Pain intensity of the thermal stimuli applied during the fMRI scans was measured again using the NRS scale.

Structural imaging analysis

We used the Freesurfer image analysis suite version 5.1.0 (http://surfer.nmr.mgh.harvard.edu/) for cortical reconstruction and volumetric segmentation. Freesurfer computes these measures in an automated approach, and technical procedures have been described extensively.[27] Each image was visually inspected and subjects with poor quality data were excluded. In subjects with small errors in the gray/white segmentation, control points, and white matter edits were added to identify and correct misclassified white matter regions. When the segmentation improved, the corrected images were used. Evaluation of surface-based cortical thickness FreeSurfer was performed using the built-in program QDEC with a smoothing filter of 10 millimeter. For the group analysis a general linear model (GLM) was fitted at each surface vertex. We corrected for age and gender and used a Monte Carlo correction ($p<0.05$) for multiple testing. Brain volumes and volume of pain related brain regions, such as the thalamus, amygdala, anterior cingulate cortex and insula,[28] were compared between cases and controls using ANCOVA analysis with correction for age, gender, and total brain volume.

Functional imaging analysis

For our functional MRI analyses, we used AFNI (http://afni.nimh.nih.gov/) for slice timing and motion correction. Runs with more than 6 mm of motion (maximum displacement) were excluded from the analyses. Functional images were co-registered to the structural image of the subject and both the functional and structural images were normalized using the Montreal Neurological Institute (MNI) 152 atlas using FSL's non-linear registration tool FNIRT. Finally, data were spatially smoothed using AFNI with an 8-mm full width at half-maximum Gaussian kernel. Following the preprocessing steps, single-subject analyses were performed using FMRIB's fMRI Expert Analysis Tool FEAT (http://www.fmrib.ox.ac.uk/fsl/feat5/index.html), comparable to a previous report of our study group.[29] The time series for the pain runs were modeled using a block design. Design matrices were created for both runs using the data from each subject's stimulus log file from the TSA. These matrices were created independently for each individual using an automated MATLAB program (MATLAB 7.1, The MathWorks Inc., Natick, MA, 2000). This modeled time series was convolved with the hemodynamic response function. Next, a general linear model was implemented using FMRIB's Improved Linear Model. The two within-subject runs were combined using a fixed effects model. The higher-level group analyses, which compared patients and controls for each of the contrasts; 46°C versus baseline, and 41°C versus baseline, were performed using FMRIB's Local Analysis of Mixed Effects. Furthermore, we corrected for multiple comparisons using random Gaussian fields and significance was set at $p<0.05$ (two-tailed).

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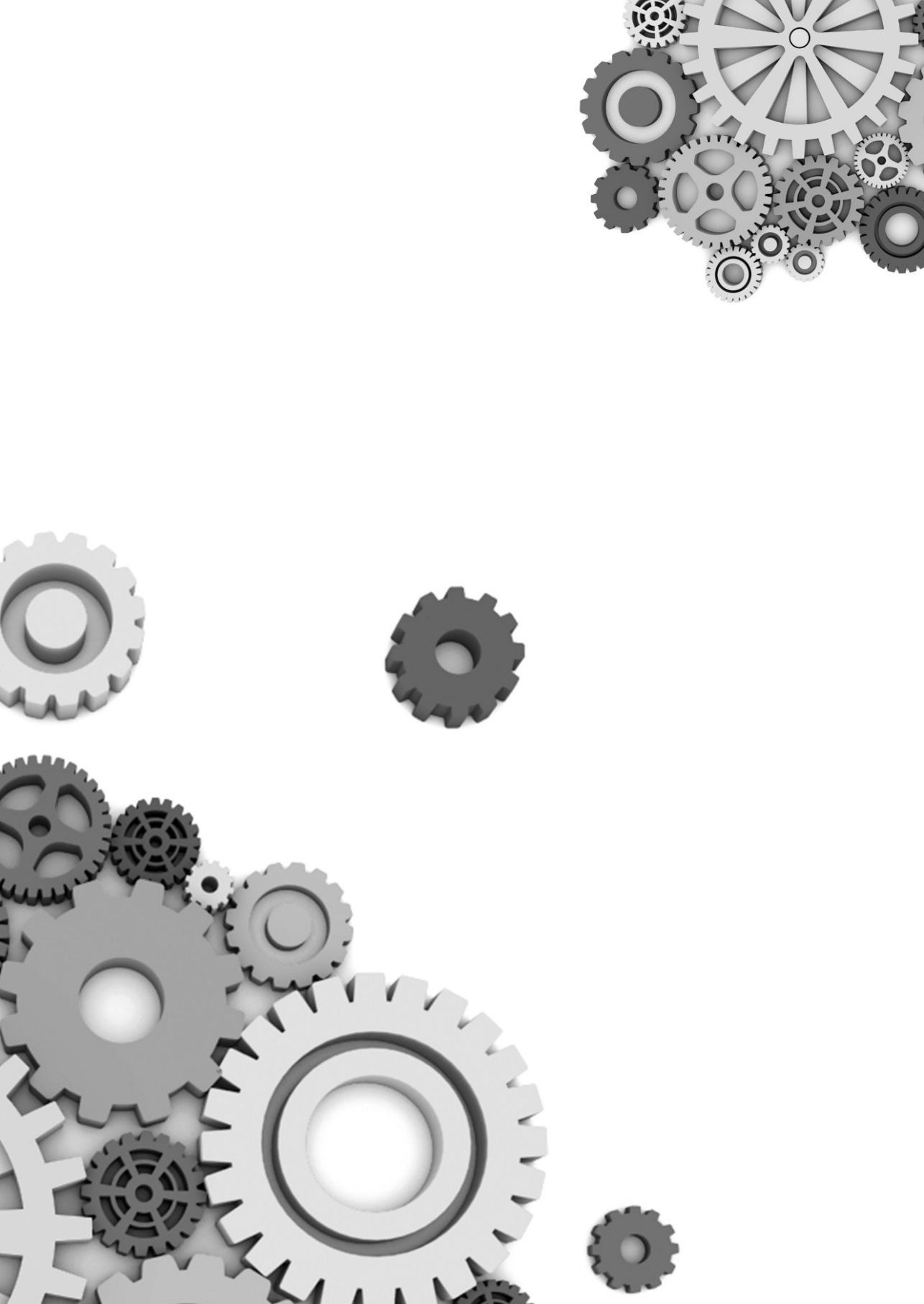

Chapter 11

Pain insensitivity syndrome misinterpreted as inflicted burns

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ABSTRACT

We present a case study of a 10-year-old child with severe burns that were misinterpreted as inflicted burns. Because of multiple injuries since early life, the family was under suspicion of child abuse and therefore under supervision of the Child Care Board for two years before the boy was burned. Because the boy incurred the burns without feeling pain, we conducted a thorough medical examination, laboratory testing, evaluated detection- and pain thresholds, and used MRI to study brain morphology and brain activation patterns during pain between this patient and three healthy age- and gender-matched controls. We found elevated detection- and pain thresholds and lower brain activation during pain in the patient, compared with the healthy controls and reference values. The patient received the diagnosis of hereditary sensory and autonomic neuropathy type IV on the basis of clinical findings and the laboratory testing, complemented with the altered pain and detection thresholds and MRI findings. Hereditary sensory and autonomic neuropathy IV is a very rare congenital pain insensitivity syndrome characterised by the absence of pain and temperature sensation combined with oral mutilation due to unawareness, fractures, and anhidrosis, caused by abnormalities in the peripheral nerves. Health care workers should be aware of the potential presence of this disease to prevent false accusations of child abuse.

INTRODUCTION

Insensitivity to pain can be caused by neuropathies due to diabetes or diseases such as leprosy. It can also be inherited and caused by congenital pain insensitivity syndromes. These congenital diseases are associated with a loss of sensory and pain discrimination and a loss of the affective-motivational response to pain.[1] The majority of these syndromes are caused by hereditary sensory and autonomic neuropathies (HSANs), of which 5 different types are recognized.[1-4] HSAN IV, or congenital insensitivity to pain with anhidrosis (CIPA), is an extremely rare autosomal recessive disease characterised by diffuse thermal and pain insensitivity and anhidrosis. Patients with HSAN IV suffer from oral mutilation, fractures, bruises and ulcerations of extremities caused by pain insensitivity.[1] These symptoms are a consequence of the absence of unmyelinated nerve fibers and a loss of small myelinated fibers in the peripheral nerves.[5] The diagnosis HSAN IV is made primarily clinically on the basis of impaired pain and temperature perception in combination with anhidrosis.[4] Additionally, an intradermal histamine test can be conducted, because a lack of a normal axon flare response is consistent with HSAN.[2,6] The diagnosis may be confirmed by a genetic test, because the related mutations and polymorphisms of the TRKA gene on chromosome 1 are identified.[7,8] In this case study we present a boy who presented with severe burns on his buttocks that were caused by an impaired temperature and pain perception.

CASE-REPORT

Patient presentation

A 10-year-old boy was admitted to the Maasstad Hospital Burn Center in Rotterdam, the Netherlands, with severe contact burns on his buttocks. He had played computer games while sitting on top of a central heating system. After a few hours he noticed severe blisters on his buttocks without experiencing pain. The parents sought medical help and were referred to our burn center. The referring hospital suspected inflicted burns, because the blisters had not been cooled and both parents and the patient did not have an explanation for the burns. After extensive questioning on what he had done before the blisters on his buttocks appeared, the central heating system was identified as the possible cause of his burns. Physical examination revealed a cooperative healthy boy with a total body surface area burn of 4%. The burns were deep dermal and surgery was needed to close the wound (Figure 1). His tongue and lips showed several scars from earlier lacerations caused by tongue biting and burns caused by drinking very hot liquids while not detecting heat or pain sensations (Figure 2). Neurological examination pointed to normal cranial nerve function, sensation of vibration, stature, proprioception, and cold/warm differentiation. Deep tendon reflexes were low.

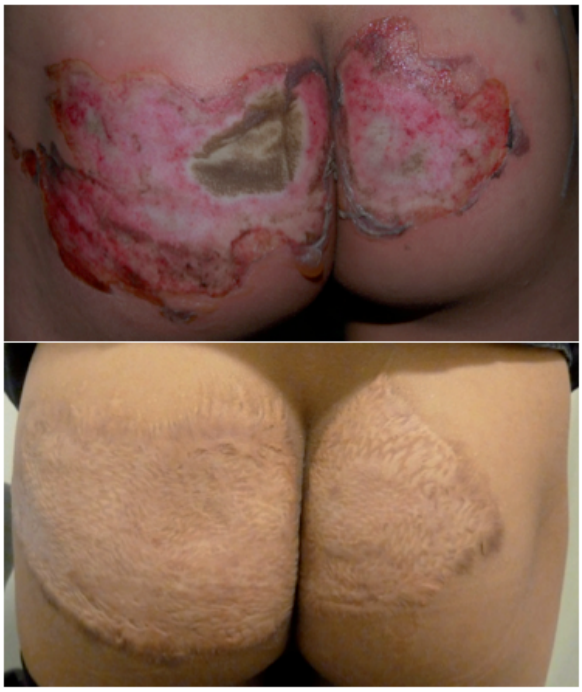

Figure 1 - The burns before surgical closure (upper panel) and the scars after surgical closure (lower panel).

This boy is the youngest child of non-consanguineous parents of Turkish ethnicity. During infancy he had no feeding or respiratory problems. After the first tooth eruptions he had lingual lacerations. Developmental milestones in the early years and learning abilities were normal, but his hyperactivity was noteworthy. After he started walking, he frequently had painless bruises, skin lacerations, and bone fractures of his legs and ankles. Furthermore, his parents noted that he did not sweat normally, that is, anhidrosis.

Due to 2 separate fractures of his lower extremities, which were unexplained at that time, the parents were already suspected of child abuse and under the supervision of the Child Care Board for 2 years before he was burned. The Child Care Board did not find evidence for psychosocial problems in the family, which are often associated with child abuse. Furthermore, the injuries occurred at different places (i.e., at school and at home). Because the boy felt no pain during the development of the burns and during admission, we looked deeper into this case and reevaluated the diagnosis of child abuse. On the basis of his medical history we considered the diagnosis of HSAN IV.

Medical tests and comparison with healthy controls

We performed a histamine flare test with an intradermal injection of histamine (0.1mg/

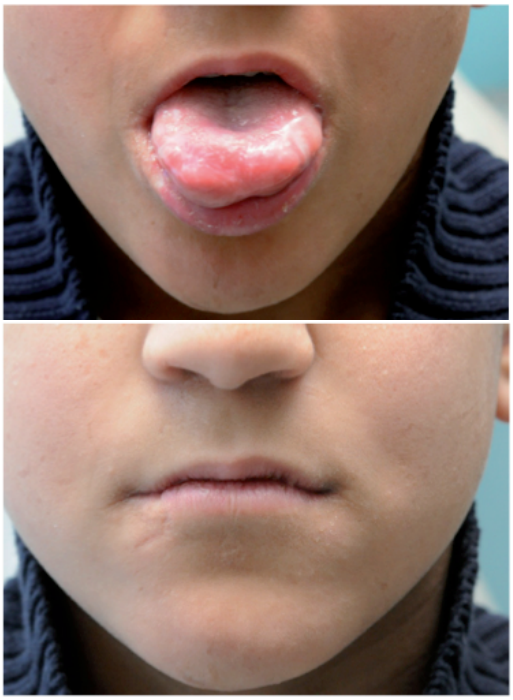

Figure 2 - The tongue (upper panel) and lips (lower panel) of the boy show several scars.

mL, 0.3mL), which showed no flare. Furthermore, an electromyogram showed no abnormalities and DNA tests revealed no gene mutations for HSAN II or for HSAN III (Riley Day syndrome).

Furthermore, we compared this patient with 3 healthy age-matched boys and conducted quantitative sensory testing (QST) to measure thermal detection and pain thresholds and compared brain morphology and brain functioning during pain by using structural and functional MRI. (For extended information regarding the methods of the QST and MRI tests, see the supplementary data.)

The patient's mean detection temperatures for cold were lower than reference values and the mean detection temperatures for warm were higher in comparison with reference values generated from 9-to-12-year-old boys[9] and compared with the 3 matched control children (Table 1 and Figure 3), suggesting hyposensitivity. We also found a lower mean threshold for the cold pain in the case in comparison with reference values and the 3 controls. The heat pain threshold temperature of the case was also higher in comparison with the reference values, but it was lower than the mean threshold of the control group (Table 1 and Figure 3).

Table 1 – Detection- and pain thresholds of the case, controls and reference group

		Thresholds Case	Mean thresholds Controls (n=3)	Reference values for boys 9-12 years old (n=32)*
Detection threshold Cold (°C (SD))	MLI	28.53 (1.15)	30.99 (0.20)	31.0 (1.6)
	MLE	26.15	31.32 (0.29)	-
Detection threshold Warmth (°C (SD))	MLI	38.86 (0.18)	33.62 (0.55)	33.4 (1.6)
	MLE	34.25	33.67 (0.53)	-
Pain threshold Cold (°C (SD))	MLI	4.45 (3.94)	12.38 (10.72)	16.27 (8.3)
Pain threshold Warmth (°C (SD))	MLI	47.19 (1.15)	48.77 (1.77)	41.24 (3.84)

* *Blankenburg et al. 2010*

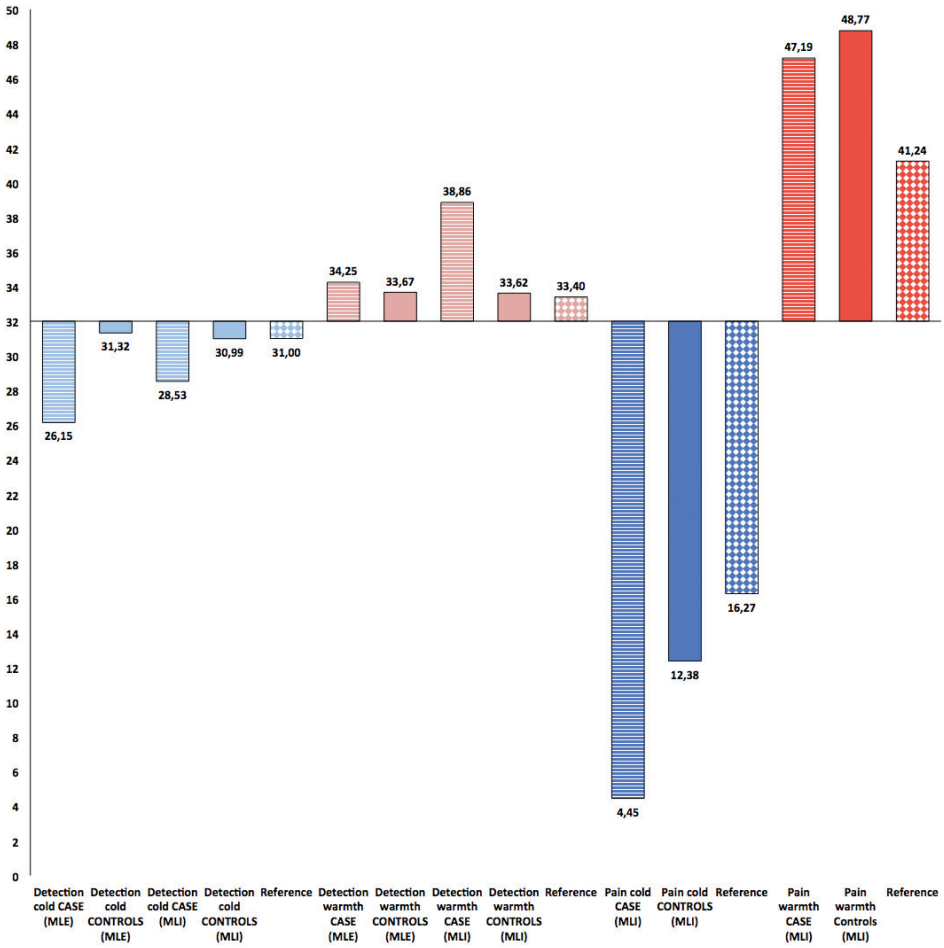

Figure 3 - Detection- and pain thresholds of the case, controls and reference group.

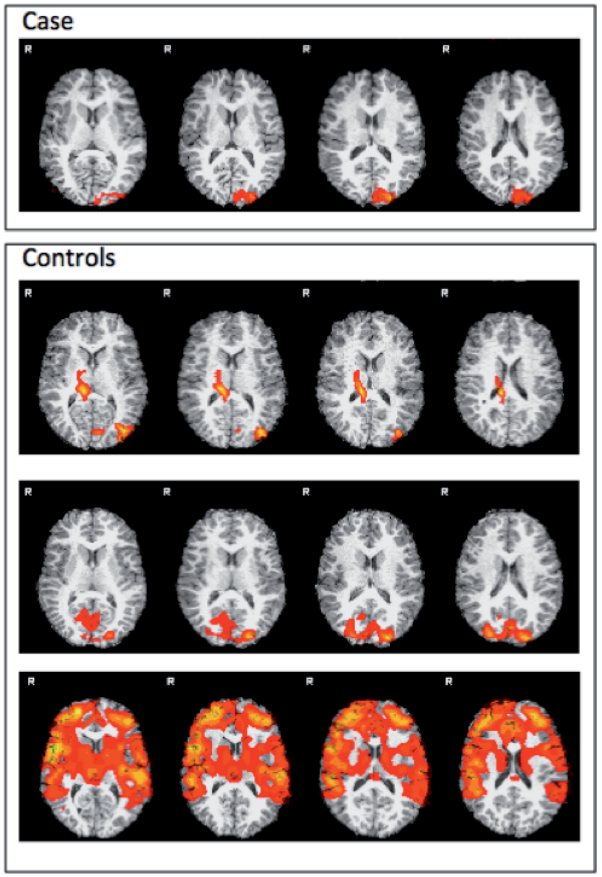

Figure 4 - The axial slices show areas of activation during pain in the case and three healthy controls (mean activation over two runs).

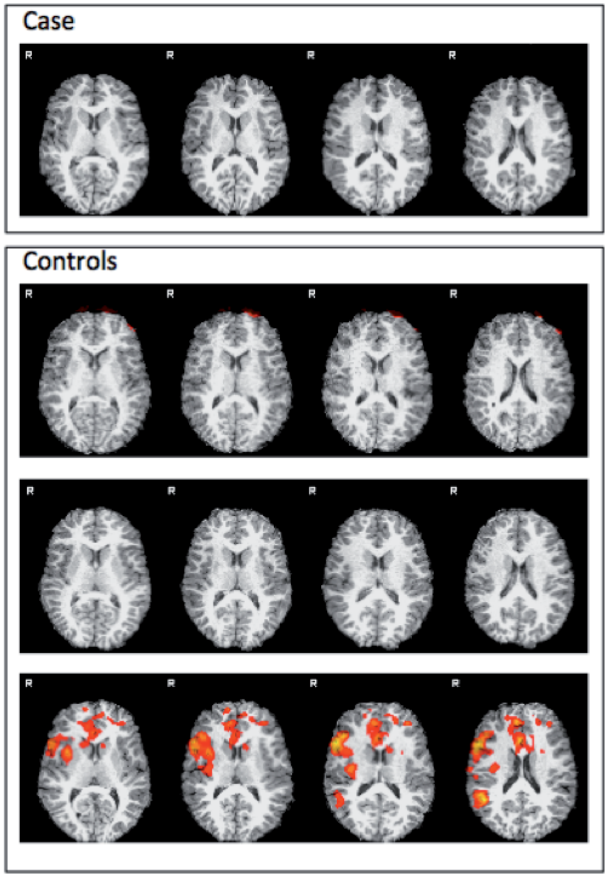

Figure 5 - The axial slices show areas of activation during warm stimuli in the case and three healthy controls (mean activation over two runs).

With regard to brain morphology, no evidence for gross brain abnormalities were found, and the total brain volume and the volumes of specific pain-related brain areas (thalamus, amygdala, anterior cingulate cortex, and the insula) were slightly smaller in the case in comparison with the 3 controls (Table 2). A painful stimulus of 46°C induced minimal significant brain activation in the patient (Figure 4). Furthermore, the activation pattern was not located in pain-related brain areas, such as the insula, and there was more significant brain activation in the controls during pain compared with the case (Figure 4). A warm stimulus of 41°C induced no significant brain activation in the case, although of the 3 controls, only 1 showed substantial significant brain activation (Figure 5).

Table 2 – Global brain volumes and volumes of pain related brain regions

		Case	Controls
Global Brain Volumes		N=1	N=3
Total Brain Volume (Mean (SD), cm^3)		1172	1246 (76)
Cerebral White Matter (Mean (SD), cm^3)		392	442 (27)
Total Gray Volume (Mean (SD), cm^3)		744	758 (47)
Cerebellum (White Matter) (Mean (SD), mm^3)	Left	11461	15460 (691)
	Right	13142	16231 (688)
Cerebellum (Cortex) (Mean (SD), mm^3)	Left	57673	59100 (2339)
	Right	53351	57751 (4185)
Pain Related Brain Regions			
Thalamus (Mean (SD), mm^3)	Left	5956	7745 (678)
	Right	6350	7503 (656)
Amygdala (Mean (SD), mm^3)	Left	1738	1986 (130)
	Right	1659	1968 (191)
Anterior Cingulate Cortex (Mean (SD), mm^3)	Left	2258	2752 (1034)
	Right	2906	2858 (619)
Insula (Mean (SD), mm^3)	Left	7722	7696 (387)
	Right	7159	7421 (523)

DISCUSSION

The diagnosis of HSAN IV or CIPA requires three clinical criteria, anhidrosis, decreased pain and temperature perception, and mental retardation.[7] However, there is wide variability in intellectual performance in these children, and mental retardation does not occur in all patients.[2,10] Furthermore, low deep tendon reflexes and hyperactivity, as in our case, are common in patients with HSAN IV.[2,5] In addition to the the clinical characteristics, the absence of axon flare after intradermal histamine injection is consistent with HSAN, as in our case.

HSAN IV is caused by mutations in the NTRK1 (TRKA) gene. This gene is located on chromosome 1 (1q21-q22) and encodes for neurotropic tyrosine kinase receptor type 1, which is autophosphorylated in response to nerve growth factor (NGF).[7] As previously described by Axelrod and Gold-von-Simson, signal transduction at the NGF receptor is impeded and NGF dependent neurons, such as the small sensory and sympathetic neurons, fail to survive as a result of mutations.[2] The numerous mutations do not allow for a straightforward diagnosis of HSAN IV. Gene expression is highly variable and may be related to the site of the mutation on the NGF receptor or whether there is genetic homo- or heterozygocity.[2,11] Unfortunately, HSAN III (Riley-Day syndrome) is the only HSAN type for which commercially available genetic testing is available.[2] The gene mutations of NTRK1 could not be determined in Dutch neurogenetic laboratories.

In our patient, medical history, clinical signs of anhidrosis, pain insensitivity, elevated detection and pain thresholds, low brain activation during warm and painful stimuli, and a negative histamine flare test sufficed to confirm the diagnosis of HSAN IV or CIPA. Even though the child appears to be hyposensitive to cold and warm detection and pain, he was able to notice pain during the QST procedure. Unfortunately we were unable to test possible habituation for pain. It is a possibility that habituation for pain in combination with hyposensitivity and distraction (computer games) contributed to the severe burns in his case, especially because video games are found to reduce behavioral distress during pain in children.[12] Furthermore, his brain activation during warm and painful stimuli was low in comparison with healthy age- and gender-matched controls. In general, more activation is visible in the brain when the stimuli are rated as more painful.[13] Low brain activation during pain in combination with greater difficulties in detecting temperature variations and pain also supported our suspicion of a pain insensitivity syndrome. On the basis of clinical findings and the histamine test, the diagnosis HSAN IV was confirmed. We then informed the family about the illness and referred the patient to a rehabilitation physician. However, it is always possible that the child has both HSAN and is a victim of child abuse, although the inspection by the Child Care Board and his medical condition did not suggest child abuse.

Makari and colleagues[14] described 2 siblings with HSAN V with a medical history of severe lacerations, fractures, and injuries. Child abuse was suggested when the girl presented with severe burns. The girl was placed in special care because of suspected child abuse. Fortunately, she was allowed to return home after the diagnosis of HSAN was confirmed in both children. Another rare disease that could be mistaken for child abuse is osteogenesis imperfecta, which should also be kept in mind with children with frequent bone fractures.[15]

CONCLUSION

Child abuse has a much higher occurrence rate than rare neuropathies. However, in selected cases with oral mucosal laceration and scars, multiple fractures, anhidrosis, and infrequently, mental retardation, a diagnosis of HSAN should be considered and thoroughly evaluated. Future diagnostic approaches may include systematic measurements of detection- and pain thresholds. Health care workers should be aware of the potential existence of the illness.

SUPPLEMENTARY DATA

Quantitative Sensory Testing

To quantify the thermal detection- and pain insensitivity, we performed Quantitative Sensory Testing (QST) at the age of 12. The obtained detection- and pain thresholds were with reference values established in the study of Blankenburg et al.[9] Furthermore, we compared our case with three healthy age- matched boys (age case: 12.87, mean age controls: 13.24 years old, range 12.53 - 14.46 years old) who participated in an ongoing study from our research group [unpublished data]. The study was performed in compliance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) and was approved by the Institutional Review Board at the Erasmus MC. Informed consent was obtained prior to participation.

To determine detection- and pain thresholds we used the Thermal Sensory Analyzer-II (TSA-II, Medoc Advanced Medical systems, Israel). The TSA-II is a precise, computer-controlled device capable of generating and recording a response to a highly repeatable thermal stimulus over a range of 0°C to 50°C. A Peltier-based contact thermode (30 x 30 mm) was placed at the thenar eminence of the non-dominant hand (left hand) to apply cold or heat to the child's skin. We determined detection- and pain thresholds using a standardized protocol, comparable with a previous study from our research group.[16] After explaining the test we first determined the children's detection- and pain thresholds for cold and warmth using the reaction time dependent Method of Limits (MLI). The test started at a baseline temperature of 32°C, which was then steadily linearly decreased at a rate of 1°C/sec. The child was asked to press the button as soon as the cold stimulus was felt. After pressing the button, the stimulus reversed to the baseline temperature of 32°C with a rate of 1°C/sec. We repeated this five times. The first two stimuli served as rehearsal stimuli. The detection threshold was calculated as the mean value of the last four stimuli. Next, the temperature was steadily increased at a linear rate of 1°C/sec to determine the detection threshold for warmth using the same method. Second, the MLI was applied to determine the pain thresholds for cold and warmth. Starting again from a baseline temperature of 32°C, the temperature was steadily decreased at a linear rate of 1.5°C/sec. The child was asked to press the button when the cold sensation started to feel painful. Now also, the temperature reversed to the baseline temperature with a rate of 10.0°C/sec. This was repeated four times. The last four temperatures obtained were used to calculate the mean pain threshold. Next, the pain threshold for warmth was determined in the same manner. When a child did not press the button before 0°C or 50°C, the test automatically terminated.

Furthermore we determined the detection thresholds for cold and warmth again, but now using the reaction time independent Method of Levels (MLE). The researcher told

the children that the thermode could either become cold, or would not change in temperature. The first thermal stimulus was 3.0°C below the baseline temperature of 32.0°C. Following each thermal stimulus the researcher asked the child if the thermode become cold or not. Dependent on the child's response, the next stimulus was increased or decreased in temperature. The test terminated when the step size of the stimulus had decreased to a level of 0.1°C. The warm detection threshold was determined in the same manner starting with a stimulus temperature of 3.0°C above the baseline temperature.

Structural MRI

To compare brain morphology between the case and the three controls, we obtained a high-resolution structural T_1-weighted image (3T) using an inversion recovery fast spoiled gradient recalled (IR-FSPGR) sequence with the following parameters: TR = 10.3 ms, TE = 4.2 ms, TI = 350 ms, NEX = 1, flip angle = 16°, readout bandwidth= 20.8 kHz, matrix 256 x 256, imaging acceleration factor of 2, and an isotropic resolution of 0.9x0.9x0.9 mm^3.[17] The scan time for the structural T_1 was 5 minutes 40 seconds. The structural analyses were performed with the Freesurfer image analysis suite, (http://ftp.nmr.mgh.harvard.edu). The technical details of these procedures are described in previous publications.[18-29]

Functional MRI

To measure brain activation during thermal stimuli we conducted two runs of a functional MRI paradigm using single-shot echo-planar imaging (EPI) T2*-weighted sequences in transverse orientation sensitive to blood oxygen level dependent (BOLD) contrast (parameters: TR/TE 2000/30 ms, flip angle 85°, 64 x 64 matrix with a field-of-view of 260 x 260 mm^2; 39 slices and voxel sizes of 3.6 x 3.6 x 4.0 mm^3). A total of 182 volumes per run were collected, (6 min. 4 sec per run). During each run of the fMRI, the TSA-II thermode induced four warm (41°C) and four painfully hot stimuli (46°C) to the thenar eminence of the child's non-dominant hand (8-16 seconds per stimulus).
The functional images were preprocessed using a combination of Analysis of Functional Neuroimages (AFNI, http://afni.nimh.nih.gov/)[30] and FSL's FMRIB's Software Library (FSL 5.0, FMRIB Software Library; FMRIB, Functional Magnetic Resonance Imaging of the Brain; http://www.fmrib.ox.ac.uk/fsl/).[31] Slice timing correction and motion correction were performed using AFNI[30]. The two within-subject runs were combined using a fixed effects model.

Unfortunately, since we compared only one case to three controls, it was impossible to conduct statistical tests to determine whether there were significant group-differences between the case and the controls. Therefore, we described the differences in the manuscript and presented the results in figures 3, 4, and 5.

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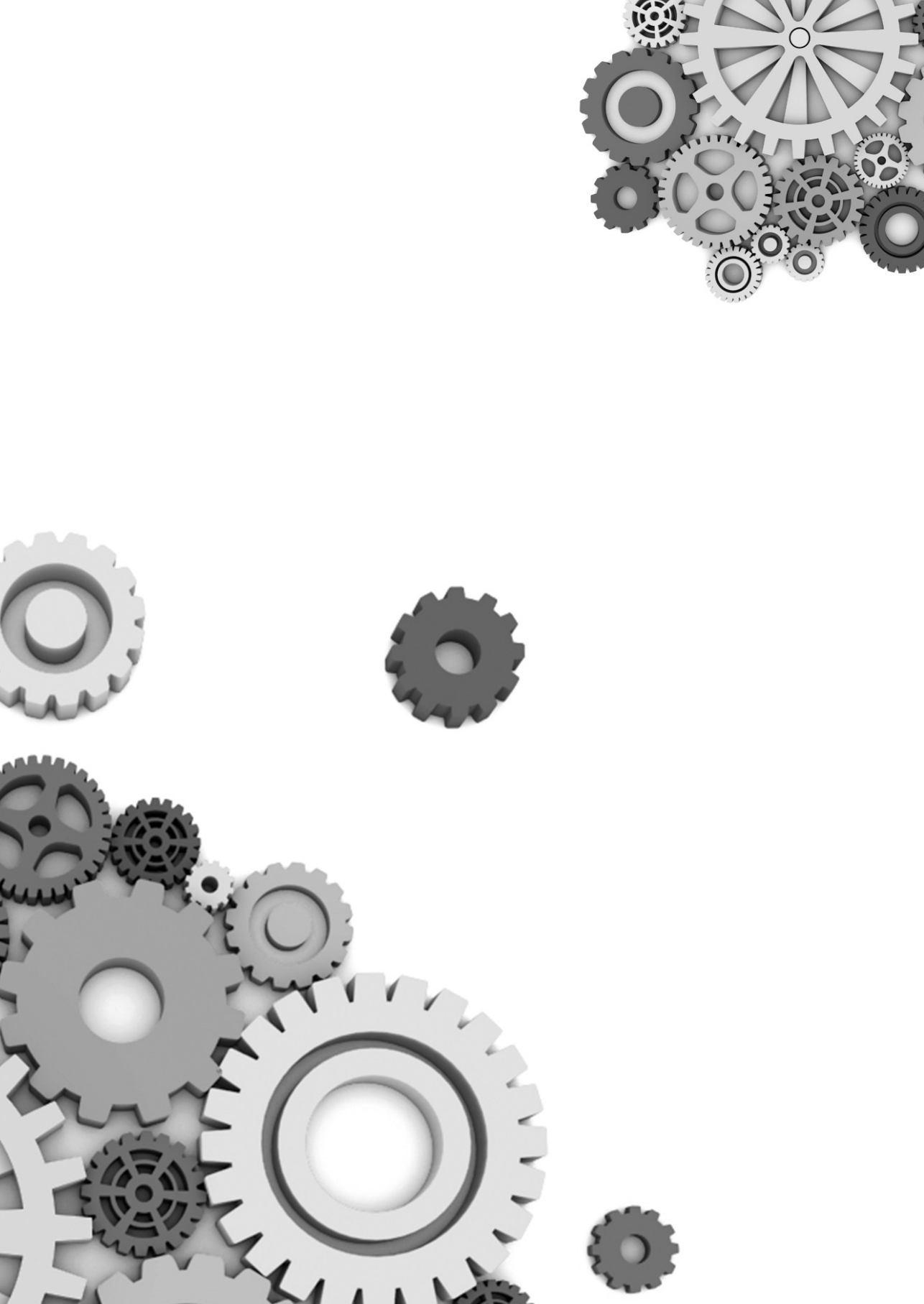
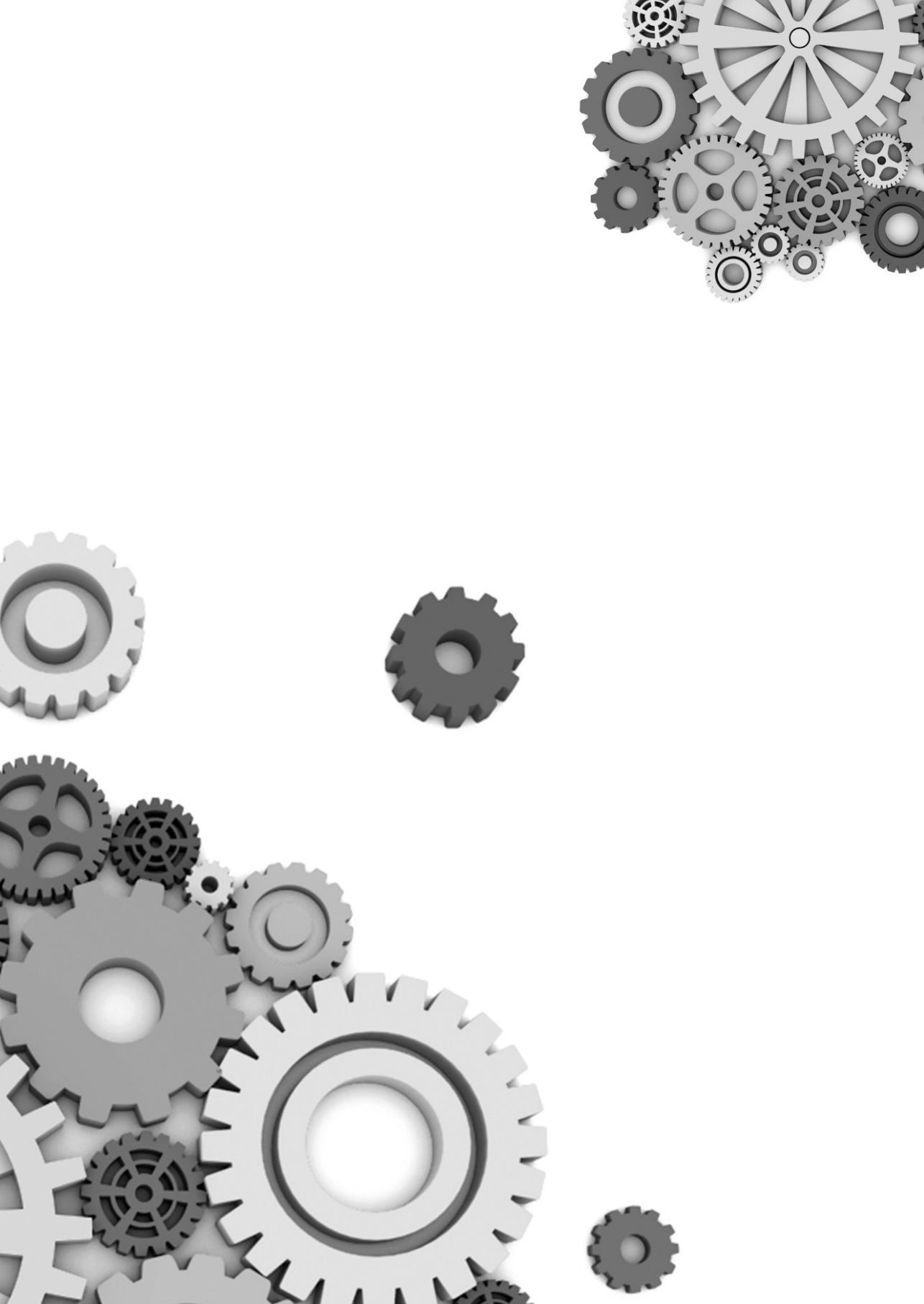


Discussion and summary



Chapter 12

General Discussion

PAIN IN HUMANS

The International Association for the Study of Pain (IASP) defined 'pain' as '*An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage'* - with the important note that *'Pain is always subjective'*.[1] Therefore self-report is the golden standard. Young children, however, are not able to self-report their pain. Through the years our department has conducted a number of studies with respect to pain, analgesia, and its long-term effects in children (Table 1).

Table 1 - Overview of a selection of studies

Author and year	Study design	Outcome measures	Results
Peters[2] 1999	RCT - continuous morphine infusion versus patient controlled analgesia	Morphine consumption Side effects	No differences in pain scores or side effects
Van Dijk[3] 2000	Prospective study	COMFORT scores	COMFORT scale reliable and valid to assess postoperative pain in neonates and infants
Bouwmeester[4] 2001	RCT - continuous versus intermittent morphine	Hormonal and metabolic stress responses	No major advantage of continuous infusion below the age of 1 year
Van Dijk[5] 2002	RCT - continuous versus intermittent morphine	Postoperative pain Actual morphine dose	No differences in postoperative pain
Peters[6] 2003	Case comparison study with respect to major surgery in the first 3 months of life	Pain responses to immunization at later age	No difference in pain response
Simons[7] 2003	RCT - morphine versus placebo in preterm newborns receiving mechanical ventilation	Analgesic effect Neurologic outcome	No support for the routine use of morphine in preterm newborns receiving mechanical ventilation
Peters[8] 2005	Cross-sectional study with respect to major surgery in the first 3 months of life	Pain sensitivity to subsequent surgery	Subsequent surgery in the same dermatome induced more need opioids, higher COMFORT and VAS scores, greater (nor)epinephrine plasma concentrations.
Schouw 2006 (unpublished)	Follow-up study of children who required surgery or ECMO therapy as neonate	Thermal detection and pain thresholds	Neonatal surgery was associated with hyposensitivity for detection and hypersensitivity for pain. ECMO survivors were hyposensitive to detection of cold and heat, but no differences in pain thresholds were found compared to controls.
De Graaf[9] 2011	5 year follow-up study of the study of Simons et al. 2003	Intelligence, visual motor integration, behavior, chronic pain and health related quality of life	Significant negative effect of morphine on the "visual analysis" IQ subtest
De Graaf[10] 2013	8/9 year follow-up study of the study of Simons et al. 2003	Intelligence, visual motor integration, behavior and executive functioning	Significant positive effect of morphine on executive functioning as rated by the parents
Ceelie[11] 2013	RCT - intravenous paracetamol versus morphine in neonates and infants receiving major non-cardiac surgery	Cumulative morphine dose, pain scores and morphine-related side effects	66% reduction of morphine in the paracetamol group and no significant differences in pain scores or adverse drug effects

RCT - Randomized controlled trial

These studies found in contrast to numerous animal studies no major short-term or long-term negative effects of pain, opioids or anaesthetics.

INTERPRETATION OF OUR MAIN FINDINGS

We designed five models to determine the long-term effects of pain, opioids and anaesthetics in humans. Figure 1 represents the models presented in the second part of this thesis: high exposure to pain, opioids and anaesthesia due to surgery in early life (model 1a,b), prolonged continuous exposure to opioids and sedatives in the absence of major pain (model 2), low intensity of pain and internationally recommended doses of opioids in preterm born children (model 3), and lastly children exposed to opioids in utero in the absence of pain (model 4). We subdivided the models in terms of pain intensity; from no pain (-) to very intense pain (+++) and with respect to opioid exposure; from no opioid exposure (-) to very high opioid exposure (+++). Figure 2 represents the most important findings per model. The figures will be discussed on the following pages.

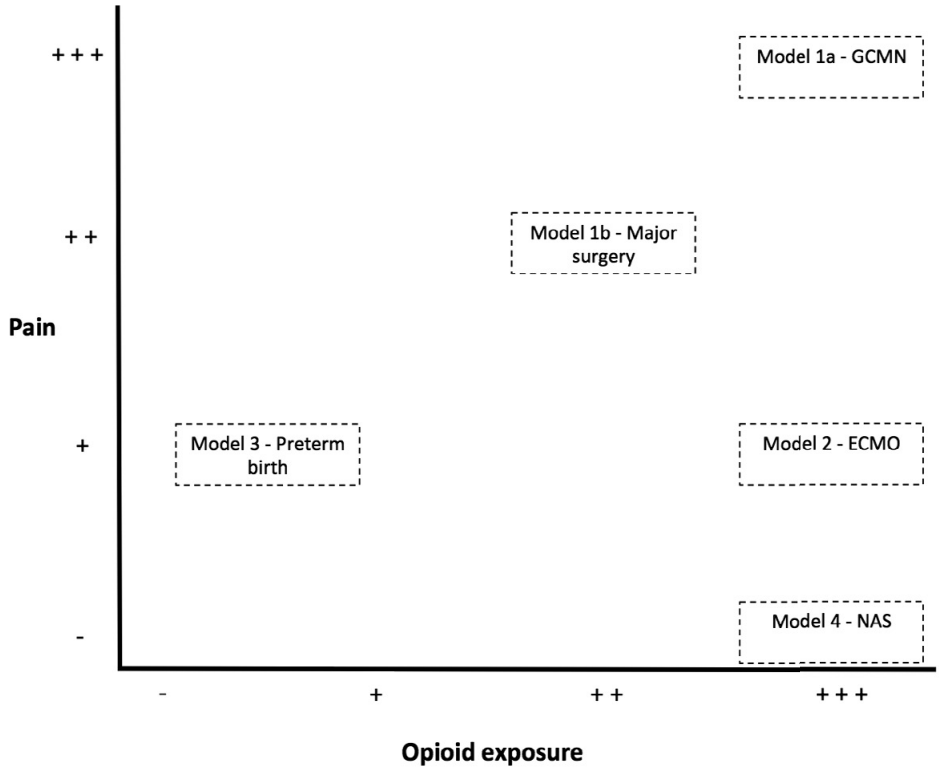
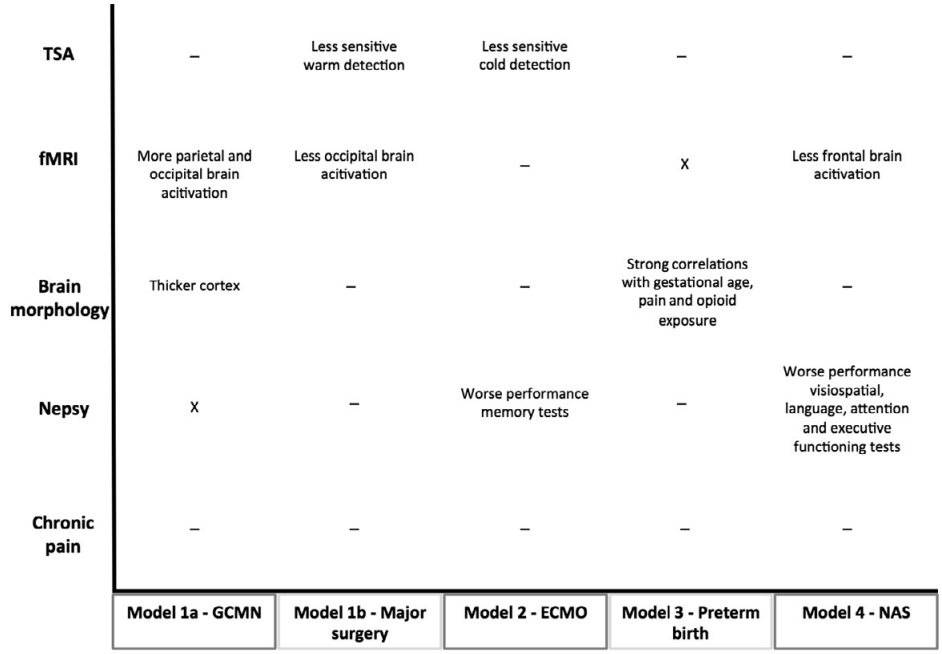


Figure 1 - Study models



X not conducted
- no differences with healthy controls

Figure 2 - Main findings per model

Model 1a - Extensive tissue damage and high dosages of opioids

Model 1b - Major surgery, general anaesthesia and opioid exposure

Extensive tissue damage and associated intense pain in combination with very high exposure to opioids induced more parietal and occipital brain activation during pain compared to healthy controls **(chapter 6)**. Less extensive tissue damage associated with major non-cardiac thoracic or abdominal surgery and lower amounts of opioids induced less occipital brain activation during pain compared to healthy controls **(chapter 7)**. Interestingly, the differences in brain activation during pain between both case groups and their controls were not specifically located in the pain centers of the brain, but rather in sensory regions. Since primary cortical areas typically develop earlier than secondary or tertiary brain regions,[12] it is possible that early exposure to pain, opioids and sedatives resulted in activity dependent neuronal changes in the primary and secondary sensorimotor cortical regions. The finding of more brain activation in model 1a and less in model 1b in the same brain region is surprising. A possible explanation could be the postnatal age differences between groups during the follow-up, but also during surgery. While children in model 1a had a median age of 31 days during surgery, children in

model 1b were younger, a median of 3.5 days old. It is also possible that after a period of hyperactivity the cells become passive due to excitotoxic neurotoxicity. Another explanation could be the fact that children in model 1a experienced more "breakthrough" pain due to the extensive tissue damage, as evidenced from the high need for opioids, while the neonates in the major surgery group were on average adequately treated - as shown from the COMFORT values - with the protocol dosage of 10 mcg/kg/hour.[5] This difference in both pain intensity and opioid exposure could have caused the difference between groups since it is known that the effects of opioids are different when given in the absence or presence of pain - at least in rodents.[13-15] Animal studies suggested a major difference in brain morphology, while the only significant finding in our patients was a thicker cortex (left rostral-middle-frontal cortex) in model 1a compared to healthy controls. From previous studies it is known that cortical thickness is associated with intelligence, in that a higher IQ is associated with faster thinning in childhood and a thicker cortex in adulthood.[16] However, the difference in thickness was minor since only a small part of the frontal lobe was involved. We therefore do not consider our findings as clinical relevant. Also, the rostral-middle-frontal cortex is not typically related to pain.

Model 2 - Prolonged neonatal opioid exposure in the absence of major pain

Prolonged continuous opioid exposure in the absence of major pain, as seen in ECMO treated newborns, induced no alterations in brain morphology **(chapter 8)**. However, it was associated with hyposensitivity for cold detection. This is in line with a previous TSA-II study showing that ECMO survivors were less sensitive for cold and warm detection (Schouw 2006, unpublished data). In this model prolonged use of opioids even in the most critically ill newborns does not result in an altered response of the central nervous system – at least as evaluated by fMRI. Our ECMO survivors' performance on the memory subtests of the NEPSY neuropsychological test was statistically significantly worse compared to healthy controls. This is in line with our own experience with regard to ECMO survivors in the outpatient follow-up clinic.[17] This finding is unrelated to the painful stimuli, but extremely important from a neurodevelopmental point of view. We have started a new study in our department this year to specifically determine the mechanism of memory deficits in neonatal ECMO survivors. This fMRI study will determine brain activity during a working memory task similar to our paradigm described in **chapter 5**. Children will be treated with a working memory program and possible effects will be measured.

Model 3 - Prematurity, opioid exposure and neonatal pain

Besides the use of high amounts of opioids, we were also interested in the long-term effects of low dosages of opioids (10 mcg/kg/hour) in the absence of tissue damage and substantial pain. For this model we included children who participated in the RCT of

Simons and colleagues as a neonate.[7] This well-defined cohort of preterm born children was comprehensively studied in two other follow-up studies of our department.[9,10] These studies found that morphine exposure was significantly negatively correlated with only one IQ subtest at the age of 5 years.[9] At age 8 or 9 years, however, this negative effect had disappeared and morphine was even positively correlated to executive functioning.[10] In line with these two previous studies we did not find major negative effects of prematurity, procedural pain and routine preemptive morphine on neuropsychological functioning **(chapter 9)**. Moreover, pain sensitivity had not been influenced, whereas a previous study did find evidence for hypersensitivity for pain later in life in sixty preterm born children compared to sixty controls.[18] These children were older during testing (12-18 years) than children in our group. The amount of morphine exposure in neonatal life was unfortunately not provided in that previous study.[18] If it was higher than in our study, this might perhaps explain the differences between both studies. With regards to brain morphology we found strong correlations between gestational age, pain, opioid exposure and volumes of brain regions. However, no differences between preterm born children and healthy controls were observed indicating no major clinical relevant influence on brain morphology. This is in contradiction to previous studies in preterm born morphine-exposed children that found differences at term-equivalent age and during childhood in head circumference (14 morphine treated and 5 placebo treated children born at 23-32 weeks of gestation), cortical thickness (25 preterm born children born at 26-33 weeks of gestation), brain microstructure (86 children born at 24-32 weeks gestation), and brain functioning during pain in preterm born children (9 children born before 31 weeks of gestation).[19-23] A possible explanation is that any reductions in brain volume at term-equivalent age had disappeared over time due to the inherent plasticity of the human brain associated with development. Additionally, the children in our cohort had been exposed to low doses of opioids (10mcr/kg/hour), while other cohorts had been exposed to the threefold dose without a solid pharmacokinetic base.[24] Additionally, our cohort included children of varying gestational ages at birth, similar to clinical practice, while the other studies included only extremely preterm born children, as described above, who probably as a consequence of lower gestational age received a higher cumulative dosage of opioids.

Model 4 - Exposure to opioids in the absence of pain

Since animal studies describe different outcomes of opioid exposure when given in the absence or presence of pain, we added a unique model to our study of individuals exposed to synthetic opioids in utero. We did not find differences with respect to pain sensitivity or brain activity during pain **(chapter 10)**. However, we found worse neuropsychological functioning in line with cognitive, memory and behavioral problems in rodents after exposure to opioids in the absence of pain.[25-27] This was found in combination with

less activity during pain in the frontal lobe, a region associated with attention and executive functioning.[28] This unique study is also important as the group of prenatally exposed children is increasing, especially in the US.[29,30]

Conclusion of our models

Taken all together it seems that very high exposure to opioids in the absence of pain has indeed the most negative effects especially on neuropsychological functioning (Models 2 and 4). However, in these particular circumstances a number of factors in both groups may also have contributed to worse neuropsychological outcomes such as poly drug abuse of mothers of the children in model 4 and the critical illness and associated disturbance of the mother-child relationship because of the extended hospital admission of ECMO children in model 2. Very high opioid exposure in combination with intense pain is associated with a thicker cortex, and since a higher IQ is associated with faster thinning in childhood,[16] it could also be a sign for worse neuropsychological functioning. Besides worse memory performance in ECMO survivors and worse neuropsychological performance in children exposed to opioids in utero, no major long-term effects of pain, opioids and anaesthetics are observed in all of our models indicating no major negative effects of pain, opioids and anaesthetics.

So most importantly, the dramatic effects expected from animal studies do not seem to occur in humans. We can be very decisive about this human study as the animal models all suffer from a methodological flaw, in that the painful stimulus used is incomparable with daily human life. Induced inflammatory pain by carrageenan in animals is nevertheless comparable to pain from for instance osteomyelitis in humans, but this condition is very rare in human neonates. Therefore this type of stimulus is not commendable to extrapolate to human situations. An interesting study of Ruda et al. published in Science found differences in the spinal neuronal circuits of rodents after pain stimuli, but since the pain was induced by an invasive inflammatory reaction the question remains if this finding can be extrapolated to humans.[31] Furthermore, other laboratories could not repeat the results. With respect to opioid and anesthetic studies there are differences between animal and human data as well. Animals often receive supratherapeutic high dosages of opioids or anaesthetics and mostly for prolonged periods of time.[32,33] Moreover, most of these animal studies are conducted in the absence of pain.[33] Additionally, children are carefully monitored during anesthesia in order to control for hypoxia and hypotension for example, while in animal studies physiologic derangement may often occur.[33] Furthermore, the manifestation of peak synaptogenesis may occur at different periods among species, and the window of vulnerability between animals and humans may be different.[34] Therefore we consider our human studies as the proof of principle.

Missing models

While we did study five different models, some important models are still missing. For ethical reasons it is impossible to determine the long-term effects of anaesthetics in the absence of surgery and vice versa. Moreover, it is hard to determine the long-term effects of pain in the absence of analgesic treatment since pain protocols are in place after the landmark studies of Anand and colleagues that underlined the importance of adequate analgesia in neonates.[35,36] Anand and colleagues found that newborns treated with fentanyl during surgery had less circulatory and metabolic complications and lower stress hormone levels after surgery compared to the newborns who only received anaesthesia and neuromuscular blocking agents in a time that analgesia during surgery was not used routinely. Still it took over 15 years before anaesthesiologists considered newborns as being able to experience pain and to treat them accordingly.[37,38] Individuals who required surgery as a neonate before the 1980s could serve as a unique model in this respect. The POPS (Project On Preterm and Small for Gestational Age infants in the Netherlands) cohort could serve as the perfect adult cohort to study the long-term effects of pain in the absence of opioids.[39] This cohort is therefore suggested for future research.

METHODOLOGICAL CONSIDERATIONS

TSA

With respect to the determination of detection and pain thresholds we used the TSA-II with a fixed protocol as described in **chapter 3**. Since the existing reference values were not user friendly[40] we added Dutch reference values, as described in chapter 3, to the existing literature. While the TSA-II is quite feasible to use in children from 8 years onwards, we suggest future studies also include the measurement of mechanical detection- and pain thresholds so as to provide a comprehensive view of somatosensory processing. If one would want to test all the different nerve fibres related to detection and pain (Aα, Aβ, Aγ, Aδ, B en C), we advise to also use electric and chemical stimuli to test for pain sensitivity. Pain tolerance would also be an informative measurement in future studies, but will be difficult in children due to ethical concerns. We obtained detection thresholds using both the Method of Limits (MLI) and the Method of Levels (MLE). To save time we suggest omitting the MLE and including the measurement of pain tolerance, for example using the cold pressor task.[41,42] In this test children immerse their hand or forearm in cold water and give pain scores for the duration of the test to indicate the experienced pain intensity. Moreover, the immersion time gives information about pain tolerance.[42] However, it is a qualitative test instead of a quantitative sensory test like the TSA test. The question remains whether pain experiments can be extrapolated to the real life situations. From our own experience we know that neuropathy can be identified with

thermal sensory tests as described in **chapter 11**. We know from studies in adults that the susceptibility for chronic pain can be predicted by experimental pain tests.[43] Future studies in children are needed in this respect.

FMRI

Functional MRI was first described in 1990 by Ogawa and colleagues.[44,45] Although relatively new, it is used frequently and offers the advantage of being non-invasive. However, it also has disadvantages since even dead matter can give brain activation when no correction for multiple testing is performed.[46] For pain related neuroimaging studies several types of stimuli can be used such as mechanical, electric and thermal stimuli.[21,47-68] We opted for thermal stimuli because our department has built experience with the use of the thermal sensory analyzer (TSA-II).[69] In **chapter 2** we observed that a standardized thermal pain stimulus induced comparable brain activation patterns in comparison with a stimulus temperature based on the individual thermal pain threshold. Therefore we used the most feasible one in children; a standardized stimulus of 46 C. In hindsight this was maybe not painful enough for all the participants since brain activity was in general not extremely high. However, if the stimulus temperature had been too high, the number of dropouts would perhaps have gone up as well. Additionally, brain activation during pain can be influenced by several factors such as fear or even pictures of a romantic partner.[70] Therefore we determined both detection- and pain thresholds using quantitative sensory testing in combination with fMRI.

Structural MRI

With regards to brain morphology no major differences between cases and controls were observed in our studies although we determined cortical thickness as well as brain volumes of several regions. Future studies involving other types of structural MRI such as Diffusion Tensor Imaging (DTI) would be very valuable to study white matter microstructure. It is possible that global brain measures are not affected but microstructural changes are detectable in the brain during childhood and adolescence. The latter is noteworthy because previous studies in preterm born neonates found that greater neonatal procedural pain was associated with reduced white matter fractional anisotropy (FA) and a slower rise in FA of the corticospinal tract at term-equivalent age.[19,71] The question remains whether these effects still exist at childhood age.

NEPSY

A previous study from our department found specific associations between neonatal morphine exposure and executive functioning during childhood age.[10] Therefore, we were specifically interested in executive functioning in our models. We decided to administer the NEPSY-II[72] rather than an IQ test for this reason. For children with spe-

cific neuropsychological problems, such as the ECMO group, the NEPSY-II test was very capable of detecting those difficulties. It is a relatively new neuropsychological test with the major advantage that it can assess several different cognitive domains within a relatively short time.

LIMITATIONS OF OUR STUDIES

We included very unique, well-defined cohorts and provided a broad overview of the long-term effects of pain and opioids using several models. Still, sample sizes of the subgroups were relatively low and therefore we could not correct for possible confounders other than age and gender. Future studies are recommendable and as described in **chapter 4**, fMRI pain studies are very feasible in young children. However, our study groups were larger in comparison to the only previous fMRI pain study in children with respect to the long-term effects of pain and pain treatment which included only 9 children per subgroup.[21] Moreover, socio-economic status could have been a factor of great influence in our studies. Unfortunately, we did not have information with regards to socio-economic status of our control group.

FUTURE PERSPECTIVES

A follow-up study of neonates included in the RCT of Ceelie and colleagues[11] would be very informative to determine differences in outcome between children exposed to intravenous paracetamol versus opioids in their first year of life. These two groups underwent comparable surgical procedures in early life. A healthy control group could serve as a third group to distinguish between possible effects of opioids, paracetamol and anaesthetics by correcting for both the dosage of opioids and anaesthetics. Our research group conducted several follow-up studies in vulnerable individuals. Our excellent infrastructure for follow-up studies in combination with good knowledge of pain related outcome measures makes a study like this feasible in our setting. Since pharmacovigilance is very important, especially in vulnerable newborns, studies exploring long-term effects of drugs are important. The results of these studies are difficult to interpret given the magnitude of factors that might contribute to adverse outcome in these critically ill preterm or term newborns.

To distinguish between the long-term effects of prematurity, procedural pain, as well as opioid exposure, a twin study would provide valuable information. This will enable to determine the long-term effects of opioid exposure and pain and specifically take into

account the effect of the amount of opioid consumption and number of painful procedures, while correcting for gestational age and twin-related demographic characteristics.

Although pain-scoring devices are standardized and less prone to subjective judgment, more objective clinical biomarkers are needed. Therefore we recently started a new follow-up project among our included subjects to search for opioid and pain sensitivity related genes from saliva. This saliva was collected during our study visits to determine cortisol levels. The remaining saliva will be used for DNA analyses. The aim of these studies are twofold; to determine whether genetic variations in genes related to pain sensitivity and (endogenous) opioid metabolism correlate with stress reactivity as measured by cortisol levels and with thermal pain sensitivity as measured previously in these children (as described in this thesis)

Since animal studies are very valuable but also very hard to extrapolate to human situations, as previously described in this discussion, we suggest closing the gap between animal and human studies. A previous study from our own department already made attempts in this direction by equalizing the painful procedures used in animal studies and human daily life at the NICU. In this important work of Knaepen and colleagues needle pricks rather than inflammatory pain stimuli were used in rodents, which is in line with the human situation.[73] Instead of inducing chronic pain in rodents, acute repetitive pain as in humans should be used in experimental designs. Exposure to analgesics and anaesthetics experimental designs should be more in line with human daily life.

SO WHAT REMAINS IN THE BRAIN AFTER THE WHEELS OF TIME?

The answer to this question is that there are no major effects of neonatal pain that remain in the brain some 8-19 years later in children without major neurological problems in neonatal life. We can conclude that apart from specific neuropsychological effects that warrant further investigation, no major effects are observed with respect to thermal and pain sensitivity, brain functioning during pain, brain morphology or in the occurrence of chronic pain. Brain development seems not to be affected at later age. Although we did detect subtle differences between exposed children and healthy controls, major clinical relevant effects of pain, opioids and anaesthetics are not observed. In view of our findings, we believe that elective surgery during infancy does not need to be postponed because of fear for negative long term effects and that the use of opioids for procedural pain or intense pain because of major tissue damage does not harm the brain later in life. The question that remains is whether analgesic therapy based on opioids is still needed since paracetamol has shown to be very effective as well.[11]

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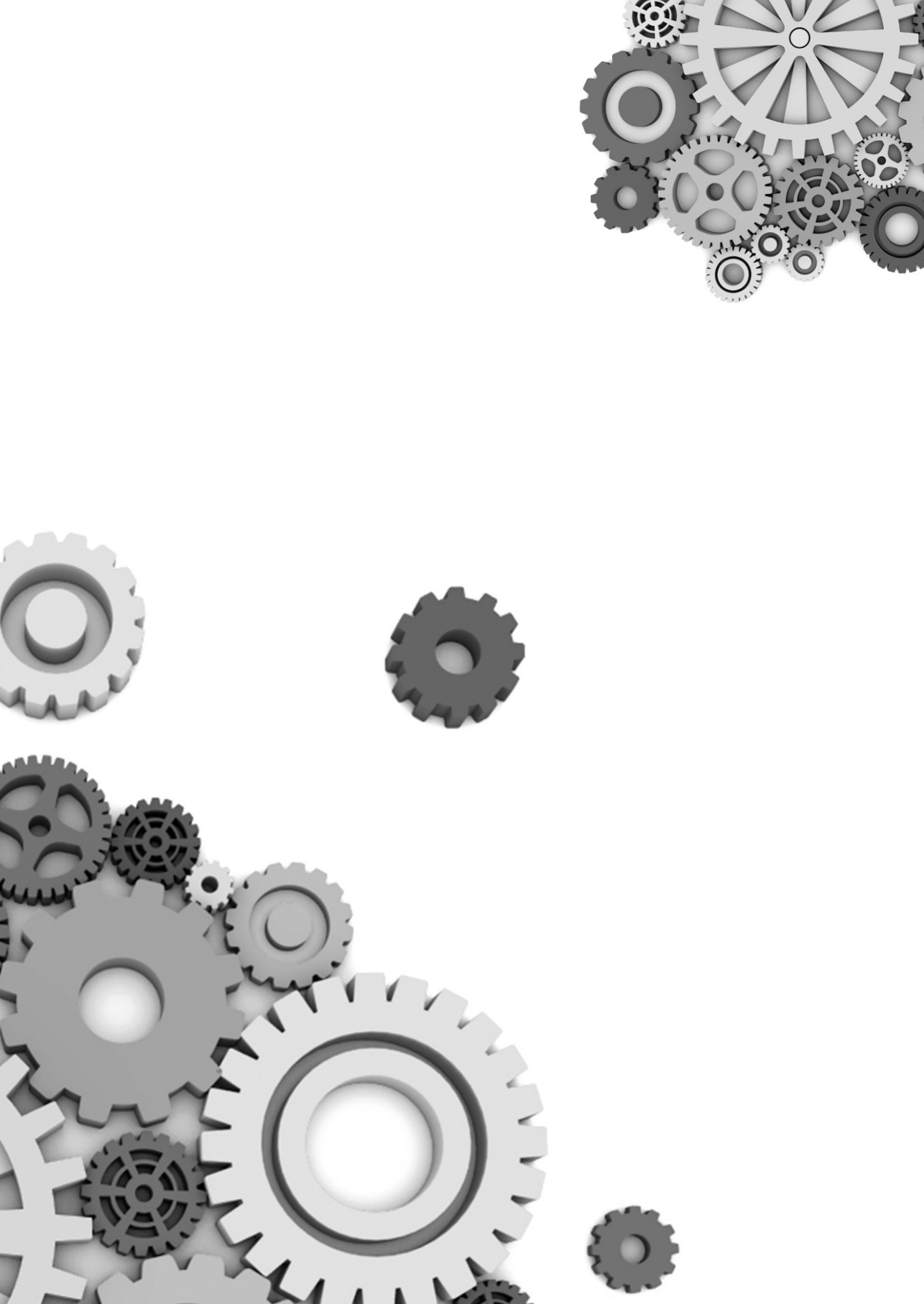


Chapter 13

Summary

Pain is defined as '*An unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage*' by the International Association for the Study of Pain (IASP) - with the note that *'Pain is always subjective'* and *'Each individual learns the application of the word through experiences related to injury in early life'*. This thesis addressed possible long-term effects of pain and opioid exposure in early life, notably with regard to pain sensitivity, brain functioning during pain, brain morphology and neuropsychological functioning later in life. We specifically chose these outcome measures since animal studies have found negative effects of neonatal pain and opioid exposure with respect to pain sensitivity, neurotoxicity and cognitive functioning. This thesis presents five human models in which exposure to pain, opioids and anaesthetics is objectified in several intensities from no pain to intense pain and no opioid exposure to very high opioid exposure.

The **first part** of this thesis focuses on the methodology for pain studies and fMRI studies in children.

In the study described in **chapter 2** we compared two different types of thermal painful stimuli during an fMRI study in adults. We observed that a standardized stimulus of 46°C induced similar brain activation patterns as a stimulus based on the subject's individual pain threshold (46°C - 48°C). Moreover, we found out that a stimulus temperature of 46°C was an adequate temperature for standardized stimulation. Since we found equal outcomes of both conditions and since the use of an individualized stimulus is more time-consuming and less practical in young children, we chose to use a standardized stimulus in our studies presented in the second part of this thesis.

In **chapter 3** we present our standardized testing protocol for the determination of detection- and pain thresholds, which appeared to be very feasible from the age of 8 years onwards. Dutch reference values were given based on a sample of 69 healthy term born children and adolescents.

FMRI is little used for pain research in children, mainly because it is thought to be too frightening for young participants. In **chapter 4** we conclude that fMRI pain research is well-tolerated and not harmful or frightening for children since 'fear' and 'fun' ratings of the child itself, a parent and the researcher indicated a high level of fun and a low level of fear. Moreover, 98% of the enrolled children were willing to undergo the MRI scan.

In **chapter 5** we studied the development of brain connectivity related to verbal working memory in normally developing children and adolescents. We present a working memory fMRI task and found age-related differences in brain connectivity during the task. It is

useful to understand the developmental trajectories in functional connectivity during working memory activation in healthy children and adolescents in order to compare this with individuals suffering from memory deficits (as described in chapter 8).

The **second part** explores the long-term effects of early pain, opioid exposure and administration of anaesthetics. Main outcome measures in this part are thermal detection and pain sensitivity, brain activity during pain, brain morphology and neuropsychological functioning.

Chapter 6 objectified the long-term effects of extensive tissue damage and high exposure to opioids in children who required surgery in early life due to a giant congenital melanocytic naevus (GCMN). Therefore these children were also exposed to opioids in early life. We compared 14 cases with 42 controls and found no differences in detection or pain thresholds. We did find greater parietal/occipital brain activity during painful stimuli, but no differences in brain volumes. A minor difference in cortical thickness was observed, although the clinical relevance is expected to be low. The dramatic neurotoxic effects of pain and opioids obtained from animal studies appear not to occur in humans.

In **chapter 7** the effects of major surgery in neonatal life and related exposure to opioids and anaesthetics are presented. In this exploratory study 10 adolescents were compared to 10 healthy controls. Cases turned out to be less sensitive for a warm stimulus (34.2°C (1.4) versus 33.1°C (0.6) in controls ($p=0.04$)) and showed less brain activation in the occipital cortex during pain. No differences with respect to brain morphology or neuropsychological functioning were observed. In this model we could also not detect the alarming findings as described in animal studies.

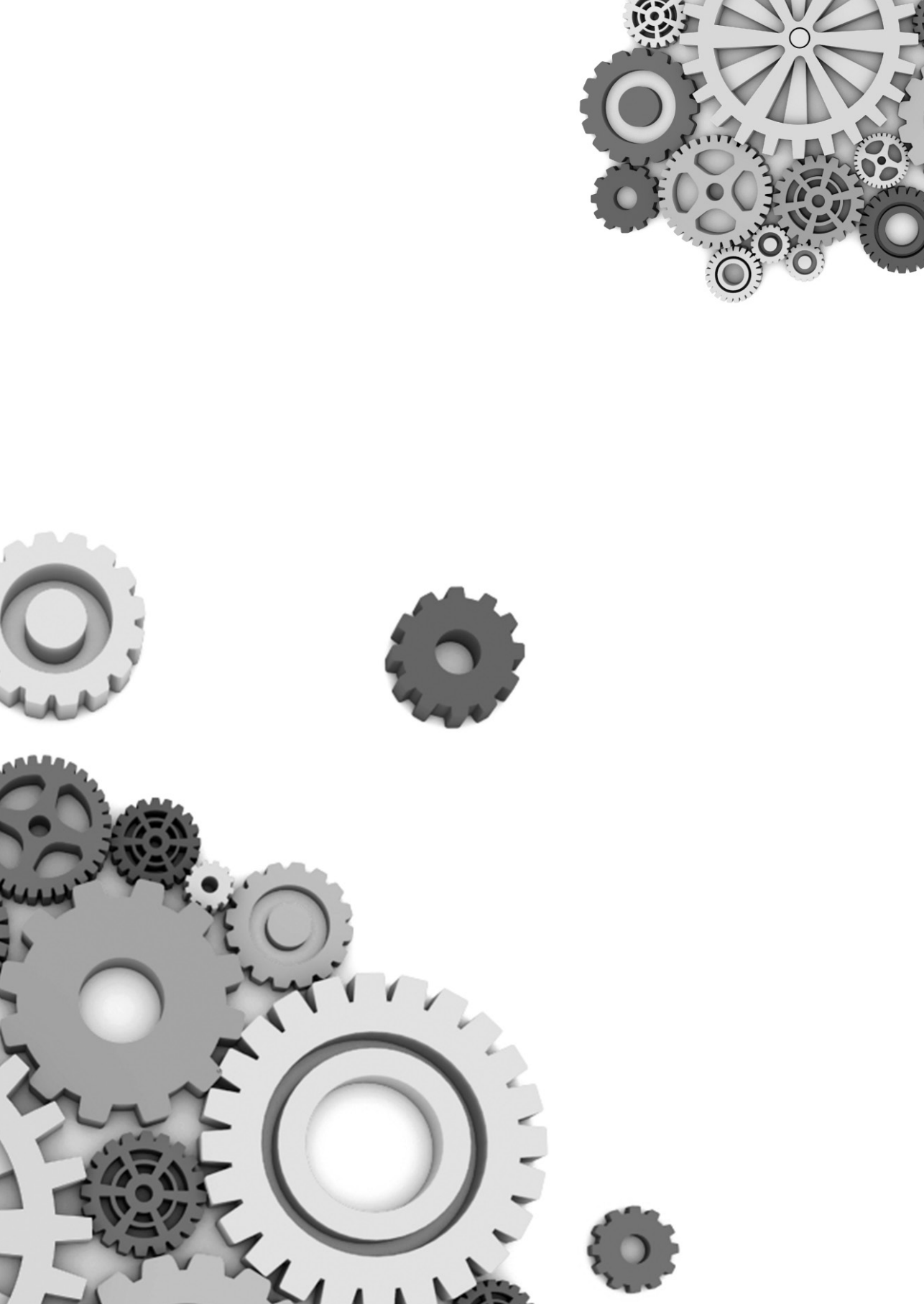
In **chapter 8** we studied the human equivalent for a proof-of-principle concept with respect to the long-term effects of prolonged neonatal opioid exposure in the absence of major pain. We compared 36 neonatal ECMO survivors (8-15 years of age) to 64 healthy controls in the same age range and found a significant difference in the detection threshold for cold (ECMO group 29.9°C (SD 1.4), control group 30.6°C (SD 0.8); $p<0.01$), However, this was only observed when measured in a reaction time dependent fashion, not when using a reaction time independent technique. No differences in pain sensitivity, brain activity during pain or brain morphology was observed. Interestingly, we observed significant memory deficits in ECMO survivors that warrant further investigation. Therefore our department recently started a new fMRI project in ECMO survivors using a similar working memory paradigm as described in chapter 5.

Besides the effects of exposure to high amounts of opioids, we also evaluated the long-term effects of internationally recommended dosages of opioids (10 mcg/kg/hour). **Chapter 9** describes the long-term correlations between gestational age, number of painful procedures, amount of opioid exposure and thermal sensitivity, brain morphology and neuropsychological functioning in former mechanically ventilated preterm born children. Strong significant correlations (coefficients 0.60-0.83) between gestational age, number of painful procedures, morphine exposure and brain volumes were observed in 19 preterm born children at 10 years of age. No major associations with respect to thermal sensitivity or cognitive functioning were detected, indicating no major effects in daily life.

Chapter 10 describes a unique human model for early opioid exposure in the absence of pain. Since opioids are not administered to paediatric patients in the absence of pain due to obvious ethical reasons, we studied children exposed to opioids in utero. Fifteen individuals (9-19 years of age), who had been exposed to heroin and methadone in utero, were compared to 71 healthy controls (8-17 years). After correction for age and gender we observed no differences in thermal sensitivity or brain functioning during pain. We did observe less brain activity during pain in the frontal lobe and poorer performance on several subtests of the NEPSY-II neuropsychological test. Since the frontal lobe is also associated with attention and executive functioning, rather than pain, we primarily observed neuropsychological long-term effects of early opioid exposure in the absence of pain.

In **chapter 11** we describe a boy who presented himself with severe contact burns on his buttocks. The family was under supervision of the Child Care Board, but since the boy also had frequent painless bruises and lacerations in combination with anhidrosis, the diagnosis hereditary sensory and autonomic neuropathy type IV (HSAN IV) was considered. Elevated detection- and pain thresholds combined with lower brain activation during pain were observed in the case in line with the diagnosis HSAN IV.

In **chapter 12** the results of our studies are discussed and recommendations for future research are given. Our overall conclusion is that that there are no major effects of neonatal pain that remain in the brain some 8-19 years later in children without major neurological problems in neonatal life. We can conclude that besides specific neuropsychological effects that warrant further investigation, no major effects with respect to thermal and pain sensitivity, brain functioning during pain or brain morphology are observed.



Samenvatting

Pijn is gedefinieerd als '*Een onplezierige sensorische en emotionele ervaring geassocieerd met daadwerkelijke of potentiele weefselschade, of beschreven als dit soort schade*' door de '*International Association for the Study of Pain*' (IASP) met de aanvulling dat pijn altijd subjectief is en ieder individu de betekenis van het woord leert kennen door ervaringen gerelateerd aan verwondingen op jonge leeftijd. In dit proefschrift worden de langetermijneffecten van pijn en blootstelling aan opioïden op jonge leeftijd beschreven. Hierbij lag de nadruk op de mogelijke effecten met betrekking tot temperatuur- en pijngevoeligheid, hersenactivatie tijdens pijn, hersenmorfologie en neuropsychologisch functioneren op latere leeftijd. We hebben specifiek voor deze uitkomstmaten gekozen, omdat dierstudies negatieve effecten van neonatale pijn en blootstelling aan opioïden hebben beschreven met betrekking tot pijngevoeligheid, neurotoxiciteit en cognitief functioneren. Dit proefschrift beschrijft vijf humane modellen waarin blootstelling aan pijn, opioïden en anesthetica zijn geobjectiveerd en waarbij de intensiteit van deze factoren uiteenliep van geen pijn tot intense pijn en geen blootstelling aan opioïden tot blootstelling aan zeer hoge doseringen op jonge leeftijd.

Het **eerste deel** van dit proefschrift betreft de methodologie van pijn- en fMRI-onderzoek bij kinderen.

De studie beschreven in **hoofdstuk 2** vergelijkt verschillende soorten thermale pijnstimuli tijdens een fMRI-experiment bij volwassenen. We vonden dat een gestandaardiseerde stimulus van 46 °C vergelijkbare hersenactivatie patronen liet zien als een stimulus gebaseerd op de individuele pijndrempel van de proefpersoon (46 °C - 48 °C). Bovendien bleek een stimulustemperatuur van 46 °C een adequate temperatuur voor gestandaardiseerde pijnstimulatie te zijn. Omdat we vergelijkbare uitkomsten hebben gevonden tussen beide soorten pijnstimuli en het gebruik van geïndividualiseerde pijnstimuli daarnaast meer tijd in beslag neemt en minder praktisch is in het gebruik bij jonge kinderen, hebben we ervoor gekozen om gestandaardiseerde pijnstimuli toe te dienen in de studies die worden beschreven in het tweede deel van dit proefschrift.

In **hoofdstuk 3** brengen we ons gestandaardiseerde protocol voor de bepaling van detectie- en pijndrempels. Dit testprotocol bleek goed uitvoerbaar bij kinderen vanaf de leeftijd van 8 jaar. Daarnaast presenteren we Nederlandse referentiewaarden gebaseerd op een groep van 69 gezonde à terme geboren kinderen en adolescenten.

FMRI wordt niet vaak gebruik voor pijnonderzoek bij kinderen, met name omdat er gedacht wordt dat het te beangstigend zou zijn voor kinderen. In **hoofdstuk 4** concluderen we dat fMRI in combinatie met pijnstimuli goed getolereerd wordt en niet schadelijk of beangstigend is voor kinderen. Dit laatste hebben we gemeten door scores voor angst

en plezier te vragen aan het kind zelf, de ouder en de onderzoeker. Deze toonden een hoog niveau van plezier en een laag niveau van angst. Daarnaast is het belangrijk om te noemen dat 98% van de kinderen na de oefenscanner de echte MRI-scan wilden ondergaan.

Hoofdstuk 5 beschrijft leeftijdsveranderingen in hersenconnectiviteit tijdens een werkgeheugentaak bij zich normaal ontwikkelende kinderen en adolescenten. We presenteren een werkgeheugentaak die voor een fMRI-experiment gebruikt kan worden en vonden aan leeftijd gerelateerde verschillen in hersenconnectiviteit tijdens deze test. Het is belangrijk om de normale ontwikkeling van functionele connectiviteit tijdens het werkgeheugen te bepalen. Dit om deze waarden te kunnen vergelijken met personen die wel geheugenproblematiek vertonen (zoals beschreven in hoofdstuk 8).

Het **tweede deel** onderzoekt de langetermijneffecten van vroege pijnervaringen, blootstelling aan opioïden en blootstelling aan anesthetica. De belangrijkste uitkomstmaten waren thermale detectie- en pijndrempels, hersenactivatie tijdens pijn, hersenmorfologie en neuropsychologisch functioneren.

Hoofdstuk 6 beschrijft de langetermijneffecten van uitgebreide weefselschade en hoge doses opioïden bij kinderen die geopereerd zijn in de eerste levensweken aan een zogenaamde 'giant congenital melanocytic naevus' (GCMN). Daarom kregen deze kinderen ook opioïden op jonge leeftijd. We hebben 14 cases vergeleken met 42 controles en vonden daarbij geen verschil in detectie- of pijndrempels. We vonden wel meer hersenactivatie tijdens pijn in de pariëtale en occipitale hersenkwab, maar geen verschil in de grootte van de hersengebieden. Een klein verschil in de dikte van de cortex van de hersenen werd gevonden, maar de klinische relevantie hiervan is waarschijnlijk laag. De dramatische verschillen die we hadden verwacht op basis van dierstudies lijken derhalve niet aanwezig bij de mens.

In **hoofdstuk 7** beschrijven we de effecten van ingrijpende operaties in de eerste levensmaand en daaraan gerelateerde blootstelling aan opioïden en anesthetica. In deze exploratieve studie hebben we 10 adolescenten vergeleken met 10 gezonde controles. We vonden dat cases minder gevoelig waren voor een warme stimulus (34.2 °C (1.4) versus 33.1 °C (0.6) bij controles (p=0.04)). Daarnaast toonden cases minder hersenactivatie in de occipitale hersencortex tijdens pijn. Er werden geen verschillen met betrekking tot hersenmorfologie of neuropsychologisch functioneren geobjectiveerd. In dit model konden we de alarmerende bevindingen zoals beschreven in dierstudies ook niet bevestigen.

In **hoofdstuk 8** bestudeerden we de menselijke equivalent voor een bewijsconcept met betrekking tot de langetermijneffecten van langdurige blootstelling aan opioïden in de eerste levensweken in de afwezigheid van intense pijn. We hebben 36 kinderen die neonatale ECMO therapie hadden gekregen (nu 8-15 jaar oud) vergeleken met 64 gezonde controles van dezelfde leeftijd. We vonden een significant verschil in de detectiedrempel voor koude (ECMO groep 29.9 °C (SD 1.4), controlegroep 30.6 °C (SD 0.8); $p<0.01$). Echter, dit verschil vonden we alleen als de detectiedrempel werd gemeten met een test die reactiesnelheid-afhankelijk was. Wanneer gemeten met de reactiesnelheid-onafhankelijke test, dan werd dit verschil niet geobjectiveerd. Daarnaast vonden we geen verschillen tussen beide groepen met betrekking tot pijngevoeligheid, hersenactivatie tijdens pijn en hersenmorfologie. Wel vonden we dat de ECMO groep significant slechter presteerden op de geheugentaken van de neuropsychologische test. Daarom zijn we op onze afdeling recent met een nieuwe fMRI-studie gestart bij ECMO-kinderen waarbij een vergelijkbare werkgeheugentaak zoals beschreven in hoofdstuk 5 zal worden gebruikt.

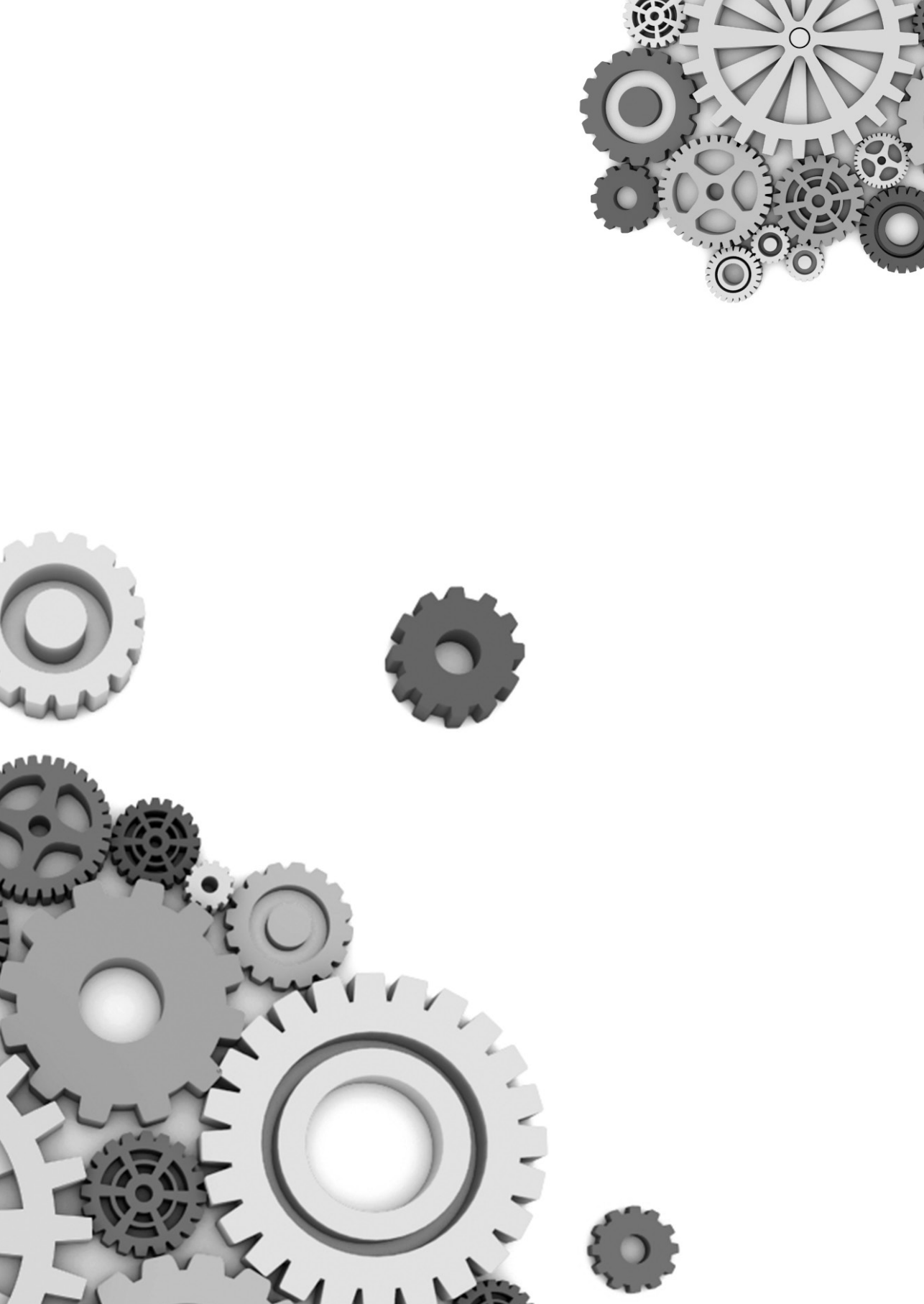
Naast de effecten van blootstelling aan hoge doses opioïden hebben we ook de effecten van internationaal aanbevolen doses van 10 mcg/kg/uur onderzocht. **Hoofdstuk 9** beschrijft de correlaties op de lange termijn tussen gestatieduur, het aantal pijnlijke procedures, de mate van blootstelling aan opioïden en de temperatuur- en pijngevoeligheid, de hersenmorfologie en het neuropsychologisch functioneren bij prematuur geboren kinderen die als neonaat beademd zijn. We vonden sterke significante correlaties (coëfficiënten 0.60-0.83) tussen gestatieduur, het aantal pijnlijke procedures, de mate van blootstelling aan opioïden en hersenvolumes bij 19 prematuur geboren kinderen op de leeftijd van 10 jaar. Er was weinig invloed van bovenstaande factoren op de temperatuur- en pijngevoeligheid of het neuropsychologisch functioneren, wat impliceert dat er geen grote gevolgen voor het dagelijks leven zijn.

Hoofdstuk 10 beschrijft een uniek humaan model voor vroege blootstelling aan opioïden in de afwezigheid van pijn. Opioïden worden op basis van ethische redenen niet aan kinderen toegediend in de afwezigheid van pijn, daarom hebben wij kinderen bestudeerd die al voor de geboorte zijn blootgesteld aan opioïden. Vijftien kinderen en jongeren (9-19 jaar oud) die in de baarmoeder al waren blootgesteld aan heroïne en methadon vanwege drugsgebruik van moeder, werden vergeleken met 71 gezonde controles (8-17 jaar oud). We vonden geen verschil tussen beide groepen met betrekking tot temperatuur- en pijngevoeligheid en de hersenmorfologie (gecorrigeerd voor leeftijd en geslacht). Wel vonden we een significant verschil tussen beide groepen met betrekking tot de hersenactivatie tijdens pijn, waarbij de cases minder hersenactivatie in de frontaalkwab lieten zien. Daarnaast presteerden de cases significant slechter op verscheidene subtesten van NEPSY-II neuropsychologische test. Omdat de frontale kwab

is geassocieerd met aandacht en executief functioneren, en niet zo zeer aan pijn, hebben we met name neuropsychologische effecten van vroege blootstelling aan opioïden in de afwezigheid van pijn geobjectiveerd.

In **hoofdstuk 11** beschrijven we een jongen die naar het ziekenhuis kwam met ernstige brandwonden op zijn billen. De familie van de jongen stond vóór het ontstaan van de brandwonden al onder toezicht van de raad van kinderbescherming. Omdat de jongen naast de brandwonden ook vaak pijnloze kneuzingen en wonden had in combinatie met niet zweten, werd de diagnose hereditaire sensorische autonome neuropathie type IV (HSAN IV) overwogen. Verhoogde detectie- and pijndrempels in combinatie met lagere hersenactivatie tijdens pijn werden geobjectiveerd, overeenkomstig met de diagnose HSAN IV.

In **hoofdstuk 12** bespreken we de resultaten van onze studies en geven we aanbevelingen voor toekomstig onderzoek. De conclusie van dit proefschrift is dat neonatale pijn geen ingrijpende effecten heeft op de hersenen 8-19 jaar later, tenminste bij kinderen zonder grote neurologische problemen als neonaat. We kunnen stellen dat afgezien van enkele specifieke neurologische effecten, die nader onderzoek behoeven, geen ingrijpende effecten met betrekking tot de temperatuur- en pijngevoeligheid, de hersenactivatie tijdens pijn en de hersenmorfologie zijn geobjectiveerd.



Chapter 14

Appendices

PhD PORTFOLIO

Name PhD student	Gerbrich E. van den Bosch
Erasmus MC Department	Intensive Care (Erasmus MC-Sophia)
PhD period	January 2010 - March 2014
Promotors	Prof. dr. D. Tibboel
Copromotors	Dr. M. van Dijk
	Dr. T. White

		Workload	
PhD training		**year**	**ECTS**
General courses	'Basiscursus Regelgeving en Organisatie voor Klinisch onderzoekers' (BROK)	2010	1.0
	CPO Minicourse	2010, 2011	0.6
	Systematic Literature Search and EndNote	2010	0.4
	MolMed - Basic Introduction Course on SPSS	2010	1.0
	MolMed - Short Introductory Course on Statistics and Survival Analysis for MD's	2010	0.5
	MolMed - R Statistical Package	2010	1.4
	Biomedical English Writing and Communication	2011	4.0
	Integrity in Scientific Research	2011	1.5
	MolMed - Research Management for PhD students	2011	1.0
	Classical Methods for Data-analysis	2011	5.7
	MolMed - Workshop Presenting Skills for Junior Researchers	2012	1.0
	MolMed - Writing Successful Grant Applications	2012	0.5
Specific courses	FSL and Freesurfer (MRI software)	2010	2.0
	MRI Safety Course	2010	0.3
	Functional MRI	2010	0.9
	Brain Anatomy	2011	0.3
	Neuroradiology and Functional Neuroanatomy	2012	1.5
	Freesurfer (MRI software)	2012	1.0
Symposia and workshops	Neuroimaging, Genetics and Endophenotypes: Development and Psychopathology	2010	0.3
	Brain Development and Developmental Disorders	2012	0.3
	NWO symposium 'Breinproducten aan de horizon'	2012	0.3
	Young Investigator Day (TULIPS/NVK)	2012, 2013	0.6
	Erasmus MC PhD days	2013	0.3
	Jackson Rees symposium	2013	0.3

International presentations	Human Brain Mapping (HBM), Quebec, Canada (2 poster presentations)	2011	1.0
	Annual meeting of the European Society of Paediatric and Neonatal Intensive Care (ESPNIC), Rotterdam (invited speaker)	2013	1.0
	International Symposium of Pediatric Pain (ISPP) (2 poster presentations; 1 highly commended)	2013	1.0
National presentations	Annual symposium for nurse practitioners and physician assistants Neonatology (invited speaker)	2013	0.3
	Research day Erasmus MC (oral presentation; first prize winner)	2013	0.3
	Several oral presentations during various research meetings at the Erasmus MC	2010-2014	1.0
Teaching			
	Teaching medial students (3rd year)	2012	0.3
	Supervising medical student master's thesis	2012	1.5
	Teaching medical students (2nd year)	2013	0.6
	Teaching medical students (3rd year)	2013	0.5
Other			
	Organization of the symposium Neuroimaging, Genetics and Endophenotypes: Development and Psychopathology	2010	0.8
	Writing F1000 evaluations (n=20)	2010-2013	2.5
	Writing several grant proposals	2011-2013	2.0
	Board of the 'Sophia Onderzoekers Vertegenwoordiging (SOV)'	2012-2013	4.0
	Pharmacology Research Meetings (multiple oral presentations)	2010-2014	2.0
	Lab Meetings KNICR	2010-2014	2.0
	KNICR-BIGR MRI meetings	2010-2014	0.5
	AMBER fMRI meetings	2010-2014	1.0

ECTS = European Credit Transfer and Accumulation System
1 ECTS represents 28 hours

LIST OF PUBLICATIONS

International

van den Bosch GE, Merkus PJFM, Buysse CM, Boehmer AL, Vaessen-Verberne AA, van Veen LN, Hop WC, de Hoog M. Risk Factors for Pediatric Intensive Care Admission in Children With Acute Asthma. Respir Care. (2012) Sep; 57(9):1391-7.

Ceelie I, de Wildt SN, van Dijk M, van den Berg MMJ, **van den Bosch GE**, Duivenvoorden HJ, de Leeuw TG, Mathôt R, Knibbe CAJ, Tibboel D. Intravenous paracetamol reduces morphine requirements in neonates and young infants undergoing major non-cardiac surgery; results of a randomized controlled trial. JAMA. (2013) Jan 9; 309(2):149-54.

van den Bosch GE*, van Hemmen J*, White T, Tibboel D, Peters JWB, van der Geest JN. Standard and individually determined thermal pain stimuli induce similar brain activations. Eur J Pain. (2013) Oct;17(9):1307-15.
** Contributed equally*

van den Bosch GE, White T, Tibboel D, van Dijk M. Functional MRI pain studies in children? Yes, we (s)can! Pediatr Radiol. (2013) Sep;43(9):1235-6.

van den Bosch GE, El Marroun H, Schmidt MN, Tibboel D, Manoach DS, Calhoun VD, White T. Brain Connectivity during Verbal Working Memory in Children and Adolescents. Hum Brain Mapp. (2014) Feb; 35(2):698-711.

van den Bosch GE, Baartmans MGA, Vos P, Dokter J, White T, Tibboel D. Pain insensitivity syndrome misinterpreted as inflicted burns. Pediatrics (2014) April 14. (Epub ahead of print)

van den Bosch GE, van Dijk M, Tibboel D, Valkenburg AJ. Thermal Quantitative Sensory Testing in healthy Dutch children and adolescents. Standardized test paradigm and Dutch reference values. (Submitted for publication)

van den Bosch GE, White T, El Marroun H, van Rosmalen J, de Leeuw TG, van der Lugt A, van der Geest JN, Tibboel D, van Dijk M. Should we be concerned about exposure to anaesthetics and opioids in neonates? A neuropsychological and neuroimaging exploratory study in humans. (Submitted for publication)

van den Bosch GE, IJsselstijn H, van der Lugt A, Tibboel D, van Dijk M, White T. Long-term effects of neonatal opioid and sedative exposure in ECMO patients. A neuroimaging study. (Submitted for publication)

van den Bosch GE, White T, El Marroun H, Schmidt MN, van der Lugt A, van der Geest JN, Tibboel D, van Dijk M. Prematurity, Opioid Exposure and Neonatal Pain: Does it affect the developing brain? (Submitted for publication)

van den Bosch GE, van Dijk M, El Marroun H, Schmidt MN, van der Lugt A, van Adrichem LNA, van der Geest JN, Tibboel D, White T. Long-term neurobiological effects of extensive tissue damage in newborns and young infants. A neuroimaging study of children with giant congenital melanocytic naevi. (Submitted for publication)

van den Bosch GE, Moelchand M, White T, El Marroun H, van der Geest JN, van der Lugt A, Sibbles BJ, van den Anker JN, van Dijk M, Tibboel D. Long-term effects of opioid exposure in utero. A neuropsychological and neuroimaging study. (Submitted for publication)

Valkenburg AJ, **van den Bosch GE**, de Graaf J, van Lingen RA, Weisglas-Kuperus N, Groot Jebbink LJ, Tibboel D, van Dijk M. Long-term effects of neonatal continuous morphine infusion on pain sensitivity: Follow-up of a randomized controlled trial. (Submitted for publication)

National

van den Bosch GE, Tibboel D. Referaat over het artikel 'Cerebral processing of pain in school-aged children with neonatal nociceptive input: An exploratory fMRI study' van Hohmeister et al. 2010. Nederlandstalig Tijdschrift Pijn en Pijnbestrijding.

van den Bosch GE, Tibboel D. Referaat over het artikel 'Influence of risk of neurological impariment and procedure invasiveness on health professionals' management of procedural pain in neonates' van Stevens et al. 2010. Nederlandstalig Tijdschrift Pijn en Pijnbestrijding.

van den Bosch GE. Referaat over het artikel '"He Says, She Says": A Comparison of Fathers' and Mothers' Verbal Behavior During Child Cold Pressor Pain' van Moon et al. 2011. Nederlandstalig Tijdschrift Pijn en Pijnbestrijding.

van den Bosch GE. Referaat over het artikel 'Reliability of the visual analog scale in children with acute pain in the emergency department' van Bailey et al. 2012. Nederlandstalig Tijdschrift Pijn en Pijnbestrijding.

van den Bosch GE. Referaat over het artikel 'Neonatal pain in relation to postnatal growth in infants born very preterm' van Vinall et al. 2012. Nederlandstalig Tijdschrift Pijn en Pijnbestrijding.

LIST OF ABBREVIATIONS

AAL	Anatomical Automatic Labeling
ADHD	Attention Deficit Hyperactivity Disorder
AFNI	Analysis of Functional NeuroImages
ANT	Amsterdam Neuropsychological Tasks
BOLD	Blood Oxygen Level Dependent
CIPA	Congenital Insensitivity of Pain with Anhidrosis
ECMO	Extracorporeal Membrane Oxygenation
EPI	Echo-planar Imaging
FEAT	FMRIB's fMRI Expert Analysis Tool
FMRI	Functional Magnetic Resonance Imaging
FSL	FMRIB's Software Library
FWE	Family-wise Error
GABA	Gamma-amino Butyric Acid
GCMN	Giant Congenital Melanocytic Naevus
HSAN	Hereditary Sensory and Autonomic Neuropathy
ICA	Independent Component Analyses
IVH	Intraventricular Haemorrhage
MLE	Method of Levels
MLI	Method of Limits
MNI	Montreal Neurological Institute
MRI	Magnetic Resonance Imaging
NAS	Neonatal Abstinence Syndrome
NEPSY	A Developmental NEuroPSYchological Assessment
NICU	Neonatal Intensive Care Unit
NMDA	N-methyl-D-aspartate
NRS	Numerical Rating Scales
PET	Positron Emission Tomography
QST	Quantitative Sensory Testing
ROI	Region of Interest
SIRP	Sternberg Item Recognition Paradigm
SPM	Statistical Parametric Mapping
TSA	Thermal Sensory Analyzer
WkM	Working Memory

DANKWOORD

Thanks are the highest form of thought, and gratitude is happiness doubled by wonder
G.K. Chesterton

De afgelopen jaren heb ik met ontzettend veel plezier aan mijn promotieonderzoek gewerkt. De samenwerking met vele mensen uit verscheidene disciplines maakt een promotietraject zo mooi en speciaal. Tijdens mijn promotie heb ik op talloze momenten aan dit dankwoord gedacht omdat veel mensen het verdienen om hier genoemd te worden.

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Een beter team van promotor en copromotoren had ik mij niet kunnen wensen.
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Dr. Monique van Dijk, lieve Monique, mijn dankbaarheid en bewondering voor jou zijn niet in een paar zinnen samen te vatten. Je bent voor mij *het* voorbeeld van hoe een copromotor hoort te zijn en een expert op het gebied van (pijn)onderzoek. Maar bovenal vind ik je een geweldig mens! Een echte 'onderzoeksmoeder' die altijd voor mij en mijn mede-promovendi klaarstaat. Ik wil je bedanken voor de geweldige samenwerking en hoop je nog vaak te zien!

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ABOUT THE AUTHOR

Gerbrich van den Bosch was born in Leeuwarden, the Netherlands, on September the 10th in 1985. She received her Atheneum degree at Regionale Scholengemeenschap Simon Vestdijk in Harlingen in 2003. In this same year she packed her belongings and left the Northern part of the Netherlands to start medical school at Erasmus University in Rotterdam, before turning 18 years old.

She finished the theoretical part of medical school in September 2007. Her graduate research focused on risk factors for paediatric intensive care admission in children with acute asthma (supervisors prof. dr. M. de Hoog and dr. P.J.F.M. Merkus). Before starting with her medical internships, she assisted in preparation of the Dutch 'Kinderformularium'. At the end of 2007 she started her medical internships and completed a final internship at the Paediatric Intensive Care Unit in the Erasmus MC-Sophia Children's Hospital and obtained her medical degree early 2010 (Cum Laude).

After presenting her graduate research project at the Dutch Intensive Care days in 2008, she met prof. dr. D. Tibboel who suggested she could start a research project at his department after finishing medical school. Without any reservation she accepted this offer and started a PhD project in 2010 under supervision of prof. dr. D. Tibboel, dr. M. van Dijk and dr. T. White. In this neuroimaging project she studied the possible long-term effects of neonatal pain and pain treatment in 8-19 year old children and adolescents, as laid down in this thesis.

Gerbrich is currently working as a paediatric resident at the Maasstad Hospital in Rotterdam (head dr. M. Groeneweg) and lives in Rotterdam with the love of her life Dick van Gendt.