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Role of descending serotonergic projections

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Role of Descending Serotonergic Projections

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General Introduction

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Neonatal pain in the neonatal intensive care unit

The World Health Organization (WHO) defines preterm birth as birth before 37 weeks of gestation [2]. Worldwide, roughly 15 million babies are born prematurely every year, accounting to one of every ten live births [3,4]. Preterm birth is the leading cause of death in children younger than 5 years [5]. Due to advances in medicine and technology the mortality rate of preterm births have decreased [6]. However, increasing survival rates of preterm birth presents a public health concern and an economic burden due to high costs of hospitalization [7]. When hospitalized in the neonatal intensive care unit (NICU), (premature) newborn babies are exposed to up to 14 painful procedures per day inherent in the required routine medical care [8-10]. The number of painful procedures only decreases slowly over time [9]. The most frequent procedures performed in neonates are heel lances and venipunctures, followed by endotracheal suctioning in those on artificial ventilation [8,11]. As the majority of NICU-patients are premature, have a more extensive average length of stay in the NICU and an increased risk of preterm complications, preterm neonates are at higher risk for neonatal pain exposure [12,13].

Neonates, even the most premature, are able to experience and respond to pain, as shown by their physiological and behavioral reactions to painful stimuli [14,15]. At the same time, the neonatal nociceptive system is characterized by its plasticity after birth and requires postnatal maturation [1,16,17]. A good understanding of the development of the neonatal nociceptive system is necessary to improve pain assessment, and to treat pain adequately in neonates. Most importantly, in light of possible long-term negative effects of neonatal pain exposure, the goal of the treatment team should be to prevent pain using the concept of pre-emptive analgesia. Within the nociceptive system, known characteristics of this postnatal maturation are: 1) peripheral sprouting and pruning of nociceptive (C) and myelination of non-nociceptive touch (A_β) fibers [16,18,19]; 2) reorganization of central connections of afferent fibers with A_β fibers withdrawing from superficial to deeper laminae of the dorsal horn and C fibers sprouting into the superficial laminae [18,20,21]; 3) change in functionality of interneurons in the spinal dorsal horn due to strengthening of glycinergic inhibition and a GABAergic switch from excitation to inhibition [22,23]; and 4) a switch from facilitation to inhibition of supraspinal input from higher brain centers [24-29]. This makes the system becoming more refined in discriminating between touch and nociception (Figure 1a). Altogether, this maturation results in more fine-tuned nociceptive reflexes and better behavioral discrimination of noxious stimuli with increasing postnatal age [1,16,30-32]. Due to the high degree of neuroplasticity in neonates, processes that intervene with this

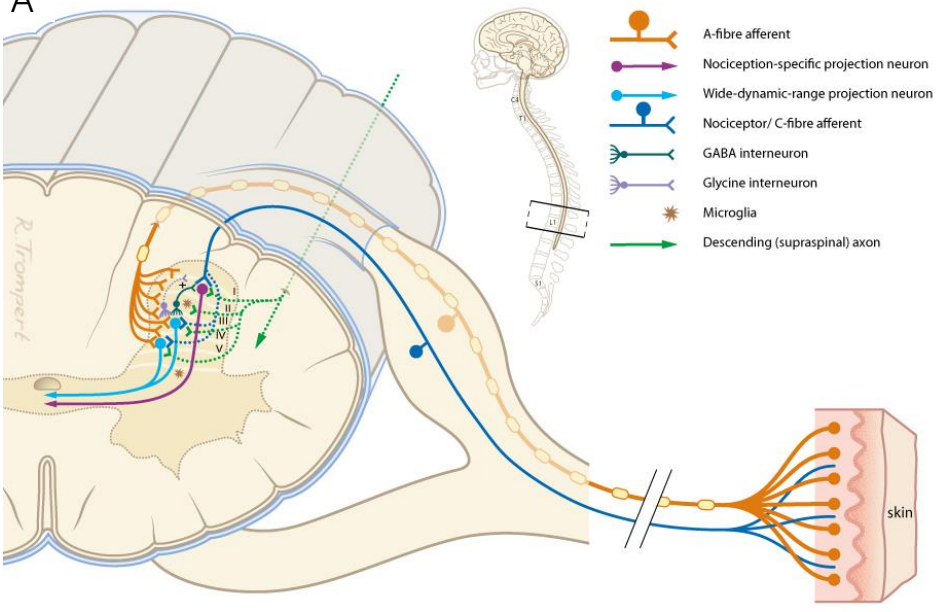
developmental sequence and events like repetitive neonatal needle pricking may not only induce acute but also long-term alterations in brain function and nociceptive behavior [1,11,33].

Clinical observations show that preterm infants display a dampened behavioral response to a painful procedure as compared to term-born infants, and this can be predicted by the number of previous painful procedures [34-36]. In contrast, the physiological responses to a painful procedure are exaggerated in pre-term infants [36,37]. Previous painful procedures are also proportional to reduced brain responses to touch and increased noxious-evoked brain potentials in newborns, suggesting a link between previous painful procedures and acute somatosensory processing [38,39]. In addition, untreated neonatal pain during NICU admittance alters pain sensitivity in later life [40-43]. The degree of change is greater in ex-preterm children and adolescents requiring longer hospitalization or additional neonatal surgery [40,41,43]. This again provides evidence for a relationship between cumulative pain exposure and long-term pain sensitivity. The presence of neonatal scars is associated with local changes in sensitivity, which may increase the risk of persistent pain if repeated surgical interventions are required in the same area. Neonatal painful procedures may also increase the risk of the development of (chronic) pain syndromes in later life [44]. Together, long-term alterations in pain sensitivity suggest changes in the underlying nociceptive system that persist well beyond infancy.

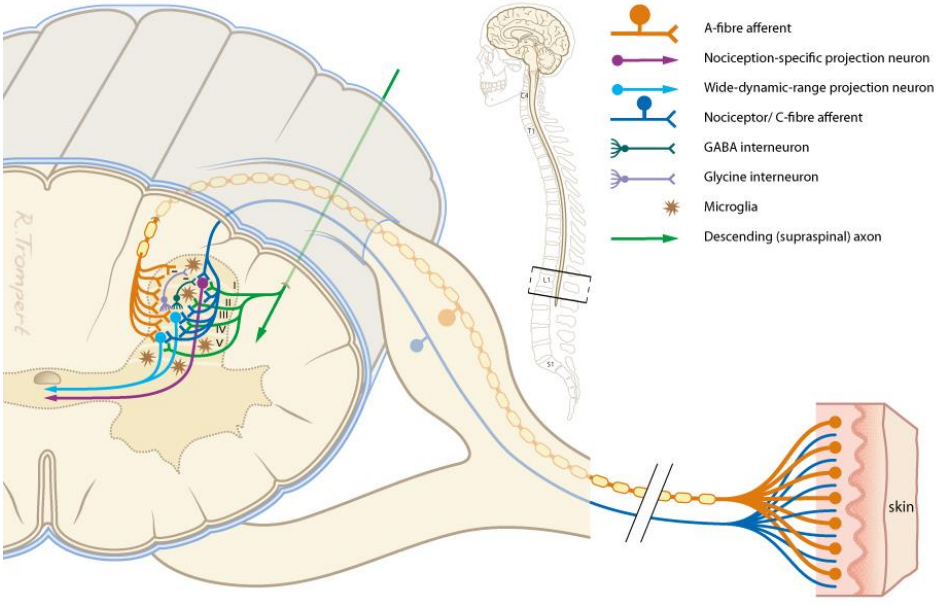
Neonatal pain as a lifespan health concern

Brain development and function in later life are shaped by neonatal pain related stress [11,45]. MRI imaging shows structural brain differences in children born very preterm including reduced subcortical grey matter, white matter volumes and total brain volumes [33,46-48]. Reduced thalamus, amygdala and hippocampal volumes, as well as microstructural alterations in thalamocortical pathways, can also be observed after neonatal pain exposure [41,47,49-51]. These structural differences in brain development as well as decrease in functional connectivity between the thalamus and sensorimotor cortices are associated with higher number of skin-breaking procedures and poorer cognitive and fear-related functioning in ex preterm children [47,49,52,53]. Brain areas involved in pain processing, including the somatosensory cortex and amygdala, also play a role in anxiety [54]. Within the complexity of anxiety behavior both innate (or trait)

A


A-fibre afferent
Nociception-specific projection neuron
Wide-dynamic-range projection neuron
Nociceptor/ C-fibre afferent
GABA interneuron
Glycine interneuron
Microglia
Descending (supraspinal) axon
skin
R. Trompert

B


A-fibre afferent
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Nociceptor/ C-fibre afferent
GABA interneuron
Glycine interneuron
Microglia
Descending (supraspinal) axon
skin
R. Trompert

Figure 1. The developing spinal nociceptive system, as present in (A) the early postnatal period and (B) the adult rat after repetitive neonatal pain. (A) Immediately after birth, nociceptive (C) and myelination of non-nociceptive touch (A_β) fibers undergo peripheral sprouting and pruning. Central connections of afferent fibers undergo reorganization, with A_β fibers (in orange) withdrawing from superficial to deeper laminae of the dorsal horn and C fibers (in blue) sprouting into the superficial laminae. Interneurons in the spinal dorsal horn are functionally inhibitory, due to immature glycinergic inhibition and a GABA-ergic switch from excitation to inhibition. Descending (i.e. serotonergic) input from higher brain centers (in green) will increase diffusely during the early postnatal period, and will switch from facilitation of nociceptive signals to a bimodal pattern of control. The nociceptive system becomes more fine-tuned in discriminating nociceptive stimuli with increasing postnatal age. **(B)** Following neonatal pain, the adult nociceptive system will show several changes at peripheral and central parts of the network. The skin will be hyper-innervated by C and A_β fibers. The C fiber afferent sprouting is increased, visible as strong projections onto projection neurons. Projection neurons, in turn, show increased sensitivity to noxious as well as non-noxious stimuli. Microglia cells are primed due to repetitive neonatal pain, increasing activity in adulthood. The effect of neonatal repetitive pain descending serotonergic projections, known to terminate in the superficial laminae of the dorsal horn, is not studied yet and could be altered in anatomy and function due to repetitive stimulation (this thesis). Adapted from van den Hoogen et al. (2017) with permission [1].

anxiety, an enduring feature of an individual that is consistent across time and context, and situation-evoked or experience-related (state) anxiety are observed [55]. Patients with clinical anxiety tend to possess greater anxious traits in comparison to healthy subjects whereas state anxiety is not related to anxiety disorders [56]. Very preterm born children are more at risk to develop generalized anxiety disorder, emotional and behavioral problems, suggesting a long-term impact of neonatal pain on anxiety behavior [57-59].

Experimental evidence for long-term effects of neonatal pain

In order to better understand the biological basis of neonatal pain and long-term effects of painful procedures on brain development, anxiety and nociceptive behavior, several preclinical models have been developed that mimicking aspects of the NICU experience [11,60]. From a developmental perspective, the rodent central nervous system at birth represents the premature human neonate during the second and third trimester [61-63]. The development of the nociceptive system in rodents and humans is fairly identical and human infants and rodent pups show similar behavioral and physiological responses to acute pain [16,64,65]. Heel lances and venipunctures, resulting in tissue damage or skin breaks, are one of the most frequent painful procedures performed in human neonates [8,13]. In 1999, Anand and colleagues presented a rodent model for repetitive procedural pain where newborn pups received one, two or four noxious needle pricks per day balanced over all hind paws

from day of birth (postnatal day, P 0) to P7 [66]. This needle pricking procedure in neonatal rats resulted in lower thermal thresholds in infant, but not in adult rats [66]. An adaptation of this model, with 4 repetitive needle pricks per day in the same hind paw from P0 to P7, results in robust acute mechanical hypersensitivity and mechanical hypersensitivity after re-injury of the same dermatome in adulthood [67,68]. Further increase of the number of needle pricking from 4 to 10 increased mechanical hypersensitivity during the neonatal period, and decreased mechanical sensitivity during development [69]. Since its introduction in 1999, the rodent model of repetitive neonatal needle pricking is widely recognized as a valid model for neonatal procedural pain [67-79]. Similar to clinical findings, repetitive neonatal needle pricking results in altered somatosensation, cognition and anxiety [11,45,60].

The pathophysiology underlying the long-term effects of neonatal procedural pain consists of a complex interplay between molecular processes across different parts of the nociceptive network, including not only peripheral afferent fibers but also central parts of the network in the spinal cord and the brain (Figure 1B) [1,11]. The perception of pain starts in peripheral nerve endings in the skin. When stimulated due to tissue damage action potentials are generated and the pain signal is transmitted via primary afferents to the spinal cord, an important hub of pain processing. Primary afferent fibers include thinly myelinated A_δ fibers (transmission of fast pain signals), unmyelinated C fibers (transmission of slow pain signals) and thickly myelinated A_β fibers (transmission of touch signals) [80]. Neonatal procedural pain does not alter C-fiber innervation in the skin of the hind paws, suggesting a lack of peripheral plasticity after neonatal procedural pain [68]. However, increased sprouting of nociceptive C-fiber afferents is observed in the spinal cord of adult animals exposed to neonatal procedural pain [68]. This structural plasticity in spinal nociceptive fibers is not accompanied by a change in the withdrawal of non-nociceptive A_β fibers in the spinal dorsal horn [68]. Thus, neonatal procedural pain only selectively affects central sprouting of nociceptive specific, but not non-nociceptive fibers. The increased sprouting of nociceptive C-fibers can lead to enhanced nociceptive signal relay onto second-order neurons, and may explain why changes in nociceptive sensitivity only emerges after re-injury and not at baseline. Wide dynamic range neurons (WDR), located in the deeper laminae (V) of the spinal dorsal horn, are responsible for conveying noxious (C fiber) as well as non-noxious (touch inputs from A_β fiber) signals to higher brain centers [80]. Neonatal repetitive tactile or noxious stimulation has been shown to result in long-term changes as it increases baseline neuronal firing to non-noxious as well as noxious input in adult WDR-neurons [72]. After re-injury to the same dermatome in adulthood WDR responses to non-noxious and noxious stimuli are further enhanced in animals previously exposed to neonatal repetitive tactile or noxious stimulation [72]. Increased structural

plasticity of nociceptive fibers as well as increased sensitivity of dorsal horn neurons in adulthood suggest a hypersensitive spinal nociceptive circuit, amplifying nociceptive signaling in later life following neonatal procedural pain. These changes in the spinal nociceptive network can contribute to the long-term effects of neonatal procedural pain.

Supraspinal brain areas that are critical for the processing of pain receive input from second-order neurons in the spinal dorsal horn and changes in these areas may also contribute to the long-term effects of neonatal procedural pain. Repetitive exposure to pain during the first postnatal week acutely increased apoptosis in frontal, parietal and cingulate cortex of neonatal rats [81]. In adulthood structural brain imaging showed that repetitive needle pricking did not result in differences in regional brain volumes in adulthood in areas involved in central pain processing, including the amygdala, prefrontal cortex, cingulate cortex [82]. Similarly, somatosensory activation at baseline is similar between animals previously exposed to neonatal tactile or painful stimulation [66]. At the same time, a decrease in the number of activated somatosensory neurons in adult animals exposed to neonatal pain is observed after acute painful stimulation [66]. This emphasizes that changes in the supraspinal brain areas may emerge only after a second painful event. Finally, after supraspinal integration of the nociceptive signal and awareness of pain descending projections from the brainstem areas project back to the spinal dorsal horn, thereby creating a feedback loop. The effect of neonatal procedural pain on these descending projections and the descending modulation of the spinal nociceptive network is still largely unexplored.

The descending serotonergic system and modulation of the spinal nociceptive network

A network of descending pathways projecting from brain structures to the spinal dorsal horn play a complex and crucial role in the modulation of nociceptive signaling, mediated via noradrenergic, opioidergic and serotonergic neurotransmission [83]. Serotonin (5-HT, 5-hydroxytryptamine) is an important regulator of pain, cognition and anxiety as well as neuronal network formations [84], areas that are affected all by neonatal procedural pain. The predominant source of serotonergic input to the spinal dorsal horn originates from the rostral ventromedial medulla (RVM) and enters the spinal cord via the dorsolateral funiculus [83]. The RVM is a key structure maintaining the balance between excitation and inhibition of the spinal nociceptive network and includes the nucleus Raphe Magnus and adjacent Reticular Formation [85]. The effects of serotonin are mediated via 15 receptors subtypes

grouped into seven receptor families (5-HT1 to 5-HT7), all expressed in the spinal cord [86]. Although anatomical projections responsible for descending modulation are already present at birth, descending inhibitory control of the spinal nociceptive network is functionally immature during the first postnatal weeks [24-27,29,87]. The descending serotonergic system switches from facilitation of nociceptive processing in the first three postnatal weeks, to predominant inhibition in adulthood [28]. This postnatal shift in descending serotonergic modulation creates a window of high neuroplasticity of the serotonergic descending RVM-spinal cord projections during the neonatal and adolescent phase in rats, which may be vulnerable to neonatal procedural pain exposure. So far no studies have investigated the effects of repetitive neonatal procedural pain on alterations in descending serotonergic projections to the spinal dorsal horn nociceptive network such as synapse formations, 5-HT sprouting and receptor modulation.

Current treatment strategies for neonatal pain

The use of pre-emptive analgesia during the neonatal phase is thought to play an important role in the prevention of both acute and long-term negative effects on pain. One has to bear in mind that major differences exist between tissue damage due to major surgical procedures such as intestinal surgery in children with for instance necrotizing enterocolitis and procedural pain such as heel lances. Consequently, the choice of analgesic therapy recommended for major surgery in neonates may be different than those recommended for procedural pain. The presently often used World Health Organization (WHO) three-step ladder for analgesic therapy [88] is originally developed to treat cancer pain in adults, but very often used in children too. In newborns, paracetamol (or acetaminophen) is often used as first step. Paracetamol is the most widely used drug for mild to moderate pain after surgery, has morphine-sparing effects as shown in term born infants following major non-cardiac surgery, but provides poor analgesia for procedural pain in neonates [89-92]. The mechanism of action is complex and includes effect of both peripheral (cyclooxygenase (COX) inhibitors) and central anti-nociceptive processes (descending serotonergic system, l-arginine/nitric oxide pathways and cannabinoid system; Table 1) [93]. For infants older than 3 months of age a non-steroidal-anti-inflammatory drug (NSAID) such as ibuprofen can be added. In preterm newborns, NSAIDs are used with caution due to limited data on side effects, pharmacokinetics and -dynamics [94]. NSAIDs reduce prostaglandin through COX inhibition. Next and second step in the WHO-ladder is the weak opioid tramadol, that is exceptionally used following major surgery in neonates and is only registered for children from 1 year of age onwards [95]. Both opioid-mediated as well as serotonin-reuptake and releasing actions are defined for the mode of action of

tramadol [96]. The last and third step at the WHO ladder is use of stronger opioids such as fentanyl or morphine. Both opioids are used in the Neonatal Intensive Care Unit for more extensive interventions [97], but at the same time may lead to acute side effects including respiratory distress [98]. The recent POPPI (Procedural Pain in Premature Infants) trial assessing the analgesic efficacy and safety of oral morphine for procedural pain in non-ventilated premature infants was stopped at an early stage due to observed harmful respiratory side effects [99,100]. This has also raised concerns related to the balance between analgesic efficacy and risk for harm in non-ventilated neonates treated with morphine during growth and development of the child [98,101-103]. While opioids and paracetamol are widely studied in the context of mild to moderate (post-operative) pain their effectiveness for procedural pain remain disputable [89,99] and many clinics around the world start with non-pharmacological approaches such as handling and/or oral sucrose. Thus, pain management for procedural pain in newborns, whether term or preterm born, remains a subject of improvement. Serotonin plays a role in the mechanism of action of paracetamol and tramadol but serotonin-mediated analgesia is often overlooked in analgesic strategies for neonates. As descending serotonergic projections to spinal dorsal horn play a pivotal role in modulation of the nociceptive signaling, anxiety and neuronal network formations, drugs acting on serotonergic receptors may be important candidates for the management of neonatal pain.

Table 1 Overview of developmental stage and the area and mechanism of action of different analgesic compounds prescribed at neonatal age

Analgesic	Area of Action	Mechanism of action	Stage of development in newborn
Opioids ((remi)fentanyl, morphine, methadone*)	Periphery Spinal cord Primary afferents (presynaptic opioid receptor) Pain transmission neurons (postsynaptic opioid receptor) Brainstem PAG RVM	Inhibition of neuro-transmitter release in the spinal gate; Inhibition of nociceptive transmission via activation of opioid descending pathways	Opioid system is facilitatory instead of inhibitory
Paracetamol	Periphery Spinal cord Pain transmission neurons Interneurons Brainstem RVM	Prevention of peripheral sensitization by inhibition of cyclo-oxygenase (COX) Central actions includes serotonergic descending pathways amongst others	Serotonergic system is facilitatory instead of inhibitory

Adapted from van den Hoogen, de Kort et al. (2019) with permission. Abbreviations: RVM, rostral ventromedial medulla; PAG, periaqueductal gray. The analgesic Methadone* functions both via an opioid and NMDA related mechanism.

Aim and research questions of the thesis

This thesis aims to investigate the acute and long-term effects of repetitive neonatal procedural pain on anxiety and pain as related to the descending serotonergic projections and their role in modulation of the spinal nociceptive network. Next it aims to modulate these serotonergic projections via pharmacological targeting of selected serotonergic receptors.

Research questions (RQs)

Based on the overall aim of this thesis, the following research questions are formulated:

1. *What are the long-term effects of repetitive neonatal procedural pain on adult trait and state anxiety?*
2. *What do we know of the physiological development of the descending serotonergic rostral ventromedial medulla-spinal cord projections and its modulatory role on the spinal nociceptive network?*
3. *Does repetitive neonatal procedural pain result in anatomical changes in descending serotonergic projections from the rostral ventromedial medulla to the spinal dorsal horn in adulthood?*
4. *Does serotonin-mediated analgesia prevent the acute and long-term effects of repetitive neonatal procedural pain on mechanical sensitivity and anxiety?*

Outline of the thesis

Following the general introduction (this chapter), the long-term effect of repetitive neonatal procedural pain on adult trait and state anxiety in rodents is described in **Chapter 2** (see RQ 1).

Chapter 3 gives an overview of the current literature on the development of descending serotonergic modulation of the spinal nociceptive network during the neonatal, pre-weaning and adult phase (see RQ 2). This review points to a rapid postnatal development of this system and its receptors, and suggests potential new therapeutic targets to treat neonatal procedural pain. From the overview provided in Chapter 3, the 5-HT_{1a} and the 5-HT_3 receptors are selected as they play an important role in the inhibition and facilitation of the neonatal spinal nociceptive network respectively.

To assess the anatomical plasticity of the descending serotonergic projections (see RQ 3), **Chapter 4** describes the effect of repetitive neonatal procedural pain on the serotonin staining intensity in the rostral ventral medulla as well as the spinal dorsal horn in adulthood.

In order to address RQ 4, a pharmacological study is described in **Chapter 5** using serotonin-mediated analgesia to prevent the acute and long-term effects of repetitive

neonatal procedural pain on mechanical sensitivity. Buspirone selectively activates the 5-HT_{1a} receptor (agonist) to produce inhibition, whereas ondansetron selectively blocks the 5-HT_3 receptor (antagonist) to prevent facilitation. The effects of these pharmacological interventions are studied after repetitive neonatal procedural pain and in context of the acute, long-term and post-operative effects on pain (mechanical hyper-sensitivity) as well as anxiety.

In the final chapter, **Chapter 6**, the key findings of this thesis are discussed along with overall strengths, limitations and suggestions for future research.

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Neonatal procedural pain affects state, but not trait anxiety behavior in adult rats

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Abstract

Introduction: The influence of neonatal experiences upon later life affective behavior is increasingly recognized, but the reported effects on anxiety are often contradictory. The observed effect may depend upon the type of anxiety (state or trait) affected. The current study aims to investigate whether neonatal repetitive needle pricking alters anxiety behavior in adulthood, by assessing both state and trait anxiety in rats.

Methods: Sprague-Dawley rat pups received four unilateral needle pricks per day, while controls received four tactile stimuli or were left completely undisturbed during the first postnatal week. Mechanical sensitivity was assessed in the neonatal phase and throughout development. State anxiety was assessed in the open field test and trait anxiety in the elevated zero maze.

Results: Repetitive needle pricking leads to acute mechanical hypersensitivity, but does not affect baseline mechanical sensitivity throughout development. In adulthood, animals previously exposed to neonatal procedural pain (including repetitive handling and removal from litter) showed lower state anxiety but did not differ in trait anxiety, as compared to undisturbed controls.

Conclusion: These findings indicate that early-life procedural pain decreases state but not trait anxiety behavior in later life in a rodent model of repetitive needle pricking.

Keywords

Neonate; Procedural pain; Needle prick; Anxiety; Long-term consequences

Introduction

Painful and invasive procedures are common for neonates hospitalized in the neonatal intensive care unit (NICU), with up to 14 painful procedures experienced each day grunau[1]. Early exposure to painful procedures has been shown to contribute to long-term negative consequences including alterations in somatosensation, anxiety, and depression [2-6]. Indeed, early exposure to painful procedures affects neurodevelopment, leading to a reduction in amygdala and thalamus volumes, which consequently can impair affective functioning [2,7-10]. Interestingly, survivors of neonatal repetitive pain show higher rates of internalizing behaviors, and higher anxiety and pain catastrophizing in childhood, infancy and early adulthood [2,5,11-13]. Although clinical evidence shows that affective behavior in infants and children is altered by early-life pain exposure, clinical data on the impact of early life pain on anxiety in adulthood remains limited.

Preclinical evidence suggests that anxiety in adolescence and adulthood is affected by neonatal procedural pain, but findings are heterogeneous [14-18]. In more detail, neonatal repetitive procedural pain has been shown to increase [18,19], decrease [14-17] or fail to show an effect on adult anxiety behavior in rodents [14,20,21]. In both humans and laboratory animals, anxiety is not a unitary phenomenon and includes both innate (trait) and situation-evoked (state) anxiety. Trait anxiety is an enduring feature that is consistent across time and context, whereas state anxiety is anxiety experienced at one particular time and can be contextual and conditioned [22]. Behavioral paradigms designed to measure trait and state anxiety have been developed in rodents. The open field test (OFT) represents normal or trait anxiety, as rodent behavior in this test reflects a natural balance between exploratory and escaping drives [23,24]. On the other hand, the elevated zero maze (EZM) models situation evoked (state) anxiety that is contextual to the anxiogenic stimuli present (including openessness and elevation). Previous studies on the long-term effect of neonatal pain used the term 'anxiety' without an a priori assumption of whether or not trait or state anxiety was represented. The type of behavioural essay used, and thus the type of anxiety (trait vs state) measured, might explain some of the contradictory findings as most often state but not trait anxiety seems affected by neonatal procedural pain. The aim of the present study is to investigate the long-term effects of repetitive neonatal procedural pain on trait and state anxiety behavior in adulthood, in a well-established animal model of repetitive needle pricks [25,26]. Trait anxiety is measured in the open field test (OFT), whereas state anxiety is measured in the elevated zero maze (EZM).

Methods

Animals

Sprague-Dawley males and females were purchased from Charles River Laboratory and were mated at Maastricht University to produce eight litters. Dams were transferred to the experimental room at gestational day (G) 16, and all pups were born on G21. Litters were culled to a maximum of N=10 to ensure equal caretaking by the dam. Each experimental litter included a balanced representation of neonatal condition, and equal distribution by sex per group (Table 1). A maximum of one or two male and/or female pups were taken from each litter for each condition, to control for any litter effects [27]. Pups were weaned at postnatal day (P)21 and randomly housed in same-sex groups of two or three in individually ventilated cages, in temperature (19-24 °C) and humidity (55 ± 15%) controlled rooms with a reversed 12:12-hr day-night cycle (lights on 7 p.m. – 7 a.m.). *Ad libitum* water and food was available for the duration of the study.

All experiments were performed in accordance with the European Directive for the Protection of Vertebrate Animal Use for Experimental and Other Scientific Purposes (2010/63/EU) and were approved by the Committee for Experiments on Animals, Maastricht, The Netherlands (DEC 2017-017).

Table 1. Distribution of sex and condition in experimental litters

	NP		**TC**		**UC**	
Litter	*f*	*m*	*f*	*m*	*f*	*m*
1	1	2	2	1		
2	2	1	1	2		
3					2	2
4	2	2	0	1		
5					2	2
6	2	2	2	2		
7	1	2	2	1		
8					3	5

Abbreviations: f, female; m, male; NP, needle prick; TC, tactile control; UC, undisturbed control

Neonatal procedures and mechanical sensitivity

A neonatal repetitive needle prick model was used as previously described [25,26]. All pups were randomly assigned to neonatal conditions at birth (using a computer-generated randomization list). Pups received either noxious needle pricks (Needle Prick (NP); *n=9* males, *n=8* females) or tactile stimulations (Tactile Control (TC); *n=7* males, *n=7* females) in the midplantar surface of the left hind-paw four times a day (at 08:00, 09:00, 10:00 and 11:00 a.m.), from day of birth (postnatal day (P0) to P7. For each procedure, the nest was briefly separated from the dam. Paw withdrawal thresholds (PWT) of the ipsi- and contralateral hind-paws were assessed before (baseline, BL) and 1, 3, and 5 hours after the last noxious or tactile stimulation using a dorsal von Frey design [28]. Ascending Von Frey filaments (bending force 0.407, 0.692, 1.202, 2.041, 3.63 (from P4 onwards) and 5.495 (from P6 onwards)) were applied 5 times to the dorsal surface of the hind-paws. The number of positive responses (withdrawal or flinching behavior evoked by the filaments) were recorded, and behavioral testing was discontinued when five positive responses were observed. A 50% PWT was calculated using sigmoidal curve fitting in GraphPad Prism 8 (GraphPad Software, San Diego, USA). Separate nests were left undisturbed during the first postnatal week (Undisturbed Control (UC); *n*=8 males, *n*=8 females).

Assessment of mechanical sensitivity throughout development

Mechanical sensitivity of the hind-paws was assessed weekly from weaning to adulthood (3-8 weeks of age; P21-P56). Animals were placed in individual Plexiglas cages on an elevated mesh floor and were allowed to acclimatize to the behavioral set-up before testing. Von Frey filaments with logarithmically increasing stiffness (bending forces 1.202, 2.041, 3.63, 5.495, 8.511, 15.136 and 28.84g; Stoelting, USA) were applied to the mid-plantar surface of the hind paws for 5 seconds. Mechanical sensitivity was assessed using the up-down method, and the mechanical force resulting in a 50% withdrawal frequency was assigned as the PWT [29].

Adult anxiety behavior testing

Anxiety-like behavior was assessed in adulthood (8 weeks of age) under dim light conditions (2 lux, 0.02 W/m^3 infrared light). Animals were allowed to acclimatize to the experimental room before testing. To control for possible carry-over effects from the open field test (OFT) to the elevated zero maze (EZM), the animals were allowed a recovery period of three days. The animal order of testing was randomized, and researchers were blinded to conditions during testing.

Trait anxiety and Open Field Test (OFT)

To assess trait anxiety, animals were introduced into the center of an open arena (100x100x40cm) and allowed to freely explore for 5 minutes. In-between each session, the arena was thoroughly cleaned with 70% ethanol. Behavior of the rats was automatically tracked and analyzed using a video tracking system (Ethovision Pro, Noldus, the Netherlands). While in the arena, time spend in the center (%), center crossings (number of entries from and to the center) and total distance travelled (locomotor path in whole arena in cm) were recorded and analyzed using Ethovision XT10 (Noldus).

State anxiety and Elevated Zero Maze

Adult animals were exposed to the elevated zero maze (EZM) [30] to assess state anxiety. The EZM is an elevated black annular arena (100 cm diameter, 10 cm path width and 70 cm above floor level) divided into four equal quadrants. Two opposite quadrants are "closed" by black Perspex walls (closed arms), while the remaining two quadrants are "open" (open arms). Animals were placed in the middle of an open arm facing one of the closed arms and allowed to explore the maze for 5 minutes. Between each session, the arena was thoroughly cleaned with 70% ethanol. Movement of the rats was recorded and scored using an infrared video camera connected to a video tracking system (Ethovision Pro, Noldus, The Netherlands). Behavioral measures recorded included: time spend in open and closed arms, latency to enter each area, and open arm entries. Anxiety-like behavior was classified as the time in the open arms as a percentage of total (corrected) trial time (= total trial duration – latency to enter first closed arm). In addition, head dips (paws in closed arm, head stretched forward into open arm) were hand scored by an experimenter, blinded to the animal's condition.

Statistical analysis

All data is presented as mean ± standard error of the mean (SEM). For the neonatal period, an area under the curve (AUC) analysis was performed. The AUC was calculated based on the PWTs for each group over the whole neonatal period (P0-7) and TC and NP animals were compared with an unpaired t-test. Differences in mechanical sensitivity between TC, NP and UC throughout development were analyzed using a repeated measures analysis of variance (ANOVA) with Tukey Post-Hoc correction (to control for multiple testing). As litters can affect anxiety-like behavioral measures, litter was used as unit of analysis, averaging data from pups of the same condition per sex for each litter [27,31]. An additional analysis was performed using pups as unit of analysis (see supplemental data). A two-way ANOVA was performed to compare the effects of condition and sex on anxiety-like behavioral

measurements. If sex effects were not observed, data was pooled by condition to increase power. All statistical analysis were conducted using Graphpad Prism 8.0 (GraphPad Software, San Diego, USA) and results were considered significant at $p < 0.05$.

Results

Mechanical sensitivity throughout development

NP animals showed a significantly decreased AUC for ipsilateral PWTs during the entire neonatal period compared to TC animals, indicating the development of acute mechanical hypersensitivity in pups who received repetitive needle pricks ($t(29) = 8.038$; $p<0.001$; figure 1a). During development from weaning (P21) to adulthood (P56), ipsilateral PWT significantly increased over time ($F(5, 220) = 52.66$; $P<0.001$), but did not differ between neonatal conditions ($F(2, 44) = 0.254$; $p=0.777$); figure 1b). Contralateral PWTs significantly increased over time ($F(5, 220) = 52.58$; $p<0.01$), but were not significantly different between conditions at any time point during the neonatal phase ($t(29) = 0.8311$; $p=0.4127$) or development ($F(2, 44) = 0.2538$; $p=0.777$, figure 2). No effect of sex on mechanical sensitivity was observed ($p>0.05$).

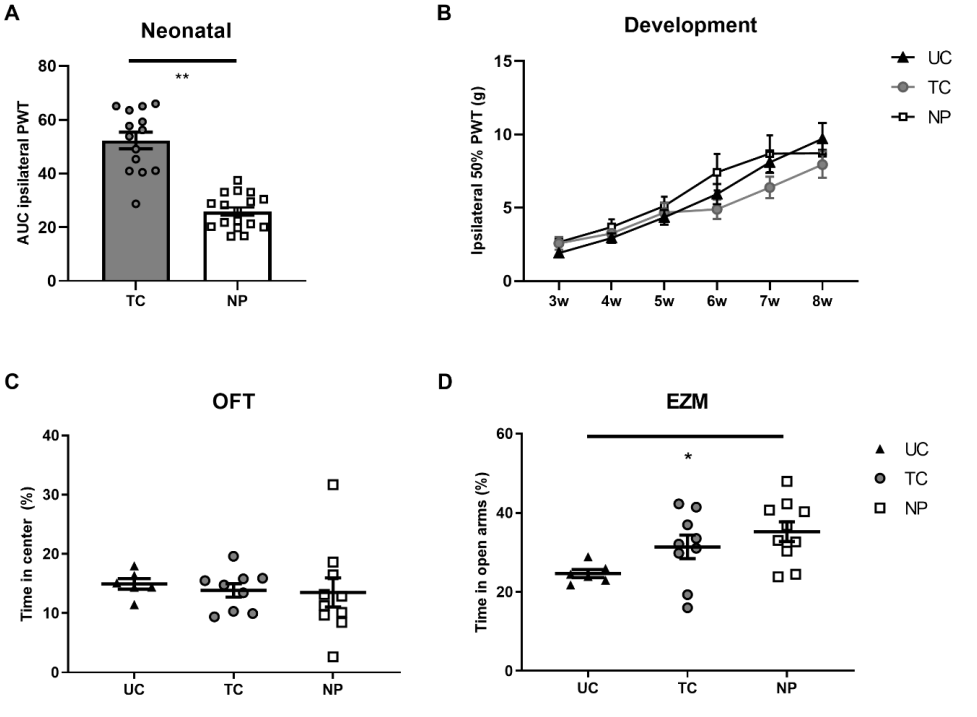

Figure 1. Mechanical sensitivity (a, b) and anxiety behavior (c, d) after repetitive neonatal needle pricking. A. Repetitive neonatal needle pricks (NP; n=17) result in a decreased paw-withdrawal threshold (PWT) in the ipsilateral paw as compared to repetitive tactile stimulation (TC; n=14), shown by a lower area under the curve (AUC) over the whole neonatal period for NP animals compared to TC animals (t (29) = 8.038; p<0.001). **B.** Ipsilateral PWTs increase over time for all groups (F (2, 220) = 52.66; p<0.01), did not significantly differ between NP, TC and undisturbed control (UC; n=16) animals (F (2, 44) = 0.254; p=0.777). **C.** The percentage of time spend in the anxio-genic (center) region of the open field test (OFT) in 8-week-old animals did not significantly differ between neonatal conditions (F (2, 19) = 0.1206; p=0.8871) or sex (F (1, 19) = 0.2284; p=0.6382). **D.** The percentage of time spend in the anxio-genic (open arms) region of the elevated zero maze (EZM) differs significantly between neonatal conditions (F (2, 19) = 3.966; p=0.0364) but not sex (F (1, 19) = 0.0001; p=0.9997). NP animals spend more time in the open arms of the elevated zero maze as compared to the UC animals (NP 35.21 ± 2.47; UD 24.63 ± 1.02; P=0.0286). Data are presented as mean ± SEM, *P <0.05 ** P<0.01.

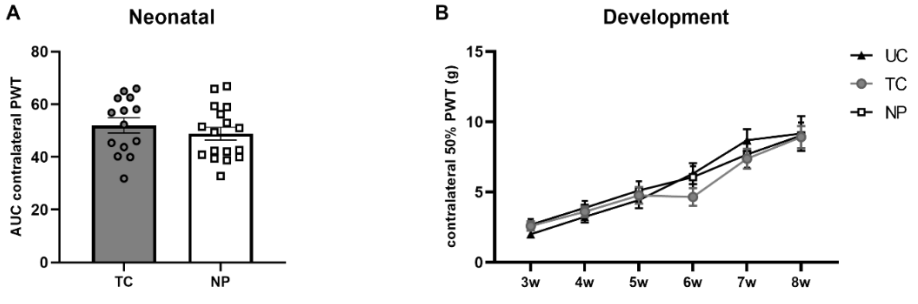

Figure 2. Contralateral mechanical sensitivity during the neonatal week and throughout development. A. Area under the curve analysis (AUC) over the whole neonatal period does not show significant differences in contralateral paw-withdrawal thresholds (PWT) between repetitive needle pricks (NP; n=17) and tactile control animals (TC; n=14) (t (29) = 0.831; p=0.4127). **B.** Contralateral PWTs increase over time for all groups (F (5, 220) = 52.58; P<0.01) but was not significantly different between conditions at any time point (F (2, 44) = 0.2538; p=0.777). Data presented as mean ± SEM, *P <0.05 ** P<0.01

Adult anxiety behavior

Trait anxiety as assessed in the OFT in adulthood showed no significant effect of condition ($F(2, 19) = 0.3218$; $p=0.7287$; figure 1c) or sex ($F(1, 19) = 0.2284$; $p=0.6382$; figure 1c) on percentage of time spend in the center of the arena. The frequency of center crossings in the OFT showed a significant interaction effect for sex*condition ($F(2, 19) = 4.194$; $p=0.0310$; figure 3), with females showing a trend to more center crossings in the TC group only ($p=0.06$). Locomotor activity was affected by sex ($F(1, 19) =6.701$; $P=0.0180$) but not condition ($F(2, 19) = 0.8271$; $p=0.4625$, with females showing higher locomotor activity as compared to males (Figure 3).

State anxiety as assessed in EZM demonstrates that the percentage of time spend in the open arms significantly differed between groups ($F(2, 19)=3.966$; $P=0.0364$; figure 1d) but was unaffected by sex ($F(1,19) = 0.0001$; $p=0.9997$). When pooling data by condition, the significant difference between neonatal conditions in the time in open arms of the EZM persisted ($F(2, 22) = 3.777$; $p=0.0389$). Post-hoc analysis revealed that 8-week-old NP animals spend significantly more time in the open arms of the EZM, compared to UC (NP 35.21 ± 2.47; UC 24.63 ± 1.02; $P=0.0303$; figure 1d). Neonatal condition did not influence exploratory behavior (head dips and open arm entries) in the EZM ($p>0.05$). However, females showed significantly more exploratory behavior, measured by the number of head dips ($F(1, 19) = 8.597$; $p<0.01$) and open arm entries ($F(1, 19) = 4.683$; $p=0.0434$; Figure 3). Using pups as unit of analysis (i.e. not averaging data of pups from the same condition per sex for each litter) did not influence behavioral outcome in the OFT and EZM (see Figure S1).

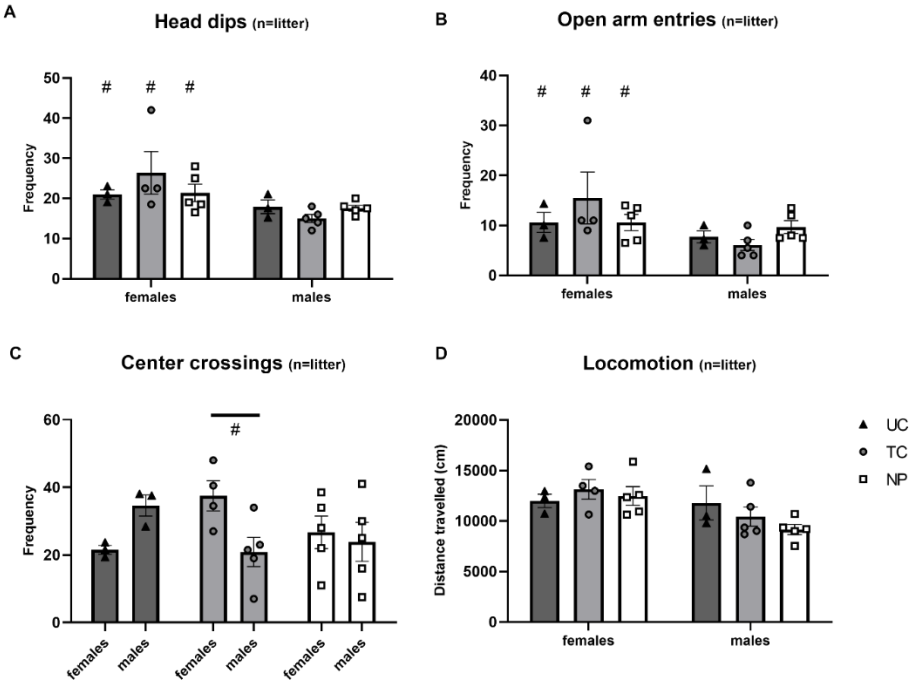

Figure 3. Exploratory behavior (head dips, open arm entries and center crossings) and locomotor behavior of adult rats exposed to needle pricks, tactile stimulation or left undisturbed as neonates. Females show significantly more exploratory behavior as compared to males, measured by the frequency of head dips (**A;** F (1, 19) = 8.597; p<0.01) and open arm entries (**B;** F (1, 19) = 4.683; p=0.0434). **C.** A significant interaction effect is observed in the frequency of center crossings (F (2,19) = 4,194; p=0.0310), with females tending to cross the center more frequently as compared to males in the TC group only (p=0.06). **D**. Locomotor activity in the open field test (OFT) was affected by sex (F (1, 19) = 6.701; p=0.0180) but not condition (F (2, 19) = 0.8271; p=0.4525), with females show significantly higher total distance travelled as compared to males. NP; needle prick animals, TC; tactile controls, UC; Undisturbed controls, Data presented as mean ± SEM, *P <0.05 ** P<0.01, # sign. effect of males vs. females.

Discussion

This study investigates the long-term effect of neonatal procedural pain on adult state and trait anxiety behavior in a well-established animal model of neonatal repetitive needle pricking. Our data show that a combination of repetitive neonatal procedural pain and handling decreases state, but not trait anxiety behavior in adult rats. At the same time, neonatal needle pricking results in acute mechanical hypersensitivity but does not produce long-term changes in baseline mechanical sensitivity.

Consistent with previous studies, limited numbers of needle pricks from P0 to P7 leads to robust acute hypersensitivity without altering mechanical sensitivity throughout development [21,25,26,32,33]. However, more widespread (all paws), prolonged (14 vs 7 days), or more intense (paw incision or 10 needle pricks per day) tissue breaking procedures lead to altered baseline sensitivity [16,34,35].

Whether neonatal procedural pain ("first-hit") affects anxiety in later life has been topic of debate. Previously, neonatal repetitive procedural pain has been shown to increase [18,19], decrease [14-17] or fail to show an effect on adult anxiety behavior in rodents [14,20,21]. The effect of neonatal repetitive procedural pain on anxiety seems to be dependent on the behavioral essay used, pointing at a distinct profile of anxiety (trait or state) affected. Rodent anxiety behavior in the OFT reflects the natural balance between exploratory and escaping drives, and therefore more likely represents inherent or trait anxiety [23,24]. Our study shows that 4 needle pricks a day between P0-P7 as a model of procedural neonatal pain does not influence trait anxiety, including time in the center of the arena and number of center crossings in the OFT, consistent with previous studies [20,21]. The open-field entries from PVC tubes, or the time in the center of a circular arena was also unaffected by neonatal procedural pain [14,36], suggesting that neonatal procedural pain does not influence trait anxiety in adulthood. Reduced exploratory behavior might confound measures of anxiety, as they depend on general locomotor activity (i.e. movement) [22]. However, locomotor activity was unaffected by neonatal condition in our study and did therefore not influence overall exploratory behavior and thus state anxiety in the OFT.

In contrast to the OFT, the EZM confronts the animal with an anxiety-provoking situation such as height and avoidance of open spaces, modelling state-induced anxiety [37]. Adult animals exposed to a combination of repetitive neonatal needle pricking and handling showed lower state anxiety levels, as they spend more time in the open arms of the EZM as compared to their undisturbed controls in this study. Previous behavioral

testing paradigms and handling can influence behavior in the EZM [38]. As all animals were exposed to similar testing protocols and handling, this effect should be similar across all groups. Previous studies using the same neonatal procedural pain model have also reported decreased state anxiety in the elevated plus maze (EPM) as compared to touched controls or irregularly stimulated animals [15,17]. One study that increased the number of needle pricks per day did not show a long-term effect on anxiety in the EPM, compared to touched or handled controls [21]. In addition, repetitive needle pricking led to an increase in anxiety behavior when tested during adolescence [18]. Therefore, inconsistencies in the manipulation procedure (4 vs 10 NP daily), the developmental stage during testing (adolescent vs adult), control groups used (touch, handling or undisturbed) as well as differences in behavioral essay used, might account for the different outcomes (Table 2). Neonatal procedural pain has also been shown to reduce later-life fear conditioning [19], suggesting that our findings are most consistent with a reduction in state anxiety in adult rodents.

The lack of concordance between tests for trait and state anxiety has previously been shown, where animals presenting high levels of anxiety in one test did not necessarily present the same levels in another test [37]. The combination of neonatal repetitive pain with a second "hit" by adding the additional factors of height and unprotected areas in the EZM, may unmask the lower anxiety levels in NP animals as compared to undisturbed controls (UC) in this test. It is important to highlight that although the combination of repetitive neonatal procedural pain and handling reduces state anxiety as compared to undisturbed animals in our study, no differences with tactile control animals are observed. This suggests that the effect is driven by handling and/or removal as well as pain rather than pain alone, and the combination results in differential state anxiety levels. Daily postnatal handling during the first two postnatal weeks also reduced anxiety-behavior in adulthood as compared to UC [39]. In addition, differences in rearing conditions between UC and NP animals may also play a role [40]. In preclinical studies, a second "hit" is often necessary for the long-term effects of neonatal pain to emerge [6]. The impact of a subsequent injury in adulthood on anxiety level of rodents previously exposed to neonatal pain has not been evaluated yet. Contrary to preclinical findings showing lower state anxiety after repetitive needle pricking, the incidence of anxiety disorder is higher in children and adolescents born premature [2,11,13,41]. Patients with clinical anxiety tend to possess greater anxious traits in comparison to healthy subjects, whereas state anxiety is not related to anxiety disorders [42]. Hence, the effects of neonatal procedural pain may therefore not always emerge in clinical context. NICU admittance alone is already stressful, but the added neonatal pain can strengthen these effects. Overall, NICU infants appear to be surprisingly robust and

adaptable; effects of neonatal pain exposure on later life behavioral adversity are subtle rather than extreme, although the effects of pain remain difficult to discriminate from anxiety or fear in a clinical setting. In this context, follow-up studies should elucidate whether differences in trait and state anxiety exist in ex-preterm patients in later-life. In conclusion, our study shows that, in a rodent model of repetitive needle pricking, early-life procedural pain (including repetitive handling and removal) decreases state but not trait anxiety behavior in later life. On top of adequate analgesia, treatment of neonatal procedural pain should therefore take these adverse effects into account.

Table 2. Summary of literature on the effect of neonatal procedural pain on adult anxiety

Model	Controls	Species	Time of modulation	Time of testing	Behavioral test	Outcome	References
RNP (4 NPs in left hind paw)	*TC, UC*	*Rats*	*P0-P7*	*Adult (P56)*	*OFT, EZM*	*↗ time in open arms* *No effect on open arm entries, head dips, time in center and center crossings*	*This study*
RNP (4 NPs balanced over all paws)	TC, UC	Rats	P0-P7	Adult (P100)	OFT, EPM	No effect on time in center or center entries ↑ time in open arms	Page 2005
RNP (10 NPs balanced over all paws)	TC, handling	Mice	P1-P6	Adult (P60)	OFT, EPM	No effect on time spend in center or time in open arms	Ranger 2014
RNP (4 NPs in left hind paw)	TC, UC	Rats	P1-P7	P24-P25	EPM	↑ time in open arms No effect on (open) arm entries	Zuke 2019
RNP (2 NPs on front and hind paw)	Sham	Mice	P8-P14	P30	EPM	↓ open arm entries* ↓ time in open arms* ↓ head dips* ↑ Stretch attend postures*	Schellinck 2003
RNP (4 NPs balanced over all paws)	TC	Rats	P0-P7	P24, P87	OFT	↑ distance in center at P24 and P86 ↑ center entries at P24 ↑ time in center at P24 and P87 ↑ total entries at P87	Chen 2016
RNP (8 NPs on P1, 6 NPs on P2, 4 NPs on P3, 2 pokes on P4, balanced over all paws)	TC, TC & isolation, UC	Rats	P1-P4	Adult (P80)	OFT	No effect on distance travelled, time in center or center entries	Mooney-Leber 2020
RNP (4 NPs in left hind paw)	TC, UC	Rats	P1-P7	P24, P45 and P66	Fear conditioning	↓ Auditory fear conditioning	Davis 2018
RNP (4 NPs, balanced over all paws)	TC	Rats	P0-P7	Adult (P65)	DWT	↑ latency to exit ↑ time in PVC tube No effect on open field entries	Anand 1999

† NP animals vs control animals. Abbreviations: DWT, defensive withdrawal testing; EPM, elevated plus maze; EZM, elevated zero maze; NPs, needle pricks; OFT, open field test; P, postnatal day; RNP, repetitive needle pricking; SD, Sprague-Dawley; TC, tactile and handled control; UC, undisturbed or unhandled controls

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Supplemental data

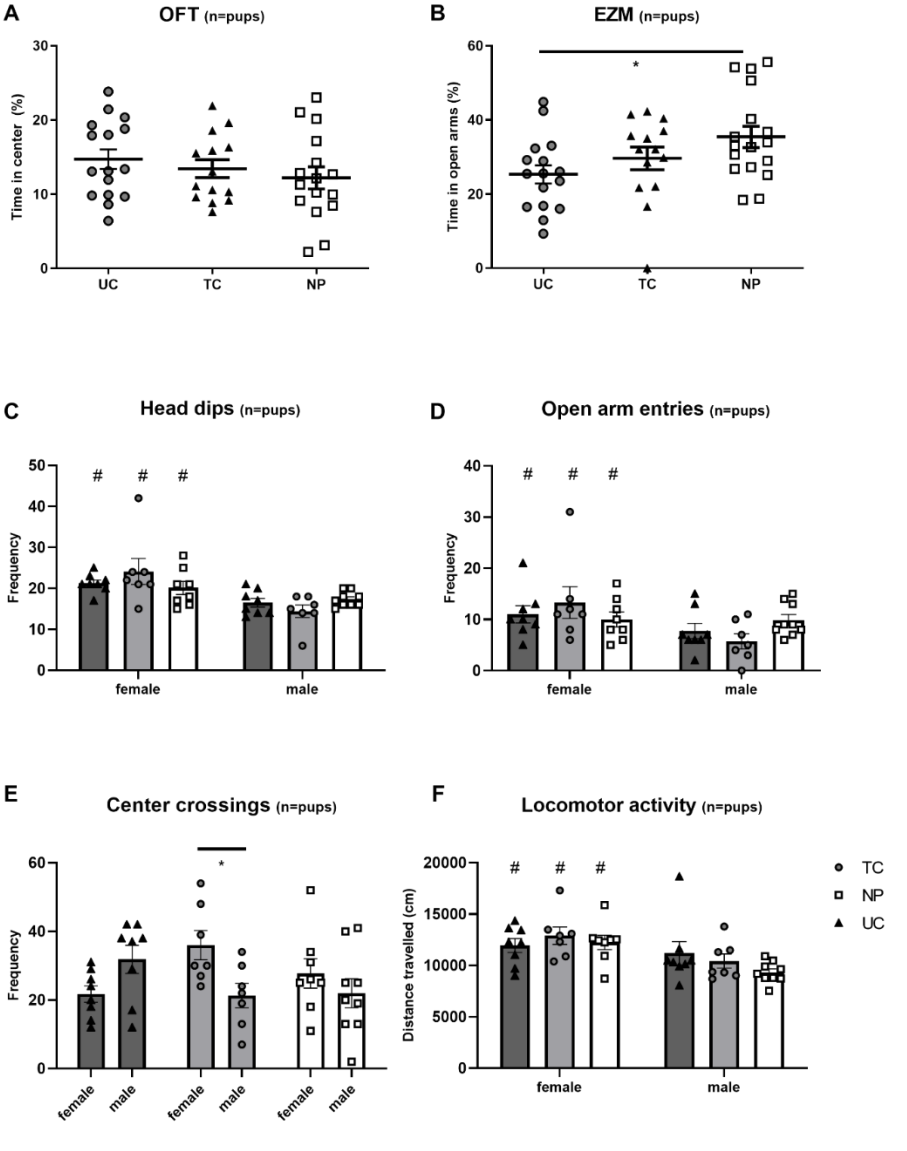

Figure S1. Anxiety behavior and exploratory behavior (head dips, open arm entries, center crossings) after repetitive needle pricking, using pups as unit of analysis. A. The percentage of time spend in the anxio-genic (center) region of the open field test (OFT) in 8-week-old animals did not significantly differ between neonatal conditions ($F(2, 41) = 0.1273$; $p=0.8808$) or sex ($F(1, 41) = 0.5261$; $p=0.4724$). **B.** The percentage of time spend in the anxio-genic (open arms) region of the elevated zero maze (EZM) differs significantly between neonatal conditions ($F(2, 41) = 3.392$; $p=0.0433$) but not sex ($F(1, 41) = 0.0001$; $p=0.9977$). NP animals spend more time in the open arms of the elevated zero maze as compared to the UC animals (NP 35.15 ± 2.88 vs UD 25.26 ± 2.48; $P=0.036$). **C.** The number of head dips in the EZM is influenced by sex ($F(1, 41) = 19.85$; $p<0.001$) but not condition ($F(2, 41) = 0.0641$; $p=0.9380$), with females showing significantly more head dips. **D.** Condition does not affect the number of open arm entries in the EZM ($F(2, 41) = 0.0.0615$; $p=0.9404$), but females show a higher frequency of open arm entries ($F(1, 41) = 6.470$; $p=0.0148$). **E.** A significant interaction effect is observed in the frequency of center crossings ($F(2, 41) = 5.056$; $p=0.0109$), with females crossing the center more frequently as compared to males in the TC group only ($p=0.0458$). **F.** Locomotor activity in the open field test (OFT) was affected by sex ($F(1, 41) = 10.95$; $p<0.01$) but not condition ($F(2, 41) = 0.8533$; $p=4334$), with females show significantly higher total distance travelled as compared to males. Data are presented as mean ± SEM, *P <0.05 ** P<0.01. # sign. effect of males vs. females.

3

The development of descending serotonergic modulation of the spinal nociceptive network: a life span perspective

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Abstract

The nociceptive network, responsible for transmission of nociceptive signals that generate the pain experience, is not fully developed at birth. Descending serotonergic modulation of spinal nociception, an important part of the pain network, undergoes substantial postnatal maturation and is suggested to be involved in the altered pain response observed in human newborns. This review summarizes preclinical data of the development of descending serotonergic modulation of the spinal nociceptive network across the lifespan, providing a comprehensive background to understand human newborn pain experience and treatment. Sprouting of descending serotonergic axons, originating from the rostroventral medulla, as well as changes in receptor function and expression take place in the first postnatal weeks of rodents, corresponding to human neonates in early infancy. Descending serotonergic modulation switches from facilitation in early life to bimodal control in adulthood, masking an already functional 5-HT inhibitory system at early ages. Specifically the 5-HT_3 and 5-HT_7 receptors seem distinctly important for pain facilitation at neonatal and early infancy, while the 5-HT_{1a}, 5-HT_{1b} and 5-HT_2 receptors mediate inhibitory effects at all ages. Analgesic therapy that considers the neurodevelopmental phase is likely to result in a more targeted treatment of neonatal pain and may improve both short- and long-term effects.

Impact

- The descending serotonergic system undergoes anatomical changes from birth to early infancy, as its sprouts and descending projections increase and the dorsal horn innervation pattern changes.
- Descending serotonergic modulation from the rostral ventral medulla switches from facilitation in early life via the 5-HT_3 and 5-HT_7 receptors, to bimodal control in adulthood.
- A functional inhibitory serotonergic system mainly via 5-HT_{1a}, 5-HT_{1b} and 5-HT_{2a} receptors at the spinal level exists already at the neonatal phase but is masked by descending facilitation.

Introduction

Pain is a powerful survival signal that can be experienced throughout the whole life span. Nevertheless, due to immaturity of the nociceptive network, the experience of pain and the processing of noxious stimuli differs in the newborn neonate as compared to infancy, childhood and adulthood [2-6]. At birth, reflex responses to noxious stimuli are exaggerated and uncoordinated, becoming more refined with increasing age in both human and rodent neonates [3,7,8]. Discrimination between touch and nociceptive stimuli is also fine-tuned during postnatal development [4,5]. These changing pain behaviors from birth to adulthood in both human and rodents mirror the maturation of the nociceptive network, responsible for processing of nociceptive signals. Postnatal structural and functional fine-tuning of the nociceptive network is an activity-dependent process, requiring input from the environment to finalize [13-15]. This, however, also leaves this system vulnerable to excessive input in early life such as neonatal pain [16-18]. Refinement of neural excitability in the spinal cord dorsal horn, the first and most important level of processing of nociceptive information [11] (see Box 1), occurs through a variety of neuronal processes including the emergence of top-down inhibitory modulation of spinal nociception [2,16].

Preclinical studies in rodents indicate that descending inhibitory modulation of the nociceptive signaling is still immature immediate around birth, especially in preterm newborns, and will switch from facilitation to inhibition during the first three postnatal weeks [9,19-22]. Notably, the rostral ventral medulla (RVM) is the major source of descending serotonergic modulation of spinal nociception, and has been shown to enhance rather than inhibit nociceptive stimuli in early life in rodents [20]. These descending serotonergic projections and their receptors are targeted for pharmacological treatment of pain in adults due to their expression in the spinal dorsal horn [23], and could also potentially be utilized to attenuate the acute effects of overstimulation of the nociceptive system at an early age. The neonatal period is unique in how it processes noxious stimuli, and develops differently after aberrant noxious stimulation, leading to changes in pain processing throughout life [16-18]. In this respect, treatment of pain should be based on the understanding of the neurodevelopment of the nociceptive system. Increased knowledge of the development of descending serotonergic RVM-spinal dorsal horn projections has significant implications for the understanding and subsequent treatment of pain throughout the whole lifespan. Here, we summarize preclinical data of the serotonergic descending modulation of the spinal nociceptive network during postnatal development, highlighting which receptors may be involved in the functionality of the descending serotonergic modulation at various phases throughout life in rodents. This will provide a better understanding of postnatal

pain processing that ultimately can identify possible pharmacological pain treatment to improve short- and long-term outcome in the vulnerable population of neonates and children.

The review starts with the newborn rodent, where the developmental phase of the nociceptive network is similar to the developmental phase in human neonates during the second and third semester (see Figure 1) and therefore representing premature newborns (> 25 gestational weeks) [24-26]. The pre-weaning phase from Postnatal day (P) 7 to P21 represents the phase of neuronal development in a human neonate at term birth (40 gestational weeks), and the developmental processes have reached a similar level as seen in human infants at weaning (1-2 years of age) [24,25]. Finally, we discuss descending serotonergic modulation from weaning into adulthood.

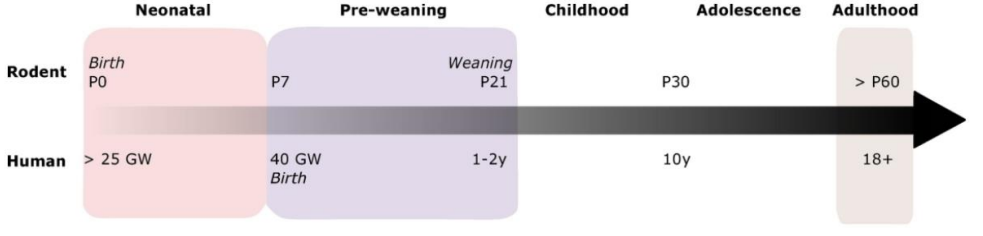

Figure 1. Developmental timelines for rodents (top) and humans (bottom), illustrating the neonatal, pre-weanling, childhood, adolescent and adult phases. (GW = gestational week, P = postnatal day, Y = years)

Serotonergic descending modulation of nociception during the neonatal phase (P0-P7)

Anatomy of descending serotonergic RVM-spinal dorsal horn projections

In the RVM, 5-HT positive neurons emerge as early as embryonic days 13–16 and do not change in absolute number with postnatal age [20,27-29]. Similarly, the proportion of spinally projecting RVM serotonergic neurons remains stable over the first postnatal week in rodents, corresponding with preterm to term human neonate, and accounts for only 3-10% of all spinally projecting serotonergic neurons [20,30]. Even though the proportion of serotonergic RVM neurons projecting to the spinal cord does not change during the first postnatal week, the number of serotonergic axons increases in the dorsal horn as fibers grow and sprout further, and innervation follows a rostral to caudal and ventral to dorsal

gradient [20,28,31-33]. Overall, spinal 5-HT axon innervation maturing from rostral to caudal could indicate serotonergic modulation of the spinal dorsal horn and therefore sensitivity develops earlier at cervical level followed by lower (thoracic and lumbar) levels in neonates [28,31,34]. The ventral to dorsal gradient ensures the first serotonergic fibers invade the deeper dorsal horn around birth in rodents, and their innervation increases progressively throughout the cord in the first few postnatal days [28,31,34]. By P7 (or term human birth), the RVM serotonergic axons project to the entire dorsal horn as they now also reach the upper laminae I and II (Substantia Gelatinosa) at all spinal cord levels [20,28,31-33] (see Figure 2).

Box 1 – Nociception: the role of descending serotonergic RVM-spinal dorsal horn projections

Nociceptive information from peripheral primary afferent C and A_δ fibers, as well as touch information from $_{A\beta}$ fibers, is transmitted to second-order projection neurons in the spinal dorsal horn [1]. These projection neurons are either nociceptive specific (NS) neurons in laminae I and II (receive input from C and A_δ fibers) or wide-dynamic range neurons (WDR) in laminae V (receive direct or indirect input from all three types of cutaneous afferents (C, Aδ and $_{A\beta}$)). Via the spinothalamic pathway, nociceptive information is perceived and integrated in the brain centers, including the prefrontal cortex, somatosensory cortex and anterior cingulate cortex [1]. Brainstem centers modulate spinal nociceptive processing via the spino-bulbo-spinal loop. The rostral ventral medulla (RVM), which includes the nucleus Raphe Magnus (NRM) and adjacent reticular formation, is part of this a spino-bulbo-spinal loop that is activated by ascending nociceptive input and drives descending modulation of the spinal dorsal horn [9]. Serotonergic neurons from the RVM project to laminae I, II and V of the spinal dorsal horn where projection neurons reside, and are therefore a major source of descending modulation of nociceptive input [10,11]. Descending serotonergic modulation of nociception is mediated via seven 5-HT receptor families (5-HT_1 to 5-HT_7) that comprise 15 receptor subtypes [12] expressed in the spinal cord and brainstem.

Functional implications of descending serotonergic RVM-spinal dorsal horn projections: effects on the nociceptive network

Facilitation of the dorsal horn nociceptive network

Descending serotonergic projections from the RVM to spinal cord, despite their low presence neonates, facilitate (enhance) both noxious and tactile spinal processing in the dorsal horn, thereby increasing the chance of signal transmission up to the brain via projection neurons [20,21,35]. This facilitation is observed in a minority of projection neurons in the dorsal horn of one-week old pups [36-39]. Electrophysiological studies using spinal cord slice preparations show that postsynaptic facilitation of dorsal horn responses in the first postnatal week occurs through the 5-HT_3 and 5-HT_7 receptors, and to a lesser extend via 5-HT_2 receptor activation (see Figure 3A top) [36,37,39]. Spinal 5-HT acts in a biphasic fashion via the presynaptic 5-HT_3 receptor, enhancing excitability of dorsal horn neurons at low concentrations, while enhancing inhibition at higher concentrations [38-41].

Inhibition of the dorsal horn nociceptive network

Although descending RVM projections predominantly facilitate spinal somatosensory processing during the first postnatal week (see 2.2.1), endogenous release and/or exogenous application of 5-HT on (isolated) neonatal spinal cord preparations has the potential to produce inhibition at both superficial and deeper dorsal horn levels in one-week old animals [36-38,40-42]. This suggests that inhibitory mechanisms are already present in neonates, but are masked by the facilitatory influence from the RVM. Primary afferent-evoked responses are inhibited via presynaptic 5-HT_{1a} and 5-HT_{2a} receptors in the spinal dorsal horn, thereby reducing the chance of signal transmission to projection neurons in the first postnatal week (see Figure 3A bottom) [38,40-42]. Postsynaptic dorsal horn responses are inhibited by means of increasing the incidence of long-term depression (LTD) via postsynaptic 5-HT_{1a} and 5-HT_{1b} mediated binding in neonatal pups (P0-7) (see Figure 3A bottom) [36-39]. Blocking the 5-$HT1_a$ receptors exclusively produces facilitation of the deep dorsal horn responses [36,37], suggesting that 5-HT_{1a} receptors tonically inhibit evoked responses in the dorsal horn of neonatal pups [36,37]. The postsynaptic 5-HT_{2a} receptor causes modest inhibition of synaptic transmission in deep dorsal horn neurons of P3-6 pups, and is not tonically active in the first postnatal week [36,37]. Next to direct modulation of primary afferent input or dorsal horn responses, local inhibitory synaptic transmission, mediated via gamma-aminobutyric acid (GABA) and glycine, can also be potentiated by the 5-HT_3 and 5-HT_{2a} receptors in the neonatal spinal cord, indirectly reducing primary afferent input [40].

Conclusions neonatal phase (P0-P7)

The first descending serotonergic axons, originating in the RVM and projecting to the spinal dorsal horn, emerge during the first postnatal week (P0-P7) and terminate mainly in the deeper, but not in the superficial laminae of the dorsal horn. Descending serotonergic RVM projections predominantly facilitate spinal nociceptive processes in neonates via the 5-HT_3 and 5-HT_7, and to lesser extend the 5-HT_2 receptor mediated binding, despite the presence of inhibitory mechanisms via the 5-HT_{1a}, 5-HT_{1b} and 5-HT_2 receptors.

Serotonergic descending modulation of nociception during pre-weaning (P7-P21)

Anatomy of descending serotonergic RVM-spinal dorsal horn projections

During the pre-weaning phase (P7-P21), the descending serotonergic system undergoes substantial anatomical changes (see Figure 2). Within the RVM, 5-HT positive neurons show an increased spatial distribution, with an increasing soma diameter and dendritic growth in the second and third postnatal week [43,44]. However, the absolute number of 5-HT positive neurons in the RVM remains unaltered [20,29,30,44,45]. Most importantly, the amount of descending projections from 5-HT positive cell bodies in the RVM significantly increases between the first and second week, reaching adult proportions around P14-16 [20,30].

Following a rapid increase in RVM-spinal cord projecting neurons between the first and second postnatal week, 5-HT axon terminals increase throughout the spinal cord over the second and third postnatal week [31-34]. Between P7 and P14, the number of 5-HT axon terminals increases significantly, particularly in laminae I and II where nociceptive specific projection neurons reside (see Figure 2) [20,28,31,34]. By P14, axon innervation evolves from a diffuse network to a more defined pattern, with 5-HT terminals only present in superficial and deep dorsal horn laminae and absent in lamina III [28,31-34]. From P14 to P21, a progressive reinforcement of 5-HT axons and terminals in the spinal dorsal horn occurs, with clear distinction between the densely innervated laminae I and II, an average density in laminae V and almost no labeling in laminae III-IV [31-34]. Although density of 5-HT projections appears higher between P7 and P21 compared with adulthood [31], quantification of retrograde labeled neurons from the RVM show similar levels of serotonergic projections to the spinal dorsal horn from P16 to P30 [20] and from P14 to P28 [30]. Possibly, sprouts are increased during pre-weaning followed by a period of pruning that results in an adult pattern of innervation.

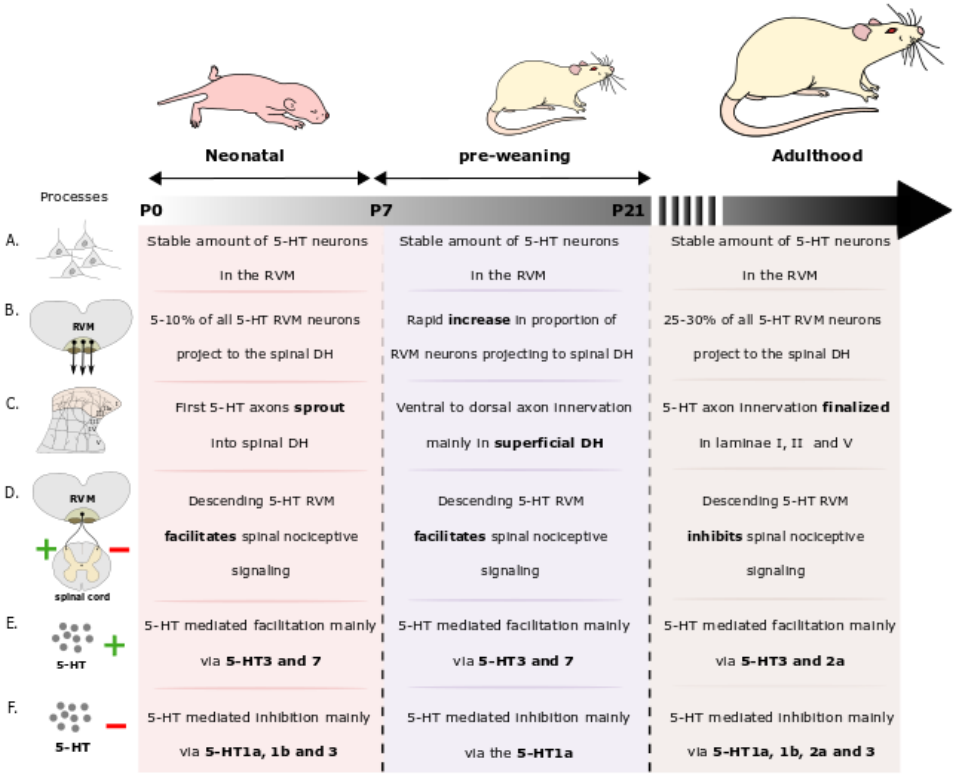

Figure 2. The development of descending serotonergic modulation of the spinal nociceptive network in neonates, pre-weanling and adult rodents for the following processes: (A) RVM cell bodies, **(B)** Descending serotonergic projections from the RVM to the spinal dorsal horn, **(C)** Spinal dorsal horn axon innervation over the laminae, **(D)** the overall effect of RVM-spinal dorsal horn serotonergic modulation of nociceptive signaling, **(E)** Receptors involved in 5-HT mediated facilitation in the spinal dorsal horn and **(F)** Receptors involved in 5-HT mediated inhibition in the spinal dorsal horn. Abbreviations: RVM, rostral ventral medulla; DH, dorsal horn; 5-HT, 5-hydroxytryptamine;

Functional implications of descending serotonergic RVM-spinal dorsal horn projections: effects on the nociceptive network

Facilitation of the dorsal horn nociceptive network

In a similar way as observed in term neonates (P8), descending serotonergic projections from the RVM continue to facilitate both spinal nociceptive and tactile processes at P21 or weaning [20,21,46]. This enhancement of both tactile and nociceptive stimuli in the dorsal horn is mediated via the 5-HT_3 receptor [20]. The increase in descending serotonergic projections during pre-weaning does not (yet) change the directionality of descending 5-HT modulation at P8 versus P21, although weak descending inhibition is first observed around P12 [20,46]. Between P7 and P21, 5-HT mediated facilitation occurs via the 5-HT_2, 5-HT_3 and 5-HT_7 receptor binding (see Figure 3B top) [37,39,47-52]. Similar to neonates, presynaptic 5-HT_3 and 5-HT_2 receptor activation increases the primary afferent evoked responses onto projection neurons in the dorsal horn during pre-weaning [47,50]. In addition, activation of the 5-HT_3 and 5-HT_7 facilitates local postsynaptic dorsal horn responses in P7-21 pups [20,37,39,47,49]. Postsynaptic 5-HT_{2a} receptor mediated effects on nociception seem only modest in pre-weanling animals, that was similar to the effect observed in neonates [37]. The low presence of 5-HT_{2a} on GABAergic interneurons together with immature GABAergic signaling in the early postnatal period, might explain the modest inhibitory effects of 5-HT_{2a} receptor activation on modulation of nociceptive transmission during development [53-55].

Inhibition of the dorsal horn nociceptive network

Despite a predominantly facilitatory effect from the serotonergic projections originating at the RVM on the spinal dorsal horn nociceptive network, 5-HT applied to the spinal cord is capable of inhibiting primary-afferent evoked responses and postsynaptic responses in the majority of dorsal horn neurons in pre-weanling animals when investigated in isolation [37,39,47,48,50-52,56-58]. 5-HT can also inhibit somatosensory input from adjacent dermatomes, thereby reducing the sensory contrast and shaping the receptive field properties of deep dorsal horn projection neurons in laminae IV and V at P8-P10 [56]. 5-HT induced inhibition of the dorsal horn nociceptive processing in pre-weanling animals is mediated by the 5-HT_1 receptor family, specifically the 5-HT_{1a} receptors both on the pre- and postsynaptic side (see Figure 3B bottom) [37,47-49,51,59]. The 5-HT_{1a} receptor is tonically activated during the pre-weaning phase, producing inhibition of nociception similar to that seen in the neonatal

phase or first postnatal week [37,47,48]. In addition, the 5-HT_{1a} is involved in thermal but not mechanical induced nociception in pre-weanling animals [59]. Unlike the involvement of 5-HT_{1a} in the 5-HT mediated inhibition, activation of 5-HT_{1b} as such does not modulate dorsal horn responses or primary afferent-evoked responses and is not involved in acute nociceptive behavior [37,48,59]. Presynaptic 5-HT_2 receptor binding, and to some extend 5-HT_3 receptor binding, also mediate inhibition of primary-afferent evoked responses in a modest way [48,50].

Conclusions pre-weaning phase

The proportion of serotonergic RVM-spinal cord projecting neurons, as well as their axons in the spinal cord, rapidly increase during pre-weaning and mainly terminate in superficial laminae I and II. The descending serotonergic RVM projections to the spinal dorsal horn continue to facilitate nociceptive processing via 5-HT_2, 5-HT_3 and 5-HT_7 receptors from first postnatal week to weaning, corresponding with human term birth to early infancy. Spinal 5-HT inhibits of the dorsal horn nociceptive network mainly via the 5-HT_{1a} receptors during pre-weaning.

Serotonergic descending modulation of nociception from infancy (or weaning) up to adulthood (P21-adult)

Anatomy of descending serotonergic RVM-spinal dorsal horn projections

The number of descending spinal cord projections originating from 5-HT positive neurons in the RVM remains similar from weaning (P21) up to adulthood and account for approximately one-third of all 5-HT neurons projecting to the spinal cord [20,30]. 5-HT axon terminals show highest innervation at the superficial laminae (Rexed laminae I and II) and deeper dorsal horn lamina V where projection neurons reside in adulthood (see Figure 2) [20,31-33].

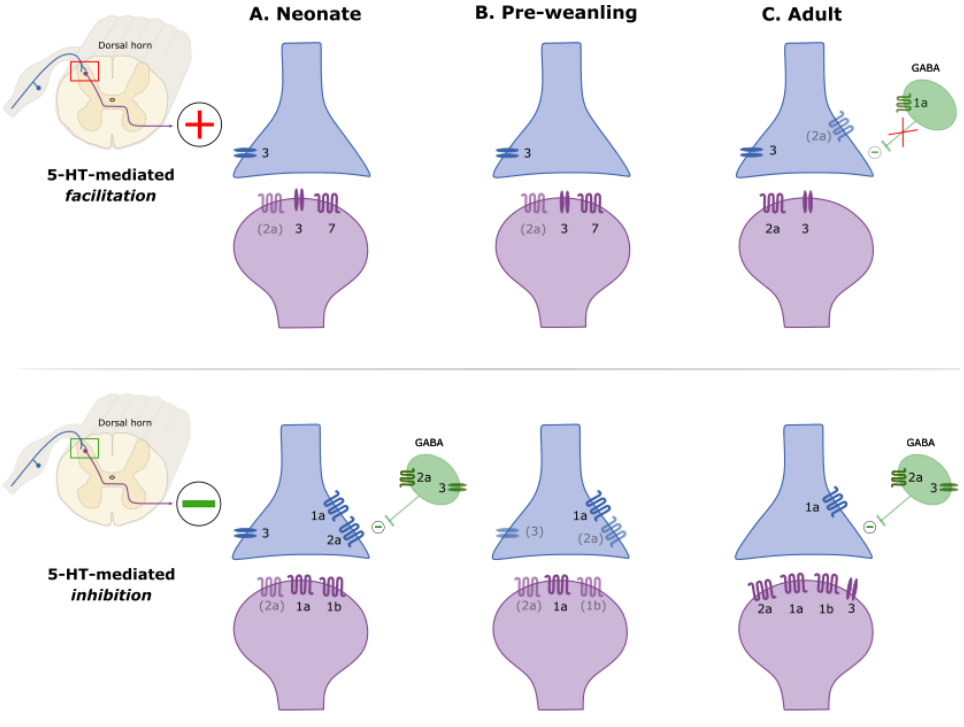

Figure 3. 5-HT receptors involved in 5-HT-mediated facilitation (top) and inhibition (bottom) of the nociceptive network in the dorsal horn in (A) neonatal, (B) pre-weaning and (C) adult phase.
5-HT mediated facilitation (top). In neonates (A) as well as in pre-weanling animals (B), 5-HT facilitates nociceptive signaling via the presynaptic 5-HT3 receptor on primary afferent terminals (in blue) and postsynaptic 5-HT3 and 5-HT7 receptors (in purple), and to lesser extend via the 5-HT2a receptor. In adulthood (C), 5-HT3 and 5-HT2a facilitate both pre- and postsynaptic signaling, whereas the 5-HT1a directly blocking GABAergic interneurons thereby causing disinhibition of projection neurons and enhancing nociceptive signaling in the dorsal horn. Unlike in younger animals, the 5-HT7 does not play a role in serotonin-mediated facilitation in adults.
5-HT mediated inhibition (bottom). In neonates (a), inhibition of primary-afferent evoked responses (in blue) occurs through the 5-HT1a, 5-HT2a and 5-HT3 receptor. 5-HT mediates postsynaptic inhibition through the 5-HT1a and 5-HT1b, and in lesser extend the 5-HT2a receptor. Both the 5-HT2a and 5-HT3 mediate GABA release of inhibitory interneurons in the spinal dorsal horn. In pre-weanling animals (b), the 5-HT1a is mainly responsible for 5-HT induced inhibition. 5-HT1b, 5-HT2a and 5-HT3 only show modest inhibition. In adulthood (c), 5-HT1a inhibits nociceptive afferent evoked responses via pre- and postsynaptic mechanisms whereas the 5-HT1b, 5-HT2 and 5-HT3 inhibit projection neurons (purple), either directly or by activation of inhibitory GABAergic signaling (green).

Functional implications of descending serotonergic RVM-spinal dorsal horn projections: effects on the nociceptive network

Facilitation of the dorsal horn nociceptive network

In adulthood, the descending serotonergic system is functionally bimodal, producing either facilitation or inhibition (see "inhibition of the dorsal horn nociceptive network") of the spinal nociceptive network by activating specific types of 5-HT receptors Stimulation of RVM at low intensities, optogenetic activation of tryptophan hydroxylase 2 neurons in the RVM or depletion of descending serotonergic terminals have been shown to facilitate nociceptive behavior or processing in healthy adult rodents in some [60-62] but not all studies [20,63-65]. At the same time, spinal 5-HT does not facilitate acute mechanical and thermal nociception, and facilitates spinal nociceptive signaling only in a minority of spinal neurons in adulthood under non pathological conditions [66,67] through activation of the 5-HT_{1a} [68-71], 5-$HT_{2a/c}$ [72,73] and 5-HT_3 receptors [71,74-81] (see Figure 3C top). 5-HT_{1a} receptors can facilitate nociceptive transmission by inhibiting GABAergic interneurons in the spinal cord [53,70]. In addition, the spinal 5-HT_{2a} receptor can directly facilitate dorsal horn responses in the spinal cord [72]. 5-HT_3 mediated descending facilitation may occur through either modulation of supraspinal brain areas [82], via spinal microglia and subsequent astrocytes activation [83], via the modulation of long-term potentiation (LTP) in NS projection neurons in the dorsal horn [74], or altering neuronal responses to natural stimuli in neurokinin-1 expressing projection neurons [81]. Activation of the 5-HT_3 receptor also consistently facilitates tactile processing in the deep dorsal horn in adulthood and this is similar to that noted during earlier developmental periods (see section 2 and 3) [20].

Of note, in pathological pain states (including neuropathic or inflammatory pain models), the descending serotonergic system has been shown to produce predominant facilitation of spinal nociceptive processing that is mainly mediated via the 5-HT_3 receptors. For more information on the facilitatory role of 5-HT in pathological pain states, the reader is referred to other reviews [84-86].

Inhibition of the dorsal horn nociceptive network

The majority of studies show a bimodal role of descending serotonergic projections from the RVM on nociceptive processing in the spinal cord in healthy adult rodents, marking a shift in modulation from the facilitation observed throughout early development (see sections 2 and 3) to bimodal control in adulthood [20,62,64]. Indeed, RVM-stimulation induced

antinociception can be abolished by spinal non-selective serotonergic antagonists [87-89]. In addition, this descending inhibition in adulthood is functionally selective for nociceptive but not for tactile spinal processing [20,35]. Depletion of descending 5-HT projections does not affect acute mechanical and thermal sensitivity [63,64,90] but abolishes RVM-stimulation induced inhibition [64], where descending serotonergic projections are part of the spinal-bulbo-spinal feedback loop modulating spinal nociception in adulthood. Electrical stimulation of the RVM increases 5-HT and 5-HIAA efflux in the spinal cord in adulthood [91]. The role of local spinal 5-HT on nociception in adulthood is mainly inhibitory [66,68,92-101]. 5-HT inhibits the dorsal horn nociceptive network in the adult rodent either directly via the 5-HT_{1a} [66-68,94,95,102,103] and 5-HT_{1b} [66,68,69,71,94,95,104] receptors, or indirectly via 5-HT_{2a} [71,92,95,97] and 5-HT_3 [67,92,95,105-109] receptor mediated activation of GABAergic neurotransmission that in turn inhibits the nociceptive network (see Figure 3C bottom). Although the 5-HT_3 receptor affects naturally evoked (mechanica and thermal) stimuli, electrically evoked deep dorsal response were unaffected suggesting that the occurance of 5-HT_3 mediated facilitation depends on the type of acute stimuli used [65,75,77,81,110]. Up to now, the role of the spinal 5-HT_7 receptor in modulation of thermal or mechanical nociception-induced responses in the spinal nociceptive network is unclear [79,92,111-114]. 5-HT_7 mediated effects are mostly studied in acute nociceptive behaviors, whereas 5-HT_7 mediated facilitation of dorsal horn responses is observed in earlier developmental periods (see section 3.2 and 2.2).

Conclusions adulthood

Serotonergic RVM-spinal cord projections terminate at the superficial laminae (Rexed laminae I and II) and deeper dorsal horn lamina V in adulthood. Although the descending serotonergic system from the RVM is functionally bimodal, spinal 5-HT predominantly inhibits acute nociceptive behavior and spinal nociceptive processing in adulthood under non pathological conditions, while it facilitates touch processing. Activation of 5-HT_{1a}, 5-HT_2 and 5-HT_3 receptors result in both inhibition and facilitation of the spinal nociceptive network in healthy rodents. Activation of the 5-HT_{1b} results in inhibition of the spinal nociceptive network. Unlike earlier developmental periods, the role of the spinal 5-HT_7 receptor in acute spinal nociceptive processing in adulthood remains unclear.

Clinical implications of the postnatal development of descending RVM-spinal dorsal horn projections

This review summarizes preclinical data on the development of descending serotonergic RVM-spinal dorsal horn projections, and its role in nociception throughout the lifespan. In short, descending serotonergic projections from the RVM develop postnatally, where they sprout to the spinal dorsal horn in neonates, continue to increase their projections and axon innervation of the nociception specific laminae I, II and V in the spinal cord in neonates and pre-weanling animals up to P21, and show highest innervation in adulthood (see Figure 2) [20,30-34]. Functionally, the descending serotonergic RVM modulation of spinal nociception undergoes a major switch: from facilitatory during neonatal and pre-weaning phases to bimodal role in later phases (>P21; or post-weaning) [9,20,21]. Despite this switch in RVM serotonergic control of spinal nociception, inhibitory mechanisms mediated via the 5-HT receptors are already present at an early age. This review also highlights the receptors involved in the functionality of the descending serotonergic modulation at various phases throughout the lifespan in rodents.

Although the majority of studies have been performed in laboratory rodents, clinical studies also suggest that descending modulation from the brainstem develops postnatally in human infants [7,115]. The changing balance between nociceptive-specific brain activity and spinal reflexes suggests that as cortical networks mature and thus nociceptive specific activity emerges, descending inhibitory modulation is activated in human infants [7,115]. Perhaps, postsynaptic facilitation mediated by 5-HT in early life may promote the formation of a functional descending modulatory neuronal circuit via transformation of silent glutamatergic synapses to functional ones for more effective sensory transmission [39]. Additionally, 5-HT mediated facilitation modulates axon branching of descending projections in the spinal dorsal horn throughout development [116,117]. A similar shift from facilitation to inhibition is seen in the developing spinal dorsal horn network in which GABAergic and glycinergic neurotransmission is involved [54,55,118]. The exact mechanism by which descending serotonergic modulation switches from facilitation to bimodal control with postnatal age is not known due to the limited preclinical studies investigating the functionality of 5-HT receptor subtypes when the switch occurs (P21 - P40), although endogenous opioidergic activity seems to play a role in the occurrence and/or timing of this switch [22]. Excessive nociceptive input during early development, such as repeated noxious stimuli occurring frequently on a daily basis in newborns admitted to NICU's and surgery in neonatal life, has been shown to augment the perception and/or processing of

nociceptive stimuli [119-123] and could consequently affect 5-HT axon branching and the formation of a functional nociceptive neuronal network.

Several 5-HT receptors are involved in the functionality of this descending serotonergic modulation within the spinal nociceptive network of rodents (see Figures 2 and 3). This information is relevant to clinical practice of preterm and term born infants, as it highlights a potential for serotonin-based pain treatment during this vulnerable period. Preclinical data shows that 5-HT_{1a} and 5-HT_{1b} receptors inhibit the spinal nociceptive network throughout life, which can point towards a potential use of 5-HT_{1a} or 5-H_{1b} receptor agonists for the treatment of pain during any developmental phase. As 5-HT_{1b} mediated inhibition of the spinal nociceptive network seems to be less robust during pre-weaning as compared to neonatal and adult phases, 5-HT_{1a} receptor modulation could be better suitable for pain management in infants (between term birth and 1-2 years of age) [37,48,59]. Additionally, the 5-HT_3 receptor plays an important role in 5-HT induced facilitation of the nociception during development (Sections 2, 3 and 4), and thus modulating this receptor is a promising additional therapeutic venue to treat pain in developing neonates and infants. In this context it is important to note that the facilitation of touch signaling is also mediated via the 5-HT3, and thus targeting this receptor may therefore impact the development of tactile processing [20]. Finally, modulating the 5-HT_7 receptor throughout development, but not in adulthood, can be promising to treat pain in early life (Sections 2 and 3). Agonists and antagonists of these receptors are currently available, but are not part of the current clinical management of pain in neonates and infants. Daily clinical practice is mainly based on prescription of paracetamol, opioids and non-pharmacological analgesia such as skin-to-skin care, non-nutritive sucking, and sucrose [124]. Painful stimuli in early life, as well as the use of pharmacological treatment such as opioids, can lead to long-term alterations lasting into adulthood [16]. Serotonin-mediated analgesia could aid the prevention of acute and long-term effects of neonatal pain, as it targets a part of the nociceptive system that is highly active at early ages. When testing any of these analgesics, side effects including anxiety and depression should be closely monitored due to the involvement of 5-HT and the expression of 5-HT receptors within the limbic system [125,126].

Despite numerous years of research, several gaps in knowledge still exist. For example, the exact functionality of most serotonin receptors in an intact *in vivo* system during early age is not clear. Most studies, performed in developing rodents, assess receptor functionality in isolated spinal cord preparations or spinal slices, thereby removing the "serotonergic tone" from supraspinal areas including the RVM, which appears to be of vital importance [36-42,47-52,56-58]. As descending serotonergic modulation plays an important

role during any age, future preclinical studies should focus on this knowledge gap by investigating 5-HT receptor functioning *in vivo* rather than *in vitro*. Moreover, several drugs interacting with the 5-HT system, while studied in adulthood, should be investigated for specific postnatal age groups to assess functionality at different ages, thereby improving treatment of both procedural and surgical pain throughout neurodevelopment.

Conclusions

In conclusion, a rapid postnatal development of the descending serotonergic RVM-spinal dorsal horn projections is noted in rodents and this precedes a switch in descending modulation from facilitation before weaning to bimodal control of spinal nociceptive signaling in later life (childhood up to adulthood). More specifically, spinal 5-HT mediated effects are functional at an early age, mediating inhibition of spinal nociception via the 5-HT_{1a}, 5-HT_{1b} and 5-HT_2 receptor and facilitation mainly via the 5-HT_3 and 5-HT_7 receptor. At later phases, activation of all 5-HT receptors, with exception of the 5-HT_7, might result in either facilitation or inhibition of acute spinal nociceptive signaling. Targeting the specific receptors in early developmental phases may help improve pain management in early life, preventing acute and long-term consequences of neonatal pain.

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3

Anatomical changes in descending serotonergic projections from the rostral ventromedial medulla to the spinal dorsal horn following repetitive neonatal painful procedures

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Abstract

Excessive noxious stimulation during the critical neonatal period affects the nociceptive network lasting into adulthood. As descending serotonergic projections from the rostral ventromedial medulla (RVM) to the spinal dorsal horn develop postnatally, this study aims to investigate the long-term effect of repetitive neonatal procedural pain on the anatomy of the descending serotonergic projections involved in the control of the spinal nociceptive network. A well-established rat model of repetitive noxious procedures is used in which neonatal rats received four noxious needle pricks or tactile stimulation with a cotton swab per day in the left hind paw from day of birth to postnatal day 7. Control animals were left undisturbed. When animals reached adulthood, tissue was collected for quantitative immunohistochemical analysis of serotonin (5-hydroxytryptamine, 5-HT) in the RVM and spinal dorsal horn. Both repetitive noxious and tactile procedures in the neonate decreased the 5-HT staining intensity in the adult ipsilateral, but not in the contralateral spinal dorsal horn. Repetitive neonatal noxious procedures resulted in an increased area covered with 5-HT staining in the adult RVM ipsilateral to the side of injury, whereas repetitive neonatal tactile stimulation resulted in increased 5-HT staining intensity in both the ipsi- and contralateral RVM. The number of serotonin positive cells in adult RVM is unaffected by neonatal conditions. This detailed anatomical study shows that not only neonatal noxious procedures, but also repetitive tactile procedures result in long-lasting anatomical changes of the descending serotonergic projections from brainstem RVM to spinal dorsal horn.

Keywords

Neonatal pain; Tactile stimulation; Serotonin (5-HT); Spinal cord; Rostral ventromedial medulla; Descending modulation

Introduction

The developing somatosensory system of both humans and rodents is highly responsive to both tactile and noxious stimulation, and painful stimuli experienced in early life affect the normal maturation and fine-tuning of this system [1-4]. More specifically, repetitive neonatal procedural pain causes long-lasting structural changes, including hyper-innervation of the injured skin by peripheral nociceptive and non-nociceptive fibers, and increased central terminal densities of nociceptive (C)-fiber terminal densities in the adult spinal dorsal horn [5-8]. In addition, neonatal pain leads to increased firing of spinal somatosensory neurons upon noxious and non-noxious stimulation in adulthood [9,10]. The latter suggests that neonatal triggering of the nociceptive spinal network results in a hypersensitive spinal nociceptive network in the adult, amplifying nociceptive signaling in later life. The spinal dorsal horn is an important hub for the integration of nociceptive and non-nociceptive input [11-13]. Serotonergic descending projections from brainstem to spinal cord play an important role in modulation of the nociceptive spinal network throughout the life span (see review [14]). Hence, developmental changes induced by repetitive procedural pain during the critical neonatal period may interfere with the normal development of the serotonergic descending projections and its modulation of the spinal nociceptive network. This is likely to be of key importance in the long-term effects of excessive painful stimulation in early life [15].

The major source of descending serotonergic projections to the spinal dorsal horn is the Raphe Magnus, located in the brainstem rostral ventromedial medulla (RVM) [16,17]. The RVM is part of a spino-bulbo-spinal loop that is activated by ascending nociceptive input and drives serotonergic descending modulation in adulthood [18]. Descending inhibition from the RVM is functionally immature at birth [19-21], and descending serotonergic RVM-spinal dorsal horn projections facilitate rather than inhibit nociceptive and non-nociceptive signaling in the dorsal horn during the first weeks of life [14,22]. Moreover, fine-tuning and developmental restriction of the distribution of descending serotonergic RVM-spinal cord projections and spinal innervation patterns occurs postnatally in rats [23-25], making them potentially vulnerable to excessive input such as neonatal pain in early life. Studies have suggested that the modulatory role of the RVM is altered after neonatal incision [26] or inflammation [27], which suggest the underlying cause may be related to interference with the process of developmental restriction of the distribution of the descending serotonergic projections. The aim of the present study is to investigate the long-term effect of repetitive neonatal noxious procedures on the anatomy of the descending serotonergic projections involved in the control of the spinal nociceptive network. Using quantitative

immunohistochemical analysis, we studied the effect of neonatal repetitive needle pricking on adult 5-HT staining intensity in both adult RVM and spinal dorsal horn.

Materials and Methods

Animals

All animal experiments are performed in accordance with the European Directive for Protection of Vertebrate Animal Use for Experimental and Other Scientific Purposes (2010/86/EU) and were approved by the Committee for Experiments on Animals, Maastricht, The Netherlands (DEC 2017-017). Male and female Sprague-Dawley rats from Charles River laboratory were mated at Maastricht University, and all rat pups were born on gestational day 21. Litters were culled to a maximum of N=10. On postnatal day (P) 21 pups were weaned and housed in groups of two or three in same-sex individually ventilated cages in a climate-controlled room (temperature 21±1 °C, humidity 55 ± 15%) with constant background music (approximately 45 decibel) under artificial lightening (12:12 reversed light/dark cycle). Animals had *ad libitum* access to water and food.

Repetitive neonatal procedural pain stimulation

As a model of repetitive procedural pain exposure in the NICU, rat pups were noxiously stimulated four times a day via unilateral 2 mm calibrated needle pricks in the mid-plantar surface of the left hind paw from P0 to P7 (Needle Prick, NP; N=7) as described before [7,28]. Control littermates received four daily tactile stimuli at the same hourly intervals as the NP animals, by stroking their left hind paw with a cotton-tipped swab (Tactile Control, TC; N=8). For each procedure, the nest was briefly separated from the dam and returned shortly after. Separate nests were left undisturbed during the first postnatal week (Undisturbed Control, UC; N=8). Researchers were blinded to treatment groups throughout all experimental procedures and tissue processing.

Tissue isolation and preparation

When animals reached adulthood (aged 8 weeks), all animals were terminally anesthetized with pentobarbital (100 mg/kg) and transcardially perfused with ice-cold tyrode buffer and fixed with Somogyi fixative (15% picric acid and 4% paraformaldehyde in 0.2M phosphate buffer saline, (PBS; pH 7.6)). Brains and lumbar spinal cord regions L4 and L5 were isolated, post-fixed overnight at 4°C and cryoprotected (10% sucrose solution for 24h, 25% sucrose solution for 72h in 0.1M PBS). Tissue was frozen using solid carbon dioxide and stored at -80°C. Transverse cryosections of spinal cord (30 µm) and coronal cryosections (30 µm) of brainstem including the RVM (Bregma -9.16 to -11.30, 30 µm) were cut, mounted on gelatin-coated glass slides and stored at -20°C.

Immunohistochemical detection of 5-HT

Spinal and RVM sections were stained for 5-HT immunoreactivity as described previously [29]. Slides were thawed at room temperature for 2 hours before being washed with Tris-buffered saline (TBS, 0.1M, pH 7.6) including 0.3% Triton X-100 (TBS-T), TBS and TBS-T. Thereafter, sections were incubated with primary rabbit anti-5-HT polyclonal antibody for 72 hours (1:20,000 RVM, 1:10,000 SC, diluted in TBS-T with 0.1% Bovine serum albumin (BSA); Steinbusch, Maastricht University, the Netherlands). After rinsing unbound primary antibody with TBS-T, TBS and TBS-T (15 min each), sections were incubated for 2 hours with donkey anti-rabbit biotinylated secondary antibody (1:800, diluted in TBS-T with 0.1% BSA; Jackson Immunoresearch Laboratories, West Grove, PA, USA). Following TBS-T, TBS and TBS-T (15 min each), all sections were incubated with an avidin-biotin-peroxidase complex (Elite ABC-kit, 1:800 diluted in TBS-T, Vector Laboratories, Burlingame, CA, USA). Sections were then again washed in TBS and Tris-hydrochloride (Tris-HCL buffer, 0.05M, pH 7.6) before incubation with 3,3'-diaminobenzidine tetra-hydrochloride (DAB)/Nickel-chloride solution to visualize the immune complex of horseradish peroxidase reaction product (10 min for SC, 20 min for RVM). After rinsing with Tris-HCL buffer (0.05M at pH 7.6), sections were dehydrated in ascending ethanol concentrations and coverslipped with Pertex (VWR International, Radnor, PE, USA).

Quantification of immunostaining

Photomicrographs of ipsi- and contralateral dorsal horns for the spinal levels L4 and L5, and the RVM region of the brainstem (Bregma -9.16 to -11.60) were taken, using a Provis AX70 microscope (Olympus, Hamburg, Germany) connected to a black and white camera equipped with CellP © imaging software (DP70, Olympus). Images were merged using Adobe Photoshop (Adobe Inc., San Jose, USA), and mean grayscale values of intensity of 5-HT immunostaining were determined after manual background subtraction (blinded for treatment) using ImageJ free software. For the spinal cord, regions of interest (ROI) were made of Rexed lamina I-III of the dorsal horn as descending serotonergic fibers terminate here [14]. Grayscale values were obtained for these laminae, based on atlas coordinates and delineation as described previously [30]. In addition, mean intensity was measured in 20µm x 30µm boxes in lamina I, II, III and IV-V of the dorsal horn to obtain grayscale values per laminae [22]. For the RVM, ROIs were determined according to the atlas of Paxinos and Watson, divided into ipsilateral and contralateral sides by the midline (triangle height 1000µm, width 800µm). Six sections per lumbar level (L4 and L5) and per RVM were averaged to create one data point per animal.

Stereological quantification in the RVM

Design-based stereology was performed using a stereological computer microscopy system with Stereo-investigator software (Microbrightfield, Williston, VT, USA), as described previously [31]. The RVM was defined as an isosceles triangle that lies at the level of the facial nucleus, with a base that was between the left and right boundaries of the pyramidal tracts, and height equal to half of the width of the base. The RVM extends from the rostral end of the inferior olive to the caudal end of the trapezoid body [32]. ROIs were delineated at 12.5x magnification on live microscopic images displayed on the monitor. The number of cells were counted separately for each half of the RVM (i.e. ipsilateral and contralateral to the noxious or tactile stimulation), separated by the midline. Systematic random sampling was used to choose the sections and points within the RVM to be evaluated; the entire RVM was sampled. Unbiased stereological methods for cell counting (including use of a counting frame of 150µm by 150µm and optical dissector method) were used to count cells at all sections, to estimate the total numbers of 5-HT positive neurons in the ipsilateral and contralateral RVM. Neurons that were positive for 5-HT and had a stained cytoplasm and a single distinct nucleus were included for cell counting. Cells were counted at a 400x magnification with the optical fractionator method (MBF Bioscience, Williston, USA).

Statistical analysis

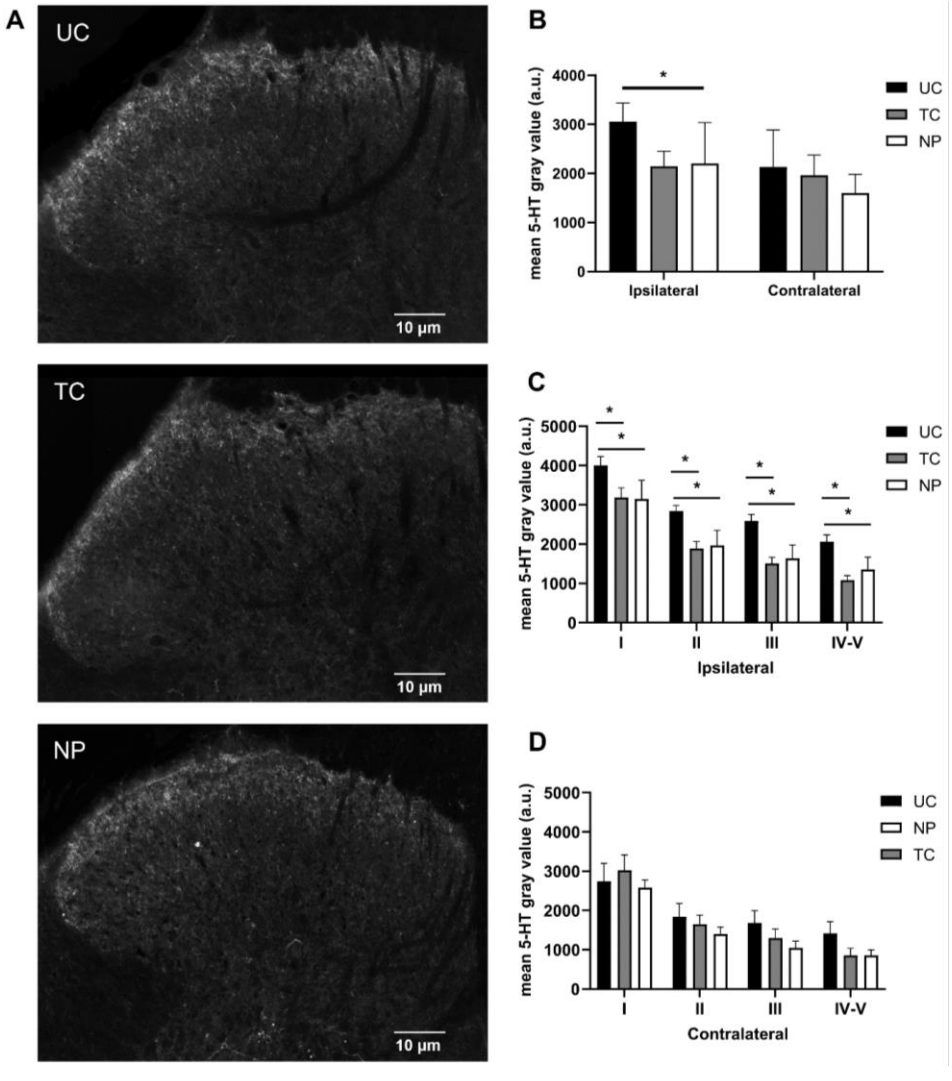
All data is presented as means ± standard error of the mean (SEM) and plotted using Graphpad Prism 9 (GraphPad Software, San Diego, USA). The Shapiro-Wilk test for normality was passed for all data ($p>0.05$). For comparisons of grayscale or stereology values between neonatal conditions (NP, TC and UC) and levels (L4 vs. L5), ipsi- and contralateral differences, or Rexed laminae (I-V), a two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used. A p-value < 0.05 was considered statistically significant.

Results

Spinal dorsal horn 5-HT immunostaining

The anti-5-HT immunohistochemical analysis revealed a strong intensity of 5-HT immunostaining, most pronounced in laminae I to III of the lumbar spinal dorsal horn (Figure 1A). 5-HT shows a uniform distribution from the medial to lateral dorsal horn, and within laminae. The intensity of 5-HT immunostaining, measured by mean grayscale values, did not significantly differ between lumbar levels L4 and L5 ($F(1, 25) = 0.5237$; $p=0.4760$). Therefore, data of L4 and L5 were pooled. Intensity of spinal 5-HT immunostaining significantly differed between the ipsilateral and contralateral dorsal horn ($F(1, 26) = 7.622$; $p=0.0104$; Figure 1B) and neonatal conditions ($F(2, 26) = 4.723$; $p=0.0178$; Figure 1B). Post-hoc analyses revealed a significant decrease in the intensity of 5-HT immunostaining in the ipsilateral dorsal horn of NP animals ($p=0.0416$) and a marginal decrease in TC animals ($p=0.0530$) as compared to UC, but no differences in intensity of contralateral 5-HT immunostaining were noted between neonatal conditions ($p>0.05$).

Within the ipsilateral dorsal horn, intensity of 5-HT immunostaining significantly differed between laminae I-IV ($F(2, 39) = 26.24$; $p<0.01$) as well as neonatal conditions ($F(2, 39) = 12.18$; $p<0.01$; Figure 1C). More specifically, the intensity of 5-HT immunostaining was highest in lamina I and significantly differed from lamina II ($p<0.01$), lamina III ($p<0.01$) and laminae IV-V ($p<0.01$). The intensity of lamina II 5-HT immunostaining was significantly higher as compared to lamina IV-V ($p=0.0185$). In addition, the intensity of 5-HT immunostaining was significantly lower in NP animals ($p<0.01$) and TC animals ($p<0.01$) as compared to UC, evident in all laminae of the ipsilateral spinal dorsal horn (Figure 1C). In the contralateral DH, significant differences in intensity of 5-HT immunostaining were observed between laminae ($F(3, 52) = 20.76$; $p<0.01$) but not between neonatal conditions ($F(2, 52) =2.802$; $p=0.0699$; Figure 1D). Similar to the ipsilateral dorsal horn, lamina I showed highest intensity of 5-HT immunostaining that significantly differed from lamina II ($p<0.01$), lamina III ($p<0.01$) and laminae IV-V ($p<0.01$).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NP
TC

Figure 1. 5-HT immunostaining in the spinal dorsal horn of undisturbed controls (UC), repetitive tactile stimulation (TC) and repetitive needle pricking animals (NP). A. Representative photomicrographs of anti-5-HT staining in the upper laminae of the spinal dorsal horn of UC, TC and NP animals. Scale bar = 10µm. **B.** 5-HT immunostaining, measured by mean grayscale value in laminae I-III of the spinal dorsal horn, is significantly different between neonatal conditions ($F(2, 26) = 4.723$; $p=0.0178$) and the ipsilateral and contralateral dorsal horn ($F(1, 26) = 7.622$; $p=0.0104$). The intensity of 5-HT is significantly decreased in the ipsilateral dorsal horn of NP animals ($p=0.0416$) and marginally decreased in TC animals ($p=0.0530$) as compared to UC. No differences in contralateral 5-HT staining intensities are observed. **C.** Within the ipsilateral dorsal horn, intensity of 5-HT immunostaining significantly differed between laminae I-IV ($F(2, 39) = 26.24$; $p<0.01$) and neonatal conditions ($F(2, 39) = 12.18$; $p<0.01$). In all laminae, the intensity of 5-HT immunostaining was significantly lower in NP and TC animals as compared to UC. **D.** In the contralateral dorsal horn, significant differences in 5-HT staining intensities were observed between laminae ($F(3, 52) = 20.76$; $p<0.01$) but not neonatal conditions. * $p<0.05$ # $p<0.10$.

5-HT immunostaining in the RVM

The intensity of 5-HT immunostaining, measured by grayscale values, showed a significant effect of neonatal condition ($F(2, 24) = 9.158$; $p<0.01$) without a significant difference between the ipsi- and contralateral RVM ($F(1, 24) = 3.735$; $p=0.0652$). Post-hoc multiple testing showed that TC animals had significantly higher 5-HT staining intensity in the RVM as compared to UC, evident in both the ipsilateral ($p=0.0169$) and contralateral RVM ($p=0.0144$). Pooled data of ipsi- and contralateral RVM 5-HT immunostaining showed a significant difference between conditions ($F(2, 12) = 4.8333$; $p=0.0289$; Figure 2C), with higher 5-HT staining intensity in the RVM of TC animals as compared to UC ($p=0.0229$).

The proportion of RVM-area that is immune-reactive for 5-HT, measured by the percentage area fraction, significantly differed between the ipsi- and contralateral RVM ($F(1, 24) = 18.84$; $p<0.01$) and neonatal condition ($F(2, 24) = 11.06$; $p<0.01$; Figure 2D). Post-hoc analyses showed a higher area fraction of 5-HT staining in the ipsilateral RVM of both TC ($p=0.0230$) and NP animals ($p=0.0355$) as compared to UC. In the contralateral RVM, an increased area fraction of 5-HT immunostaining was noted in TC as compared to UC ($p<0.01$).

5-HT immune-positive cells in the RVM

The number of 5-HT immunostained cells in the RVM did not differ between the ipsilateral and contralateral RVM ($F(1, 40) = 0.001$; $p=0.9698$) or neonatal condition ($F(2, 40) = 0.4897$; $p=0.6165$). When pooling data of ipsi- and contralateral RVM, no significant differences in the number of 5-HT positive cells was observed between neonatal conditions ($F(2, 20) = 2.033$; $p=0.1571$; Figure 2B).

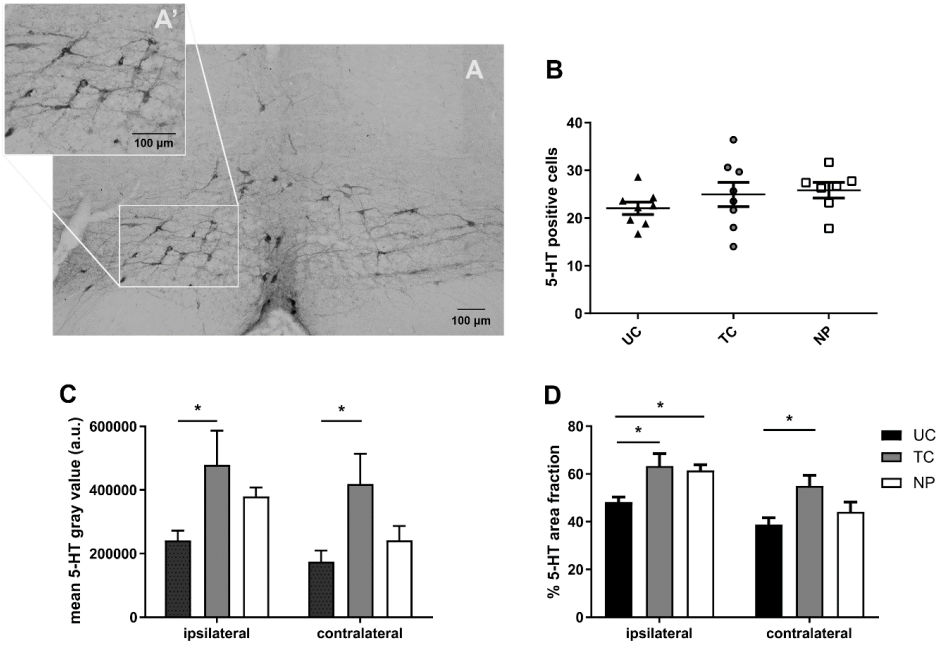

Figure 2. Intensity of 5-HT immunostaining, area fraction and number of 5-HT positive cells in the rostral ventral medulla (RVM). A. Representative photomicrograph of anti-5-HT staining in the RVM of adult animals. 5-HT staining is visible inside the cells as well as in fibres. **B.** The number of 5-HT positive cells does not differ between the ipsi- and contralateral RVM, or neonatal condition ($p>0.05$). **C.** The intensity of 5-HT immunostaining significantly differs between neonatal condition ($F(2, 24) = 9.158$; $p<0.01$) but not ipsi- and contralateral RVM ($F(1, 24) = 3.735$; $p=0.0652$). Tactile control (TC) animals showing higher intensity of 5-HT staining as compared to undisturbed controls (UC) in the ipsi- and contralateral RVM ($P<0.05$). **D.** The proportion of 5-HT immunostaining within the RVM, measured by % area fraction, is significantly affected by the side of the RMV (ipsi vs. contra; $F(1, 24) = 18.84$; $p<0.01$. In the ipsilateral RVM, TC and NP animals show a significantly higher proportion of 5-HT staining, as compared to controls ($F(2, 12) = 7.770$; $p<0.01$). In the contralateral RVM, the proportion of 5-HT immunostaining is increased as compared to TC animals or UC ($F(2, 12) = 4.987$; $p=0.0265$). * $p<0.05$.

Discussion

The present study is the first to analyze the anatomy of the descending serotonergic RVM-spinal projections in adult rats following neonatal procedural pain. Quantitative immunohistochemical analysis of the levels of 5-HT staining intensity reveals that early life noxious and tactile procedures decrease 5-HT levels in all laminae of the ipsilateral, but not contralateral spinal dorsal horn. Furthermore, neonatal noxious stimulation increases the percentage area of the RVM stained for 5-HT ipsilateral to the noxious needle pricks, whereas neonatal tactile stimulation increases 5-HT intensity and percentage of stained area in both the ipsi- and contralateral RVM. This study provides important insights into long-lasting anatomical changes in descending serotonergic projections after neonatal noxious or tactile procedures. These changes may underlie the long-term effects after excessive painful stimulation in early life.

Serotonin is a key player in the modulation of nociceptive and non-nociceptive input into the spinal dorsal horn [11]. In adulthood, approximately one-third of all RVM neurons project to the spinal cord through serotonergic projections, terminating in the superficial laminae [16,22]. Consistent with anatomical studies, our study shows that 5-HT staining levels are highest in lamina I of the spinal dorsal horn and gradually decreases in a dorsal to ventral gradient in all animals [23,33]. In adulthood, local release in the spinal dorsal horn results in predominant inhibition of nociceptive signaling [14,34-38]. The reduced spinal 5-HT staining intensity after neonatal noxious procedures in our study suggests a decreased level of 5-HT that could lower the potential for pre- and post-synaptic inhibition in the spinal nociceptive network. The nociceptive network in the spinal cord is known to be under developmental change after neonatal procedural pain [1,3,4]. In fact, nociceptive C-fiber terminal innervation and firing of somatosensory second-order neurons in the spinal dorsal horn are increased after neonatal noxious procedures, suggesting a hypersensitive spinal nociceptive network in adulthood [7,10]. Serotonergic receptors, including the 5-HT1a, are expressed on both C-fiber terminals and second-order projection neurons and have the ability to regulate nociceptive processing the release of 5-HT [14,39]. The decrease in spinal 5-HT staining intensity may contribute to the sensitized spinal nociceptive network after neonatal procedural pain. Despite the observed anatomical and biochemical changes in the spinal nociceptive network after procedural neonatal pain, mechanical sensitivity at baseline is unaltered after neonatal procedural pain [7,40-42]. In line with this, depletion of descending serotonergic projections does not alter mechanical or thermal sensitivity [43-45]. A decrease in spinal 5-HT staining intensity after neonatal procedural pain may contribute

to subthreshold postsynaptic excitability changes that are unmasked by re-injury later in life, resulting in an enhanced duration of mechanical hypersensitivity [7,28,41].

An important observation of our results is that repetitive tactile stimulation also decreases 5-HT staining intensity in the ipsilateral dorsal horn at a level comparable to neonatal painful procedures. Tactile input has the ability to guide nociceptive synaptic organization during early development, even in the absence of noxious input [46]. More importantly, noxious procedures also activate low-threshold touch receptors and play an important role in acute nociceptive signaling and the development of mechanical paw withdrawals [47]. Anatomical localization of 5-HT positive fibers suggests that there may be a relationship between sensory modality and the type of 5-HT innervation it receives [48]. Although serotonergic modulation of spinal processes is directed towards nociceptive processing only in adulthood, descending serotonergic projections from the RVM facilitate both nociceptive as well as non-nociceptive processing in the spinal dorsal horn in a non-modality specificity during the first three weeks of life in rats [22]. This early-life facilitation of descending RVM projections is selective for sensory inputs from A-fibers, but not C-fibers [49]. Hence, both noxious and tactile stimulation in neonates is facilitated by descending serotonergic projections to a similar degree and has the potential to induce similar effects on the spinal nociceptive network. Although neonatal noxious procedures have a distinct behavioral and functional effect, repetitive tactile procedures also increase firing of somatosensory projection neurons upon noxious and non-noxious stimulation in adulthood at baseline, although to a lesser extend [10].

Descending serotonergic modulation of spinal somatosensory and nociceptive processing is mediated via seven 5-HT receptor families [50]. The net effect of changes in 5-HT levels depends on which receptor subtype is activated, to what degree the receptor is activated and on which cells these receptors are located [14,51]. Therefore, their respective balance in the RVM and spinal dorsal may be altered differently after repetitive noxious as compared to tactile procedures. Expression levels of several 5-HT receptors, including the 5-HT1, 5-HT2, 5-HT4 and 5-HT5, are upregulated after neonatal pain in spinal cord but also in the periaqueductal gray, the latter an important regulator of RVM serotonergic modulation [52,53]. Future studies should aim to link the observed anatomical changes related to the development of the descending serotonergic projections to the role and modulation of specific serotonin receptors to explain the long-term effects in following neonatal noxious or tactile procedures.

In the RVM, we observed an increase in the 5-HT-stained area of the ipsilateral RVM in adult animals previously exposed to unilateral neonatal noxious stimulation, without differences in the proportion of 5-HT neurons and staining intensity. The observed increase in 5-HT-stained area might be related to an effect of neonatal noxious stimulation on sprouting of serotonergic fibers in the RVM. How, and to what extend this drives changes in descending modulation is an important venue of future research. Changes in functionality of the descending control from RVM to the spinal nociceptive network have been identified after neonatal skin incision [26] and neonatal inflammation [27], but the role of descending serotonergic fibers in these functionality changes is yet to be investigated. Neonatal injury-induced alterations in RVM descending control were prevented by perioperative anesthetic nerve block at time of injury, indicating the importance of early-life nociceptive input in driving these long-term changes in brain function and pain control [26]. This also suggests that descending serotonergic projections from the RVM are part of an activity-dependent system that is vulnerable to excessive input in early life. As the increase is unilateral to the site of injury, it is likely directly related to the repetitive noxious stimulation. In contrast, the effect of repetitive tactile stimulation on 5-HTstaining intensity in the RVM appears to be more general as both the ipsilateral and contralateral side are affected. Moreover, both 5-HT grayscale values as well as percentage area fraction are increased (see Figure 2). The global effect on both ipsi- and contralateral 5-HT staining intensity in RVM suggests that stress due to maternal separation, rather than unilateral tactile stimulation, is responsible [54,55].

Conclusion

This detailed anatomical study demonstrates that neonatal noxious, but also tactile procedures result in long-lasting anatomical changes of the descending serotonergic projections from brainstem RVM to the spinal dorsal horn. These changes may underlie the long-term effects following painful stimulation in early life.

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5

Selective targeting of serotonin 5-HT_{1a} and 5-HT_3 receptors attenuates acute and long-term hypersensitivity associated with neonatal procedural pain

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Abstract

Neonatal painful procedures causes acute pain and trigger long-term changes in nociceptive processing and anxiety behavior, highlighting the need for adequate analgesia during this critical time. Spinal serotonergic receptors 5-HT_{1a} and 5-HT_3 play an important role in modulating incoming nociceptive signals in neonates. The current study aims to attenuate acute and long-term hypersensitivity associated with neonatal procedural pain using ondansetron (a 5-HT_3 antagonist) and buspirone (a 5-HT_{1a} agonist) in a well-established rat model of repetitive needle pricking. Sprague-Dawley rat pups of both sexes received ondansetron (3 mg/kg), buspirone (3 mg/kg) or saline prior to repetitive needle pricks into the left hind-paw from postnatal day 0-7. Control animals received tactile stimulation or were left undisturbed. Acute, long-term, and post-operative mechanical sensitivity as well as adult anxiety were assessed. Neonatal 5-HT_{1a} receptor agonism completely reverses acute hypersensitivity from P0-7. The increased duration of postoperative hypersensitivity after re-injury in adulthood is abolished by 5-HT_3 receptor antagonism during neonatal repetitive needle pricking, without affecting baseline sensitivity. Moreover, 5-HT_{1a} and 5-HT_3 receptor modulation decreases adult state anxiety. Altogether, our data suggests that targeted pharmacological treatment based on the modulation of spinal serotonergic network via the 5-HT_{1a} and 5-HT_3 receptors in neonates may be of use in treatment of neonatal procedural pain and its long-term consequences. This may result in a new mechanism-based therapeutic venue in treatment of procedural pain in human neonates.

Keywords

Neonate; Procedural pain; Treatment; Serotonin; Ondansetron; Buspirone

Introduction

Newborns admitted to the neonatal intensive care unit (NICU) can be exposed to repetitive nociceptive input during a vulnerable time frame for the central nervous system, as part of their care and treatment. The neonatal period is unique in how it processes nociceptive stimuli, and the nociceptive network undergoes developmental changes in early life [1-3]. Due to its activity-dependent maturation, the nociceptive network develops differently when exposed to excessive noxious input in early life [4-7]. Both clinical and fundamental studies have provided evidence for neonatal painful stimulation induced alterations in the developing nociceptive network, resulting in subsequent long-term alterations in pain sensitivity [3,8-11] and exacerbated sensitivity after re-injury in adulthood [12-17]. Neonatal procedural pain induced effects are not limited to the nociceptive network but also affect anxiety behavior amongst others [18-23], suggesting a common neurocircuitry that regulates both pain and anxiety behavior is involved.

Serotonin (5-hydroxytryptamine, 5-HT) is a key modulator of anxiety behavior and perception of pain, and its ascending and descending fibers are distributed throughout the brain and spinal cord [24]. Descending serotonergic modulation of the spinal nociceptive network, originating in the rostral ventromedial medulla (RVM), is immature in early life and undergoes developmental changes in anatomy and functionality. Importantly, the functionality switches from facilitation to inhibition of nociceptive signaling in early life [25,26]. Since treatment of neonatal pain is still a clinical challenge, analgesic therapy that considers the neurodevelopmental phase may result in more targeted treatment of neonatal procedural pain [27]. During the neonatal period, facilitation of nociceptive signaling is mediated via the 5-HT_3 receptors (R) in the dorsal horn [28-30] whereas the 5-HT_{1a}R plays a role in inhibition of nociception [25,28,29,31,32]. Therefore, activation of the 5-HT_{1a}R or inactivation of the 5-HT_3R in neonates may provide neurodevelopmental relevant analgesia, and could restore injury-evoked disruptions in the normal balance of excitation and inhibition in the spinal dorsal horn [17,33-35]. In addition, early postnatal signaling of the 5-HT_{1a}R is required for normal development of anxiety-related brain areas and behavior [36,37]. Antagonizing the 5-HT_3R in adulthood also shows promising anxiolytic effects [38]. Targeting the 5-HT_3R and 5-HT_{1a}R in newborns may therefore attenuate both nociception and anxiety related long-term consequences of neonatal painful stimuli, while providing adequate acute analgesia.

The aim of this study is to prevent the acute and long-term consequences of repetitive neonatal procedural pain by pharmacological targeting the serotonin 5-HT_{1a}R or

5-HT_3R in neonates. Here, we used buspirone, a full agonist of the 5-HT_{1a}R to produce inhibition [39], or ondansetron, a selective 5-HT_3R antagonist to prevent facilitation [40], in a well-established rat model of neonatal repetitive needle pricking. The effects of these interventions are studied in the context of acute, long-term and post-operative pain (or mechanical sensitivity) as well as adult anxiety.

Materials and Methods

Animals

All experiments and procedures were performed in line with the European Directive for Protection of Vertebrate Animal Used for Experimental and Other Scientific Purposes (2010/63/EU), and were ethically approved by the Committee for Experiments on Animals, Maastricht University, Maastricht, the Netherlands (DEC protocol 2017-017). Adult Sprague-Dawley rats were purchased from Charles River Laboratory (Sulzfeld, Germany), and were mated at Maastricht University. Animals were housed under reversed day-night conditions (12:12hr, lights on 7 p.m. – 7 a.m.), in temperature (21±1°C) and humidity (55 ± 15%) controlled rooms with food and water available ad libitum. Pups were collected and sexed at birth (postnatal (P) day 0), and litters were culled to a maximum of 10 pups to ensure equal caretaking by the dam. Conditions were distributed equally over litters and sexes (Table 1). Pups were weaned at P21 and randomly housed in same-sex groups of two or three in individually ventilated cages for the remainder of the study. The animal order of behavioral testing was randomized, and researchers were blinded to treatment groups throughout the entire experimental protocol. Behavioral testing and surgeries were performed by the same researcher.

Neonatal procedures

On the day of birth (P0) newborn pups were randomized, using a computer-generated randomization list, to one of four conditions; 1. Repetitive needle pricking (needle pricking, NP; n=12), 2. NP with buspirone treatment (NP+BUS; 3 mg/kg; n=12), 3. NP with ondansetron treatment (NP+OND; 3 mg/kg; n=13) or 4. Repetitive tactile stimulation (TC; n=12). A separate litter was left completely undisturbed (UC; n=10). Rat pups (conditions 1-3) were noxiously stimulated with repetitive needle pricks (NP) four times a day at 09:00 a.m., 10:00 a.m., 11:00 a.m. and 12:00 p.m. from P0 to P7 as previously described [13,14]. Each noxious stimulation consisted of a single 2 mm deep needle prick with a 25G needle in the mid-plantar surface of the left hind paw. Age-matched littermates used for the tactile stimulation (TC) (condition 4) received four tactile stimulations by stroking the left hind haw with a cotton tip swab at similar intervals. Drug doses (50 µl) were administered subcutaneously (sc.) 10 minutes before the first and third NP to cover the entire period of repetitive needle pricking. TC and NP animals received sc. saline to control for injection procedures and handling. Mechanical sensitivity was assessed before (baseline, BL) and 1, 3 and 5 hours after the last needle prick or tactile stimulation from P0 to P7, using dorsal

application of calibrated Von Frey filaments (bending force 0.407, 0.692, 1.202, 2.041, 3.63 (from P4 onwards) and 5.495 (from P6 onwards)).

Table 1. Distribution of sex and condition in experimental litters

	UD		TC		NP		NP+BUS		NP+OND	
Litter	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*	*F*	*M*
1			1	1	1	1	3	3		
2			1	1	1	1	3	3		
3			1	1	1	1			3	3
4			1	1	1	1			3	3
5	5	5								
6			2	2	2	2				2

Abbreviations: F, female; M, male; NP, needle prick animals; NP+BUS, needle prick animals receiving buspirone; NP+OND, needle prick animals receiving ondansetron; TC, tactile controls; UD, undisturbed control

Drugs

For 5-HT3R antagonism, the selective 5-HT_3R antagonist Ondansetron (OND; ondansetron hydrochloride dihydrate, Sigma-Aldrich, O3639) was dissolved in sterile saline in a concentration of 2 µg/µl. For 5-HT_{1a}R agonism, the selective agonist Buspirone (BUS, buspirone hydrochloride; Sigma-Aldrich, B7148) was dissolved in sterile saline to a concentration of 10 µg/µl. Pilot studies testing 1, 3 and 5 mg/kg BUS and 0.3, 1 and 3 mg/kg OND showed that 3 mg/kg BUS and 3 mg/kg OND were most promising in preventing acute hypersensitivity associated with neonatal repetitive needle pricking (see figure S1), without adverse side effects (including body weight changes or mortality). Based on pup's body weight per postnatal day, 50 µl solution containing OND (3 mg/kg) or BUS (3 mg/kg) was injected sc. twice daily (at 9 a.m. before the 1st needle prick and 11 a.m. before the 3rd needle prick). TC and NP animals received an equivalent volume of saline.

Developmental assessment

To assess the effect of neonatal 5-HT_{1a}R agonism and 5-HT_3R antagonism on general postnatal development, physical developmental milestones such as pinna detachment (unfolding of the external ear), incisor eruption (of lower and upper teeth), fur development, complete opening of both ears and complete opening of both eyes were expressed as the number of postnatal days required for the appearance of these milestones [41]. These

milestones were assessed every 24h at 1 p.m. (after VF testing to reduce separation), until all pups of a given litter developed all milestones. The surface righting reflex was tested from P1-P5, to assess reflex development and motor function. Pups were placed in a supine position on a flat surface and then released. The righting reflex is defined as the number of seconds required for a newborn pup to turn over on all four limbs, and was considered completed if the pup did so within 30 seconds [42].

Adult re-incision model

To assess acute post-operative hypersensitivity, a plantar hind-paw incision surgery (or Brennan model for postoperative pain) was performed on all animals [43]. Briefly, under isoflurane anesthesia (4-5% induction, 2% maintenance), a 1 cm longitudinal incision through the skin and fascia of the ipsilateral paw was made, followed by the elevation and incision (1-2mm at midline) of the plantaris muscle. The skin was closed using two mattress sutures (5.0 Ethicon Ethylon, Somerville, New Jersey).

Von Frey test for mechanical sensitivity

Mechanical sensitivity was assessed by determining the paw withdrawal thresholds (PWT) of the hind paws to calibrated Von Frey filaments. Briefly, animals were placed in a transparent box resting on an elevated mesh floor. After a 15-minute acclimation period, a series of von Frey filaments (bending forces 1.202, 2.041, 3.63, 5.495, 8.511, 15.136 and 28.84g; Stoelting, USA) were applied to the plantar surface of the hind paw for 5 seconds using the up-down method [44]. Mechanical sensitivity was assessed weekly from 3 to 8 weeks of age, and every other day after adult re-injury (at 1, 3, 5 and 7 days post-incision). The animal order of behavioral testing was randomized, and researchers were blinded to treatment groups during behavioral testing and surgery throughout the entire experimental protocol.

Open Field Test

The open field test was used to evaluate locomotor activity and trait anxiety in adulthood (at 8 weeks of age) [23]. Briefly, individual animals were placed into the center of a plexiglass square arena (100x100x40cm), and allowed to explore the environment for 20 minutes. The arena was thoroughly cleaned with 70% ethanol in between sessions. A camera mounted directly above the maze recorded the behavior of the animal, analyzed by the image tracking system (Noldus Ethovision XT software, Noldus Information Technology, the Netherlands). Variables included time spend in the center (%) and center crossings

(number of entries from and to the center) for the first five minutes, and the total distance travelled (locomotor path in whole arena in cm) for a 20-minute duration.

Elevated Zero Maze

The Elevated Zero Maze (EZM) was used to assess state anxiety behavior [23]. Briefly: a black annular maze (100 cm diameter, 10 cm path width) was placed 70 cm above floor level, with four equal quadrants (two opposite quadrants are "closed" by black Perspex walls, two remaining quadrants are "open"). Animals were placed in an open arm facing one of the closed arms and allowed to explore the maze for 5 minutes. The arena was thoroughly cleaned with 70% ethanol between sessions. An infrared camera mounted directly above the maze recorded the behavior of the animal, analyzed by the image tracking system (Noldus Ethovision XT software, Noldus Information Technology, the Netherlands). Variables included time spend in open and closed arms, latency to enter each arm, and open arm entries. The time spend in the open arms as a percentage of total (corrected) trial time (= total trial duration – latency to enter first closed arm) was classified as anxiety-like behavior.

Statistical analysis

All data are presented as mean ± SEM and plotted using Graphpad Prism 9 (GraphPad Software, San Diego, USA). A P value < 0.05 was considered statistically significant. Mechanical force resulting in a 50% withdrawal frequency was assigned as the PWT and was calculated using a sigmoid curve fitting in Graphpad Prism 9. For the neonatal period, an area under the curve (AUC) analysis was performed. The AUC was calculated based on the PWTs for each group over the entire neonatal period (P0-7) and statistically compared using an unpaired Student t-test. Differences in PWT in the first neonatal week, mechanical sensitivity throughout development, and post-operative mechanical sensitivity were analyzed using a repeated measures analysis of variance (ANOVA) with Bonferroni Post-Hoc correction. Both within- and between-group analysis were run. A non-parametric Kruskal-Wallis tests was used to compare differences in developmental milestones between conditions. A two-way ANOVA was performed to compare the effects of condition and sex on anxiety-like behavioral measurements.

Results

Early postnatal developmental milestones are unaffected by neonatal 5-HT_{1a}R agonism or 5-HT_3R antagonism

Pinnae detachment, lower and upper incisor eruption, fur development, ear opening and eye opening are unaffected by neonatal condition ($p>0.05$; Table 2). In addition, no significant differences between conditions in their surface righting reflex ($F(3, 46) = 0.0232$; $p=0.9951$; Table 3) or weight ($F(3, 46) = 0.6406$; $p=0.5928$; Figure 2) during the first postnatal week are observed.

Table 2. Developmental milestones

	Pinnae detachment	Lower incisors eruption	Upper incisors eruption	Fur development	Ear opening	Eye opening
TC	1.42 ± 0.15	5.00 ± 0.00	9.58 ± 0.26	7.00 ± 0.00	11.17 ± 0.27	13.50 ± 0.23
NP	1.33 ± 0.14	5.00 ± 0.00	9.17 ± 0.24	7.00 ± 0.00	11.17 ± 0.32	14.00 ± 0.25
NP +BUS	1.83 ± 0.17	5.00 ± 0.00	9.92 ± 0.23	7.00 ± 0.00	11.75 ± 0.13	13.17 ± 0.21
NP+ OND	1.43 ± 0.14	5.00 ± 0.00	9.57 ± 0.17	7.00 ± 0.00	11.36 ± 0.20	13.71 ± 0.27

Abbreviations: NP; needle prick animals, NP+BUS; needle prick animals receiving buspirone, NP+OND; needle prick animals receiving ondansetron, TC; tactile controls. Data is presented as mean ± SEM.

Table 3. Surface righting reflex from P1 to P5

	TC		NP		NP+BUS		NP+OND	
	Mean	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*	*Mean*	*SEM*
P1	7.375	1.550	6.091	1.028	6.889	0.809	8.929	1.737
P2	5.091	4.784	9.000	2.603	9.917	2.864	6.214	1.661
P3	5.333	1.912	6.727	1.794	3.333	0.678	3.500	0.749
P4	2.833	0.474	2.083	0.379	2.083	0.229	3.500	1.114
P5	3.166	1.065	2.250	0.579	1.833	0.297	1.500	0.188

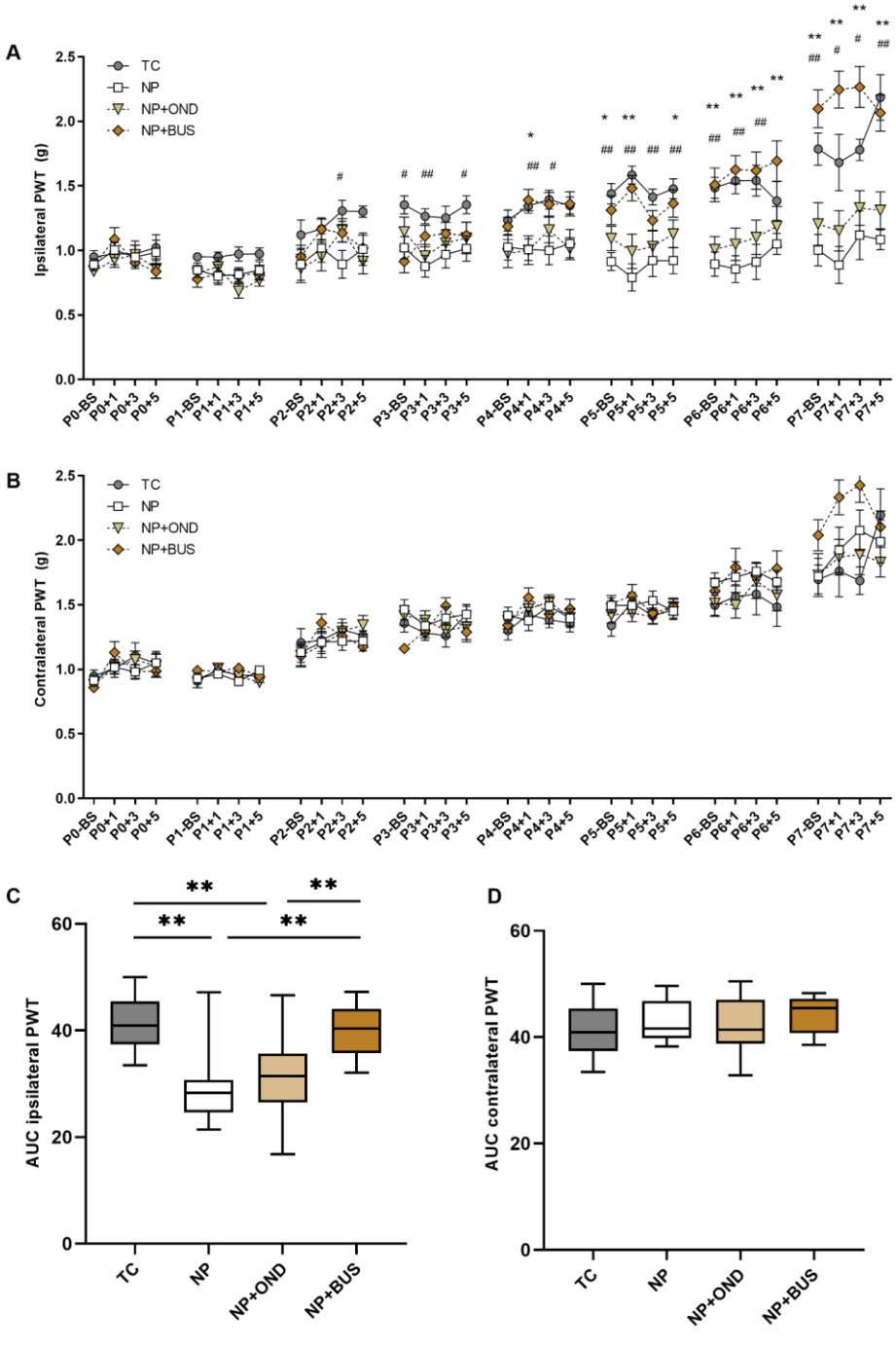
Abbreviations: NP; needle prick animals, NP+BUS; needle prick animals receiving buspirone, NP+OND; needle prick animals receiving ondansetron, P(1-5); postnatal day 1 to 5, TC; tactile controls. Data is presented as mean ± SEM.

Neonatal 5-HT_{1a}R agonism, but not 5-HT_3R antagonism, prevents acute mechanical hypersensitivity after repetitive neonatal needle pricking

During the first postnatal week ipsilateral mechanical sensitivity increases over time, but not equally for all conditions (interaction effect: $F(93, 1426) = 4.798$; $p<0.001$). Post hoc analysis revealed that repetitive NP results in significantly lower PWTs as compared to repetitive tactile stimulation from postnatal day 2 onwards (Figure 1a). Neonatal 5-HT_{1a}R agonism using buspirone reverses the drop in PWTs after repetitive NP in NP+BUS animals from P4 onwards, whereas the drop in PWTs is still present after neonatal 5-HT_3R antagonism using ondansetron (NP+OND; Figure 1a). The AUC analysis over the entire neonatal period reveals a significant decreased ipsilateral AUC in NP animals and NP+OND, as compared to both TC and NP+BUS animals (effect of condition: $F(3, 46) = 10.79$; $p<0.01$; Figure 1b). Contralateral mechanical sensitivity increases over time (effect of time: $F(10.02, 460.7) = 62.68$; $p<0.01$) but is not significantly different between conditions (effect of condition: $F(3, 46) = 0.9085$; $p=0.444$; Figure 1c). Similarly, contralateral AUC are not significantly different between conditions (effect of condition: $F(3, 46) = 0.9818$; $p=0.4096$; Figure 1d).

Neonatal 5-HT_{1a}R agonism, but not 5-HT_3R antagonism, affects mechanical sensitivity during development from weaning to adulthood

During development from 3 to 8 weeks of age, PWT based on mechanical sensitivity to von Frey-filaments significantly increases over time for both paws (ipsilateral, $F(3.048, 164.6) = 105.2$, $p<0.01$; contralateral, $F(23.319, 179.2) = 81.69$; $p<0.01$). The PWT increase over time seems dependent upon neonatal condition for the ipsilateral (interaction effect: $F(20, 270) = 2.865$, $p<0.01$; Figure 3a) as well as the contralateral paw (interaction effect: $F(20, 270) = 2.104$, $p<0.01$; Figure 3b). NP+BUS animals show significantly lower ipsilateral PWTs compared to other groups at various time points: 3 weeks (NP+BUS 1.824 vs NP 2.992; vs NP+OND 3.411; vs TC 3.189; vs UD 3.576), 4 weeks (NP+BUS 3.103 vs NP 3.626; vs NP+OND 5.565; vs TC 3.908; vs UD 4.770) and 5 weeks (NP+BUS 3.876 vs NP+OND 5.925). Contralateral PWTs of NP+BUS animals are significantly lower at 3 weeks (NP+BUS 1.787 vs NP 3.103; vs NP+OND 3.658, vs UD 3.642) and 4 weeks (NP+BUS 2.876 vs NP+OND 5.538, vs UD 4.889). This effect ceases as NP+BUS animals grow older (>4-5 weeks of age). In addition, PWT of both hind-paws are significantly increased in NP and TC animals as compared to UD animals at 8 weeks of age (UD 6.682 vs NP 9.622; vs TC 10.90), suggesting differences in PWTs before incision.


A
TC
NP
NP+OND
NP+BUS
Ipsilateral PWT (g)
B
Contralateral PWT (g)
P0-BS
P0+1
P0+3
P0+5
P1-BS
P1+1
P1+3
P1+5
P2-BS
P2+1
P2+3
P2+5
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P5+3
P5+5
P6-BS
P6+1
P6+3
P6+5
P7-BS
P7+1
P7+3
P7+5
C
AUC ipsilateral PWT
D
AUC contralateral PWT
TC
NP
NP+OND
NP+BUS

Figure 1. Mechanical sensitivity to von Frey filaments during first neonatal week. A. Repetitive needle pricking (NP; n=12) results in a decreased paw-withdrawal threshold (PWT) in the ipsilateral paw as compared to repetitive tactile stimulation (TC; n=12) from postnatal day (P) 2 onwards, indicating the development of acute mechanical hypersensitivity in NP pups. Neonatal 5-HT_{1a}R agonism (NP+BUS; n=12), but not 5-HT_3R antagonism (NP+OND; n=14), is able to reverse the acute mechanical hypersensitivity after repetitive needle pricking (interaction effect: F (93, 1426) = 4.798; p<0.001). **B.** The area under the curve (AUC) over the entire neonatal period is significantly different between neonatal conditions for the ipsilateral paw-withdrawal thresholds (PWT) (F (3, 46) = 10.79; p<0.01). NP as well as NP+OND animals show a decreased AUC as compared to TC animals, whereas NP+BUS reverses the decreased AUC back to TC control values. **C.** Mechanical sensitivity of the contralateral paw increases over time all groups, but was not affected by neonatal condition (F (3, 46) = 0.9085; p=0.444). **D.** The area under the curve analysis (AUC) over the entire neonatal period does not show significant differences in contralateral PWTs between conditions (F (3, 46) = 0.9818; p=0.4096). Abbreviations: BS; baseline measurement, NP; needle pricking animals, NP+BUS; needle prick animals receiving buspirone, NP+OND; needle prick animals receiving ondansetron, PWT; paw withdrawal threshold, P(0-7); postnatal day 0 to 7, +1, +3, +5; measurement 1, 3 and 5 hours after needle prick or tactile stimulation, TC; tactile controls. Data is presented as mean ± SEM, NP vs NP+BUS *P <0.05 ** P<0.01, NP vs. TC # P<0.05 ## P<0.01.

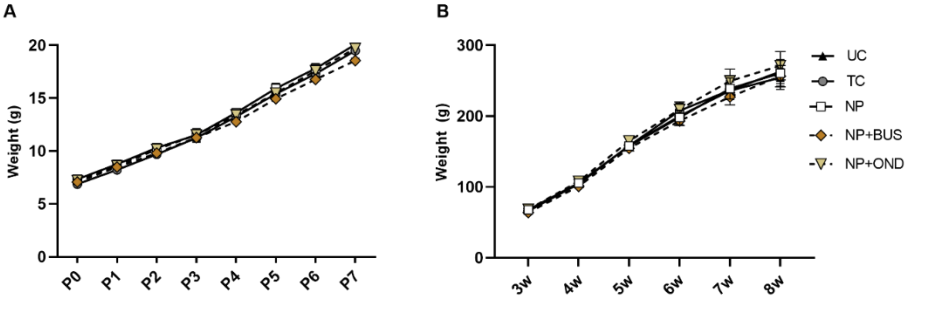

Figure 2. Weight during the first postnatal week and during development from 3-8w of age. A. Weight during the first postnatal week significantly increases over time (F (2.541, 116.9) = 2628.0; p<0.01), but is not significantly different between conditions (F (3, 46) = 0.6406; p=0.5928). **B.** Weight increases significantly with time during development (F (1.105, 59.65) = 579.6; p<0.01) but does not significantly differ between conditions (F (4, 54) = 0.3339; P=0.8540). Abbreviations: NP, needle prick animals; NP+BUS, needle prick animals receiving buspirone; NP+OND, needle prick animals receiving ondansetron; PWT, paw withdrawal threshold; TC, tactile control animals; UD, undisturbed control animals. Data is presented as mean ± SEM, *P <0.05 ** P<0.01.

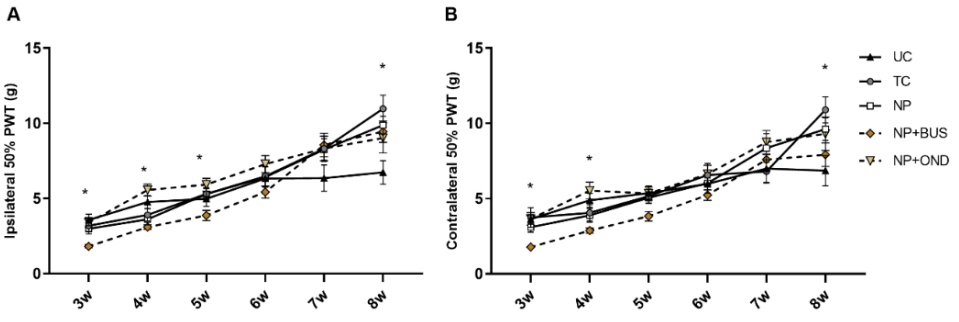

Figure 3. Mechanical sensitivity to von Frey filaments throughout development. A. Ipsilateral PWTs increase over time for all groups (F (3.048, 164.6) = 105.2, p<0.01), but show a significant time*condition interaction (F (20, 270) = 2.865, p<0.01). Post-hoc analysis revealed a lower PWTs for the NP+BUS animals as compared to NP (at 3 and 4 weeks), NP+OND (at 3, 4 and 5 weeks), TC (at 3 weeks) and UD animals (at 3 and 4 weeks). NP and TC animals show higher PWTs at 8 weeks as compared to UD animals. **B.** Contralateral PWTs increase over time for all groups (F (23.319, 179.2) = 81.69; p<0.01), but show a significant time*condition effect (F (20, 270) = 2.104, p<0.01). Lower PWTs for the NP+BUS animals as compared to NP (at 3 weeks), NP+OND (at 3 and 4 weeks) and UD (at 3 and 4 weeks) are noted. Significantly higher PWTs are noted in NP and TC animals as compared to UD animals at 8 weeks. Abbreviations: NP, needle prick animals; NP+BUS, needle prick animals receiving buspirone; NP+OND, needle prick animals receiving ondansetron; PWT, paw withdrawal threshold; TC, tactile control animals; UD, undisturbed control animals. Data is presented as mean ± SEM, *P <0.05 ** P<0.01.

NP+OND animals do not show any differences in mechanical sensitivity from 3-8 weeks of age (p>0.05).

Neonatal 5-HT_{1a}R agonism, but not 5-HT_3R antagonism, affects mechanical sensitivity during development from weaning to adulthood

The effect of re-injury to the same dermatome on the PWT is measured in adulthood from 1-day post-incision (PI+1) to 7 days post-incision (PI+7). All animals develop acute hypersensitivity 1 and 3 days after incision, however a significant interaction effect of time*condition is observed (F (16, 216) = 2.915; p<0.01; Figure 4). Within group analysis shows that UD PWTs return to pre-incision values by 5 days post-incision, whereas NP and TC animals return to baseline values by 7 days post-incision. This increased duration of mechanical hypersensitivity after re-injury is abolished in NP+OND, but not NP+BUS animals, indicated by a recovery to pre-incision values at PI+5 rather than PI+7.

A significant interaction between time and condition is noted in contralateral PWTs ($F(16, 216) = 1.891$; $p=0.0225$), with UD animals showing lower contralateral PWTs at PI+7 as compared to NP, NP+BUS and NP+OND animals. The injury-induced change from baseline PWTs is not significantly affected by condition and only shows a significant effect of time ($F(2.877, 155.3) = 5.099$; $p=0.0025$).

Neonatal 5-HT_{1a}R and 5-HT_3R modulation affects adult state anxiety behavior in males but not females

In the OFT, assessing trait anxiety, no significant effect of condition ($F(4, 49) = 1.972$; $p=0.1135$) or sex ($F(1, 49) = 0.001$; $p=0.9772$; Figure 5b) on the percentage of time spend in the center of the arena is observed. In addition, the number of center crossings does not significantly differ between sex ($F(1, 19) = 0.3304$; $p=0.5681$) or condition ($F(4, 49) = 0.8757$; $p=0.4853$; Figure 5d). Locomotor activity significantly differs between males and females ($F(1, 49) = 21.20$, $p<0.01$) but is unaffected by neonatal condition ($F(4, 49) = 1.177$; $p=0.3325$).

In the EZM, assessing state anxiety, the percentage of time spend in the open arms of the EZM significantly differs between males and females (effect of sex: $F(1, 49$; Figure 5a$) = 19.75$; $p<0.01$). Females do not show differences between conditions in the time spend in the open arms of the EZM (effect of condition: $F(4, 24) = 0.2669$; $p=0.8963$). In males, UD spend significant less time in the open arms of the EZM as compared to NP+BUS and NP+OND animals (effect of condition: $F(4, 25) = 3.545$; $p=0.0201$). The number of open arm entries in the EZM reveals a significant effect of sex ($F(2, 49) = 16.62$; $p<0.01$) and condition ($F(4, 49) = 2.788$; $p=0.0365$; Figure 5c). NP+BUS animals exhibit significantly more open arm entries as compared to UD animals, in males only (NP+BUS 13.50 vs UC 6.00; $P=0.0266$).

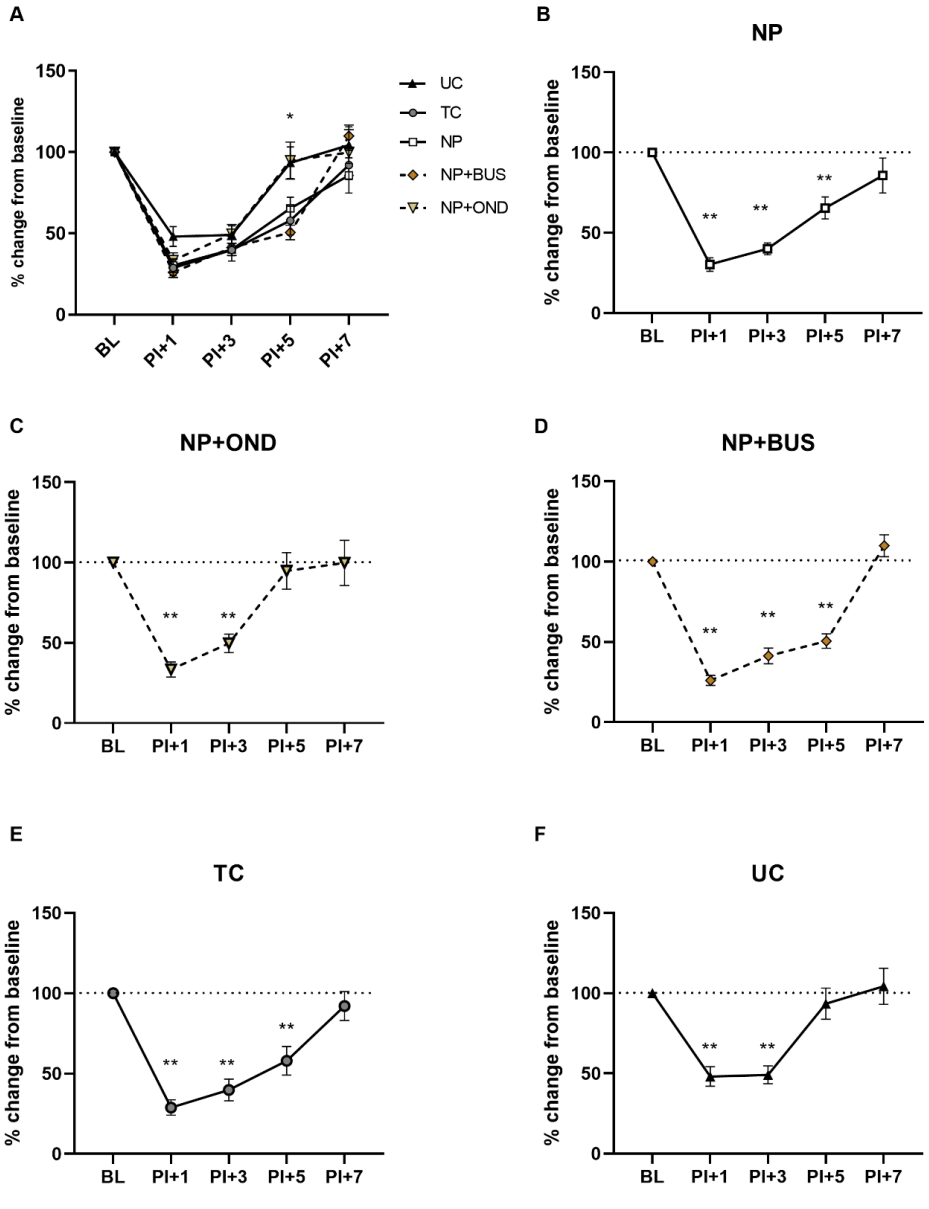
A
% change from baseline
150
100
50
0
BL
PI+1
PI+3
PI+5
PI+7
UC
TC
NP
NP+BUS
NP+OND
*
B
NP
**
C
NP+OND
D
NP+BUS
E
TC
F
UC

Figure 4. Mechanical sensitivity after re-injury in adulthood. A. All groups show mechanical hypersensitivity 1 and 3 days after incision in adulthood, but differences emerge between groups at 5 and 7 days post-incision. Return to pre-operative base-line PWT levels is noted in UD animals by 5 days postoperative **(F),** while PWTs of NP **(B)** and TC animals **(E)** are recovered to pre-operative baseline by 7 days postoperative. NP+OND **(D)**, but not NP+BUS **(C),** abolishes this longer recovery time after incision. Abbreviations: BL; baseline, NP, needle prick animals; NP+OND, needle prick animals receiving ondansetron; NP+BUS, needle prick animals receiving buspirone; PI; paw incision, +1, +3, +5; measurement 1, 3 and 5 days after paw incision, TC, tactile stimulation animals; UD, undisturbed control animals. Data is presented as mean ± SEM, *P <0.05 ** P<0.01 as compared to their respective baseline.

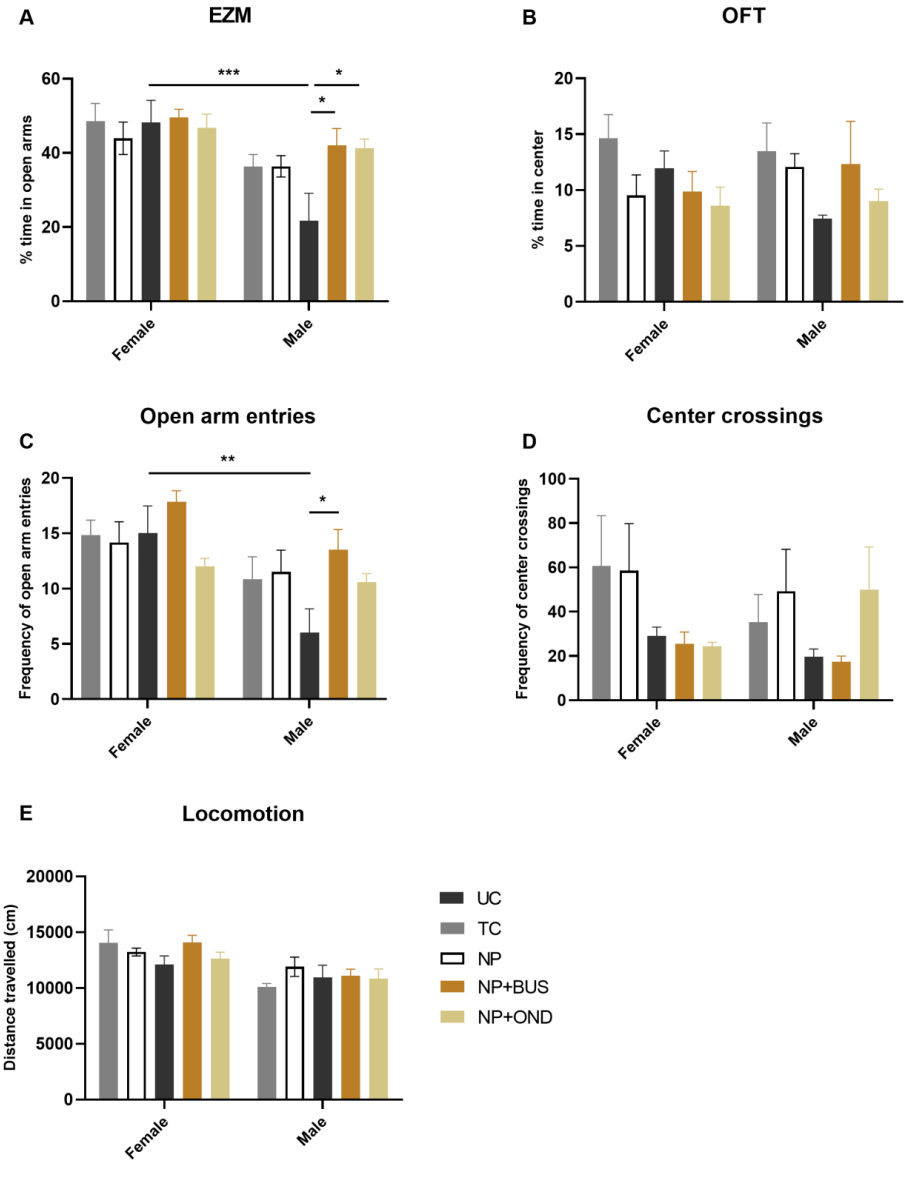
A
EZM
% time in open arms
60
40
20
0
***
*
*
Female
Male
B
OFT
% time in center
20
15
10
5
0
Female
Male
Open arm entries
C
Frequency of open arm entries
20
15
10
5
0
**
*
Female
Male
Center crossings
D
Frequency of center crossings
100
80
60
40
20
0
Female
Male
E
Locomotion
Distance travelled (cm)
20000
15000
10000
5000
0
Female
Male
UC
TC
NP
NP+BUS
NP+OND

Figure 5. Neonatal conditions and adult anxiety behavior. A. The percentage of time spend in the anxio-genic (open arm) region of the elevated zero maze (EZM) showed a significant difference between males and females ($F(1, 49) = 19.75$; $p<0.01$). No differences between conditions are observed in females, whereas UD males spend significant less time in the open arms as compared to all other neonatal conditions ($F(4, 25) = 3.545$; $p=0.0201$). **B.** The percentage of time spend in the anxio-genic (centre) region of the open field test (OFT) is not influenced by sex or condition**. C.** The frequency of open arm entries is significantly affected by condition ($F(4, 49) = 2.788$; $p=0.0365$) and sex ($F(2, 49) = 16.62$; $p<0.01$). UD animals exhibit significantly less open arm entries as compared to NP+BUS animals in males only. **D.** The number of centre crossings in the OFT is not affected by sex ($F(1, 19) = 0.3304$; $p=0.5681$) or condition ($F(4, 49) = 0.8757$; $p=0.4853$). **E.** Locomotor activity significantly differs between males and females ($F(1, 49) = 21.20$, $p<0.01$) but is unaffected by condition ($F(4, 49) = 1.177$; $p=0.3325$). Abbreviations: NP, needle prick animals; NP+OND, needle prick animals receiving ondansetron; NP+BUS, needle prick animals receiving buspirone; TC, tactile stimulation animals; UD, undisturbed control animals. Data is presented as mean ± SEM, *P <0.05 ** P<0.01 *** P<0.001.

Discussion

Neonatal repetitive needle pricking leads to acute hypersensitivity and increased duration of post-operative recovery after re-injury in adulthood [12-17,23]. Here, we show that selective neonatal agonism of the 5-HT_{1a}R using buspirone reduces the acute hypersensitivity associated with neonatal procedural pain, whereas antagonizing the 5-HT_3R using ondansetron attenuates the long-term consequences of neonatal procedural pain. Moreover, modulation of both neonatal 5-HT_{1a}R and 5-HT_3R is associated with decreased adult state anxiety. Altogether, our data suggests that targeted pharmacological treatment directed at the serotonergic 5-HT_{1a} and 5-HT_3 receptors in neonates is possible and may be of use in treatment of acute neonatal procedural pain and its long-term consequences.

The 5-HT_3R and 5-HT_{1a}R are important in a variety of neurodevelopment processes [24,45]. This implies that early pharmacological interventions with buspirone or ondansetron may affect normal development of rat pups. For this reason, we controlled for major developmental milestones and possible side-effects in our study. Our results show that major developmental milestones, reflex development, weight and locomotor behavior were unaffected by early life repetitive procedures or 5HT_3R and 5-HT_{1a}R modulation, in line with earlier studies [46-50].

The observation that neonatal 5-HT_{1a}R agonism using buspirone reverses acute hypersensitivity after repetitive needle pricking in rat pups can be explained by its inhibitory role in spinal neurotransmission, previously shown in neonatal dorsal horn cells [25]. Here we show for the first time that this system can be used *in vivo* to attenuate sensitivity after noxious stimuli in neonatal animals, suggesting the inhibitory effect of the 5HT_{1a}R is present and functional during the neonatal period. This is important as descending serotonergic RVM-spinal dorsal horn have been reported to act in a facilitatory way in neonates [26]. In addition, the anti-nociceptive effect of 5-HT_{1a}R becomes more apparent from postnatal day 4 onwards, and this coincides with progressive increase in acute hypersensitivity with cumulative exposure to repetitive needle pricking [14-16,23]. 5-HT_{1a}R mediated binding, via the use of selective agonists, can inhibit primary-afferent evoked responses as well as postsynaptic dorsal horn responses in neonates [28,29,31,32], thereby restoring neonatal procedural pain-evoked disruptions in the normal balance of excitation and inhibition in the spinal dorsal horn [17,33-35]. 5-HT_{1a}R is expressed on 70-80% of RVM neurons in neonatal rats [51], and may also mediate its anti-nociceptive effects by blocking descending serotonergic facilitation from the RVM observed in younger animals [25].

Our data indicates that acute hypersensitivity in rat pups exposed to neonatal procedural pain is likely not mediated via the 5-HT_3R, as the neonatal 5-HT_3R antagonist ondansetron showed no behavioral effect in our study. This is remarkable, as descending serotonergic release from the RVM are thought to play an important role in 5-HT_3R mediated facilitation of nociceptive signaling up to P21 in rats [26,52-54]. 5-HT_3R expression in the dorsal horn has been reported to be stable from P7 up to adulthood in rodents [26], but expression patterns are yet to be documented for the first postnatal week. Activation of neonatal 5-HT_3R activation *in vitro* is involved in facilitation of most dorsal horn cells, although the level of facilitation remains modest [25,28,29]. Moreover, non-noxious mechanical stimuli, as used in this study, are not facilitated by descending serotonergic projections at P8 [26]. In infant rats at P21, ondansetron reduces dorsal horn activity in response to nociceptive, non-nociceptive stimuli and mechanical stimuli [26]. Thus it is likely, that although 5-HT_3R antagonism may induce direct changes in the physiological response to nociceptive and non-nociceptive stimuli in neonates, this does not translate to a behavioral effect of 5-HT_3R antagonism on mechanical hypersensitivity after neonatal procedural pain based on von Frey testing as in our model.

Neonatal procedural pain has been associated with changes in pain sensitivity that persist beyond infancy, in both clinical and preclinical settings [4-7]. Here, we show that 5-HT3R antagonism during neonatal procedural pain prevents the increased duration of neonatal injury-induced postoperative hypersensitivity in adulthood (Figure 4). This suggests that the underlying injury-induced alteration in nociceptive pathways are regulated, at least in part, by neonatal 5-HT_3R activation. The 5-HT_3R plays a prominent role in network formation and function of the neonatal brain [45]. Within the nociceptive network, 5-HT_3R are located on primary afferent fibres, glutamate terminals and projection neurons in the dorsal horn [55-58]. Neonatal injury increases the excitatory drive onto spinal sensory neurons, resulting in enhanced glutamate signaling [35,59,60], a process known to be of pivotal importance in sensitization of the nociceptive network [61]. Descending serotonergic projections facilitate noxious spinal processing via the 5-HT_3R in the dorsal horn in early life [26]. Hence, the blocked facilitation of 5-HT_3R during early neonatal procedural pain is likely to underlie the effect of ondansetron at later stages.

In contrast to the acute anti-nociceptive effect, neonatal 5-HT_{1a}R agonism did not affect extended post-operative recovery in adulthood in our study. Chronic treatment with selective 5-HT_{1a}R agonists for 7 days, like in our study, desensitizes 5-HT_{1a} auto-receptors, leading to impaired functioning of this receptor [62-64]. Likewise, substance P that is released upon nociceptive C-fibre activation, likely to occur after repetitive needle pricking, also

desensitizes 5-HT_{1a}R and may impact receptor functioning [65,66]. Future studies should include anatomical as well as functional analysis to detect changes in 5-HT_{1a}R functioning after neonatal targeting. During development, we observed a temporary increase in mechanical sensitivity in NP animals when exposed to neonatal 5-HT_{1a}R agonist buspirone that ceased with increasing age (>5 weeks). Repetitive activation of the 5-HT_{1a}R from P0 to P7 may result in long-term depression [28], leading to altered synaptic plasticity in the spinal nociceptive circuit which consequently may alter basal nociceptive behavior.

Serotonin signaling also regulates the normal development of emotional behavior, including anxiety [24]. Hence, sensitive windows in development through which serotonergic signaling is altered may differentially encode anxiety behaviors. Neonatal 5-HT_{1a}R agonism decreased adult male state anxiety in the EZM as compared to undisturbed males, but did not affect trait anxiety in the OFT (Figure 5). 5-HT_{1a}R have been reported to be central players in controlling anxiety [36], and thus interference with early postnatal functioning of the 5-HT_{1a}R (buspirone) may affect normal anxiety behavior [36,37]. Neonatal 5-HT_3R antagonism using ondansetron also shows some anxiogenic properties in males in the EZM, albeit small. Previous studies on the role of 5-HT_3R antagonism related to anxiety remain contradictory [38,67], Of note, our results demonstrate the absence of any difference in adult state and trait anxiety levels between TC and NP animals and neonatal 5-HT_{1a}R or 5-HT_3R modulation, suggesting receptor modulation does have an additive effect on anxiety. Interestingly as related to state anxiety (EZM), a significant difference between sex in state anxiety is noted that was not observed or assessed in earlier studies using a similar neonatal model [20,22,23,49]. Lower levels of state anxiety in females compared to males, predominantly observed in the undisturbed animals in our study, is not uncommon in in rodent models that use both sexes [68].

The current study is not without limitations. First, the specificity of buspirone and ondansetron as pharmacological modulators of the 5-HT_{1a}R and 5-HT_3R, respectively, is of importance. Buspirone has high affinity for 5-HT_{1a}R but also increases dopamine levels via D_2 inactivation as well as noradrenaline levels via α_2R in the frontal cortex [69,70]. Therefore, buspirone-mediated effects in our study may not be uniquely related to 5-HT_{1a}R functioning, but can also be (partially) the result of D_2R or α_2R binding. Ondansetron is a potent, highly selective antagonist of the 5-HT_3R [71]. Although it shows some affinity for 5-HT_{1b}, 5-HT_4, opioid and α1-adrenergic receptors, it has a selectivity of 1000:1 towards 5-HT_3R [40]. Hence, the effects of ondansetron as observed in this study are most likely exclusively mediated by 5-HT_3R binding. Next, ondansetron and buspirone were administered systemically and this limits the interpretation of location-specific effects in the

spinal cord nociceptive network. Future studies should include local intrathecal administration to further specify the involvement of spinal 5-HT_{1a}R and 5-HT_3R in the observed effects. Lastly, baseline differences between animals exposed to repetitive needle pricking or tactile stimulation and undisturbed animals at 8 weeks of age, prior to re-incision were observed. Nevertheless and based on within-group analysis any possible effects related to the differences in baseline value differences on post-operative mechanical sensitivity can be excluded [14-16].

Conclusion

Our data shows that neonatal targeted pharmacological modulation of the 5-HT_{1a}R and 5-HT_3R attenuates the acute and long-term effects associated with neonatal procedural pain. These results may form the fundament of a new mechanism-based therapeutic venue in treatment of procedural pain in human neonates.

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Supplemental data

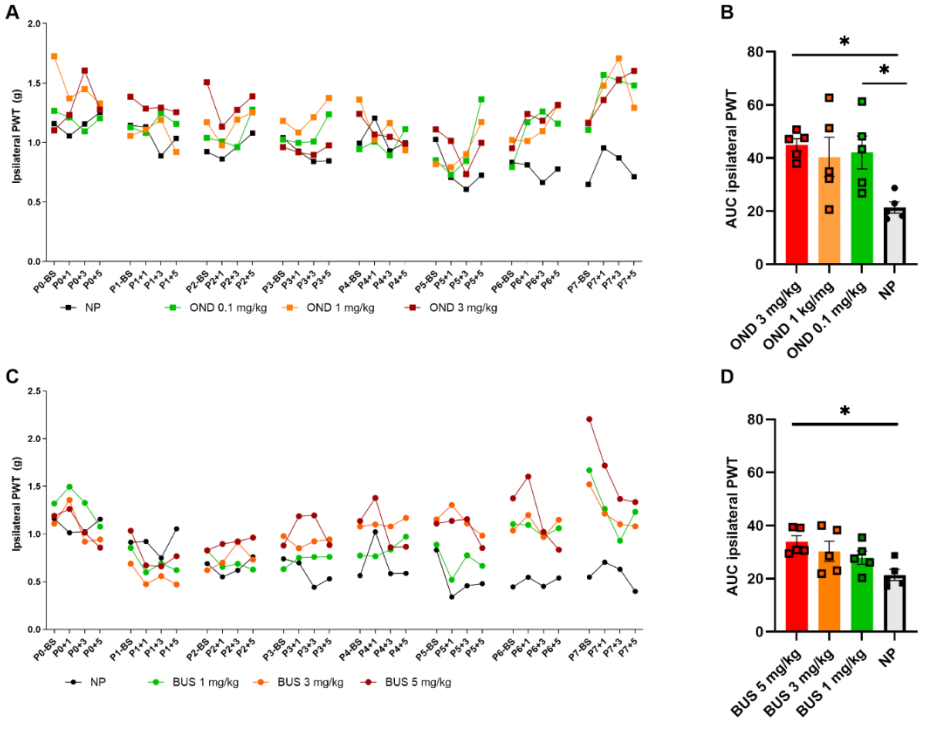

Figure S1. Dose-response pilots of ondansetron (0.1, 1 and 3 mg/kg) and buspirone (1, 3 and 5 mg/kg). A. PWTs increase over time, but not equally for all conditions ($F(93, 636) = 4.841$; $p<0.01$). Repetitive needle pricking (NP; n=12) results in a decreased paw-withdrawal threshold (PWT) over time, that was significantly different from OND 3 mg/kg from P5 onwards. **B.** The area under the curve (AUC) over the entire neonatal period is significantly different between neonatal conditions for the ipsilateral paw-withdrawal thresholds (PWT) ($F(3, 16) = 4.432$; $p=0.0189$). AUC was significantly increased in NP animals after administration of 0.3 mg/kg ($p=0.0329$) and OND 3 mg/kg ($p=0.0143$), as compared to NP animals receiving saline. **C.** PWTs increase over time, but not equally for all groups ($F(93,496) = 2.600$; $p<0.01$). Repetitive needle pricking (NP) results in decreased PWTs over time, that was significant different from BUS 1 mg/kg from P5 onwards, from BUS 3 mg/kg from P4 onwards and BUS 5 mg/kg from P3 onwards. **D.** The AUC over the entire neonatal period is significantly different between conditions ($F(3, 16) = 3.745$; $p=0.0327$). NP animals receiving BUS 5 mg/kg showed significantly increased AUC as compared to NP animals receiving saline ($p=0.0151$). Abbreviations: BS; baseline measurement, NP; needle pricking animals, BUS; buspirone, OND; ondansetron, P(0-7); postnatal day 0 to 7, +1, +3, +5; measurement 1, 3 and 5 hours after needle prick or tactile stimulation. Data is presented as mean ± SEM, NP vs NP+BUS *P <0.05 ** P<0.01, NP vs. TC # P<0.05 ## P<0.01.

General discussion

6

This academic thesis builds upon the available literature of the acute and long-term effects of repetitive neonatal procedural pain. Although significant advances have been made in the understanding, recognition and management of neonatal procedural pain, many questions remain that cannot be easily answered in human neonates [1]. In 1999, a rat model of repetitive neonatal needle pricking in the hind paws was developed to mimic the experience of heel sticks for blood sampling in human neonates, a common painful procedure in the neonatal intensive care unit (NICU) [2]. After its introduction it is now widely recognized as a robust and well-validated model for neonatal procedural pain-induced effects [3-17]. By not only standardizing the depth of needle pricking to 2mm, but also the application of repeated needle pricking in the same left hind paw from P0 to P7, our laboratory optimized the existing model [3]. This model shows that repetitive needle pricking in the same hind paw results in plasticity changes of the spinal nociceptive network which in turn leads to acute and long-term effects on mechanical sensitivity after inflammatory or surgical re-injury [3,11,13,14]. Plasticity changes in the spinal nociceptive network were evident by an increase in nociceptive-specific primary afferents, altered expression μ opioid receptors [18], and increased excitability of sensory neurons in the spinal dorsal horn [6]. Although research has made big steps towards understanding the injury-induced plasticity in developing pain pathways as well as the long-term effects of procedural pain in newborn babies, further optimization of current pharmacological pain management strategies is needed. Clinically relevant pharmacological treatments during this phase of repetitive neonatal needle pricking, such as paracetamol (or acetaminophen) or methadone, were tested for their potential not only to minimize the acute but also attenuate the long-term consequences of neonatal procedural pain [13,19]. Despite the fact that these treatments show promising results, a lack of an acute effect for paracetamol and the fear of side-effects associated with methadone hampers its use for pain management in the NICU[20,21]. In this thesis, we focused on the role of descending serotonergic projections as an important component in the mechanism underlying the acute and long-term consequences of repetitive neonatal procedural pain on later life pain and anxiety. The first part of this general discussion is related to the four research questions as formulated in the introduction **(Chapter 1)** and investigated in **Chapter 1-5** of this thesis. Following the research questions future perspectives are addressed. The discussion ends with general concluding remarks.

RQ1: What are the long-term effects of repetitive neonatal procedural pain on adult trait and state anxiety?

The first research question is answered in **Chapter 2** *"Neonatal procedural pain affects state, but not trait anxiety behavior in adult rats"*. The experiments described in this Chapter used the open field test (OFT) and Elevated Zero Maze (EZM) to study trait and state anxiety respectively in adulthood in a well-established rat model of repetitive neonatal needle pricking. First the reproducibility of this rat model of neonatal procedural pain was confirmed [3,13,14,22], as we show robust hypersensitivity in neonates without changes in baseline mechanical sensitivity after repetitive neonatal needle pricking. Then our study shows that neonatal procedural pain differentially affects adult anxiety resulting in lower state anxiety behavior as compared to controls without differences in trait anxiety. This effect of neonatal procedural pain points to a distinct profile of anxiety (state or trait) affected. Anxiety is defined as a sustained response to temporally uncertain danger, and features include anticipation of and preparation for future harm [23,24]. Behaviorally anxiety is associated with avoidance, reduced exploratory behavior, risk assessment behaviors as well as heightened vigilance and apprehension that can be assessed across species [25,26]. Whereas trait anxiety reflects the predisposition towards becoming anxious and is an enduring feature (innate), state anxiety indicates anxiety experienced in a particularly anxiety-provoking situation [25]. When investigating the effect of repetitive needle pricking on the type of anxiety, measured in different validated behavioral constructs, state anxiety but not trait anxiety seems to be affected in rodents in line with our findings [2,4,7,10,16,17]. This provides more clarity in the contradicting evidence when unstructured in the type of anxiety measured. Patients suffering from anxiety disorders tend to have more anxious traits as compared to healthy subjects while state anxiety is unrelated [27]. This is similar in school-aged children where trait anxiety was correlated to anxiety disorder symptoms [28]. The latter suggests that differences in state anxiety after early life pain exposure as described in Chapter 2 do not necessarily translate to increased risk to develop clinical anxiety disorders or related symptoms at later stages.

In the neonatal intensive care unit (NICU) pain is not the only stressor that can affect changes in neurodevelopment. Preterm birth as well as lower body weight at birth and lower gestational age are all risk factors for developing anxiety disorders [29-33]. In a clinical setting of neonatal pain it remains a challenge to differentiate the effects of procedural pain from those related to stress and discomfort in neonates on later life affective disorders. Research groups, including those of Grunau and colleagues, have opted

to refer to this experience as neonatal pain-related stress instead [34,35]. Testing different profiles of anxiety in former premature infants exposed to neonatal procedural pain during different stages of development would provide us with important insights whether differences in state or trait anxiety do exists, an area hardly investigated in a structured manner. Spielberger and colleagues have provided an important groundwork for the assessment of state and trait anxiety in healthy and clinical populations via the use of the state-trait anxiety inventory [36]. The latter may help identify somatosensory and affective factors that influence pain experience and future risks or both.

In adulthood anxiety disorders and (chronic) pain share a strong co-morbidity. This is not surprising: both signal impending danger and necessity for action that are essential for survival. Adaptations in anxiety often arises as a consequence of chronic pain or can be an important risk factor in the exacerbation of pain experiences and chronification of postoperative pain [37-39]. Central to biological functions in both anxiety disorders as well as in (chronic) pain is serotonin (5-hydroxytryptamine (5-HT)), especially in brain areas and connections involving limbic and brainstem circuits. The neural substrate of both pain processing as well as (state and trait) anxiety constitutes of similar brain regions. These include the anterior cingulate cortex, thalamus, prefrontal cortex, amygdala and nuclei in the brainstem which are all innervated by 5-HT and its projections [40-44]. State anxiety is related to functional brain connectivity in these regions whereas structural neural features are linked to state anxiety [43,44]. Neonatal procedural pain has been shown to shape the plasticity and development of these brain regions [33,45-48], suggesting that neonatal procedural pain alters the underlying pathways in both affective and nociceptive processing and 5-HT may play an important role. The spinal dorsal horn is the first and most important level of central nociceptive processing [49]. The descending serotonergic pathways originating in the rostral ventromedial medulla (RVM) play a key role in maintaining the balance of excitation and inhibition in the spinal dorsal horn and regulate anxiety-induced changes in pain behavior [50,51]. The descending serotonergic pathway from the RVM to the spinal dorsal horn may provide a first read-out of injury-induced plasticity in supraspinal areas involved in processing of both pain and anxiety. Before experiments focused on descending serotonergic projections and their role in modulation of the spinal nociceptive network after neonatal procedural pain, a complete and detailed understanding of the physiological development of descending serotonergic projections and their modulation of the spinal nociceptive network is needed (see RQ 2 in Chapter 3).

RQ2: What do we know of the physiological development of the descending rostral ventromedial medulla-spinal dorsal horn projections and its modulatory role on the spinal nociceptive network?

In a review paper in **Chapter 3** *"The development of descending serotonergic modulation of the spinal nociceptive network: a life span perspective"*, the second research question is answered and all available literature on the normal physiological development of the descending serotonergic system from the rostral ventral medulla (RVM) to the spinal dorsal horn in rodents is reviewed and presented. Studies in laboratory animals are imperative to understand the development of descending serotonergic projections. During the neonatal and pre-weaning phase, changes in the anatomy of 5-HT RVM neurons projecting to the spinal cord are reported, as well as local sprouting of descending serotonergic projections from the ventral to dorsal spinal cord in rodents. Electrophysiological studies showed that spinal 5-HT mediated effects are functional at an early age. Facilitation of spinal nociception is mediated via the 5-HT_3 and 5-HT_7 receptor and inhibition via the 5-HT_{1a}, 5-HT_{1b} and 5-HT_2 receptor. In adulthood the activation of all 5-HT receptors results in a complex interplay of either facilitation or inhibition of acute spinal nociceptive signaling. Our review is structured around various phases throughout the life span of rodents including the neonatal, pre-weaning and adult phases. The next step is to translate these preclinical findings in rodents to the descending serotonergic system in humans. The developmental phase of a newborn rodent corresponds with human neonates during second and third trimester, whereas pre-weaning represents neuronal development of human term born baby up to infancy [52-54]. This classification is based upon the developmental timing in both rodents and humans, and includes developmental events within the 5-HT system [52]. When aligning developmental phases of rodent and humans one has to keep in mind that the developmental timing may differ between different brain structures (i.e. spinal cord and brainstem vs. cerebral cortex) [52] or the specific developmental process investigated [53]. As presented in Chapter 3 the predominant changes in anatomy of descending serotonergic projections occur between P7 and P21. This corresponds to term birth up until early infancy in humans. Similarly, functionality of descending serotonergic modulation switches from facilitation to inhibition of spinal nociception after P21 in rodents and thus implying a high level of neuroplasticity at a developmental timing that is similar to human birth to early infancy [55]. This highlights important developmental differences in function at different developmental periods in rodents, and the need to use age-appropriate interventions

when targeting this descending serotonergic projections. It needs to be stressed that the majority of findings were based on *in vitro* studies and this hampers the possible translation to an intact descending serotonergic system not only in rodents but certainly also in humans. Expansion and validation of current findings in animal studies is necessary as the studies published up till now vary with respect to the use of different pharmacological agonists and antagonists, different ages during testing and different methods of investigation. Especially during the critical window in development, where a switch in functionality in descending serotonergic modulation is observed, the replication of pioneer studies is necessary. The presence of a switch in functional modulation from the RVM to the spinal dorsal horn nociceptive network is well-established [56-59]. It is important to note that descending projections from the RVM not only include serotonergic but also GABAergic, glycinergic and glutamatergic pathways[60]. The unique contribution of descending serotonergic projections and its serotonergic receptors to this switch remains relatively unexplored.

Based on the findings within each developmental period, our review identified distinct 5-HT receptor subtypes that play a role in facilitating or inhibiting the spinal nociceptive network during different phases of neurodevelopment. Even though further validation and functional examination of these 5-HT receptors remains necessary along the life span of both rodents and humans, the identification of localization and functionality of 5-HT receptors may provide novel molecular leads that deepen our knowledge on underlying disease mechanisms. In turn this aids the use of mechanism-based therapeutic interventions in the treatment of neonatal procedural pain. For example, in neonatal rats the 5-HT_3 as well as the 5-HT_7 plays a prominent role in facilitation of the spinal nociceptive network [61-63]. If one aims to reduce enhanced facilitation of the neonatal nociceptive system due to for example repetitive procedural pain, blocking these receptors may be of use in a clinical setting. On the other hand as inhibition of nociceptive processing appears possible in neonates, targeting the 5-HT_{1a} or 5-HT_{1b} may also be of interest due to their role in inhibition of the spinal nociceptive network. With use of our review describing this life span frame of physiological functioning of descending serotonergic projections over postnatal age in rodents, the changes in plasticity and function of these serotonergic projections can be studied after repetitive procedural pain in early life (see RQ3 in Chapter 4). Moreover, mechanism-based targeting of developmentally identified 5-HT receptors can be tested for their potential to mitigate the acute and long-term consequences of neonatal procedural pain in a well-established rodent model (see RQ4 in Chapter 5).

RQ3: Does repetitive neonatal procedural pain result in anatomical changes in descending serotonergic projections from the rostral ventromedial medulla to the spinal dorsal horn in adulthood?

The third research question is addressed in **Chapter 4** *"Anatomical changes in descending serotonergic projections from the rostral ventromedial medulla to the spinal dorsal horn following repetitive neonatal painful procedures"*. This is investigated based on a detailed quantitative immunohistochemical analysis of 5-HT in the RVM and in the spinal dorsal horn where the effect of repetitive neonatal procedural pain, tactile stimulation as well as in normal undisturbed development are studied. Our study revealed a decreased 5-HT staining intensity in the ipsilateral dorsal horn in adulthood after repetitive noxious and tactile procedures in early life. Within the RVM animals exposed to repetitive tactile stimulation showed an increased 5-HT staining in ipsi- and contralateral RVM while repetitive noxious procedures increased the area of 5-HT staining in the ipsilateral area of RVM.

This is the first time that anatomical alterations in 5-HT levels in the RVM and spinal dorsal horn are described after neonatal procedural pain, measured by staining intensities. As reported in Chapter 3, 5-HT plays a pivotal role in maintaining the balance of excitation and inhibition in the dorsal horn through 5-HT receptor-mediated binding in adulthood. Anatomical studies have reported that approximately 30% of neurons in the RVM project to the spinal dorsal horn through serotonergic projections that relay in the superficial laminae of the dorsal horn [55,64]. These descending projections are the prominent source of spinal 5-HT. Upon noxious, electrical or pharmacological activation of the RVM, 5-HT is released in the dorsal horn and produces inhibition of nociceptive signaling [65-70]. Local release of 5-HT in the spinal nociceptive network is predominantly inhibitory in adulthood (see Chapter 3). The decrease in spinal 5-HT staining intensity after repetitive noxious or tactile stimulation could therefore point to lower 5-HT levels and thus a lower potential for local inhibition of the spinal nociceptive network in the dorsal horn. Neonatal noxious and tactile procedures have been shown to increase firing in spinal sensory neurons in the dorsal horn upon noxious and tactile stimulation in adulthood, indicating increased excitation [6]. Other models of neonatal pain have also shown a disrupted balance of excitation or inhibition on the spinal nociceptive network in dorsal horn [71-75]. The decrease in 5-HT levels in the dorsal horn as observed in our study may go hand in hand with dorsal horn neuron hypersensitivity after repetitive nociceptive procedures. The latter

resulting in a hypersensitive spinal neuro-circuitry, amplifying nociceptive signaling in later life following neonatal procedural pain. The increased excitatory drive after neonatal procedural pain may be of pivotal importance in sensitization of the spinal nociceptive network [76]. Excitability of spinal projection neurons after neonatal procedural pain can be regulated by 5-HT due to 5-HT receptor mediated binding in the spinal dorsal horn [77]. As described in Chapter 3, most 5-HT receptors (including the 5-HT_{1a}, 5-$HT_{2a/c}$ and 5-HT_3) have the ability to induce both inhibition and facilitation of the spinal nociceptive network in adulthood. Therefore, as expression of these receptors may be altered differently after neonatal noxious or tactile procedures, this may result in a functionally different system that leads to altered descending modulation of the spinal nociceptive network. Importantly, a decrease in spinal 5-HT staining intensity indicates structural changes in the descending serotonergic system and are therefore likely to be long-lasting. This knowledge on long-lasting alterations in the (serotonergic) nociceptive network after painful neonatal procedures may lead clinicians to aim for minimizing the amount of such procedures. Future studies should be designed to study real-time changes in 5-HT neurotransmission during neonatal repetitive needle pricking or around re-injury in adulthood. This may provide more insights into the acute injury-induced 5-HT release in various regions that are regulated by descending serotonergic projections.

The increased 5-HT stained area in the RVM ipsilateral to needle pricking suggests a local and not global effect and may interfere with RVM-mediated serotonergic modulation. More chronic pain models have suggested that increased 5-HT levels in the RVM mediate descending facilitation [68,78]. Connecting functional techniques like electrophysiology, optogenetics or calcium imaging to the anatomical findings discussed here is necessary to understand the implications of these changes. A first step could be to assess differences in functional descending inhibition after neonatal procedural pain. An example is given by Wei and colleagues, who have used optogenetic activation of serotonergic neurons expressing tryptophan hydroxylase 2 (TPH-2) in the RVM and were able to show a facilitatory role of these projections after persistent pain [68]. Other future options include the selective ablation of serotonergic projections as reported in developing rats with no prior neonatal procedural pain [55]. Combining this approach with RVM-stimulation at different amplitudes, where lower amplitudes (5-20 μA) normally initiate facilitation and higher amplitudes induce inhibition (50-200 μA) in adult rats, one can assess whether the pattern of serotonergic modulation is altered by early life pain exposure as a direct read-out of functionality [57]. 5-HT mediates its facilitatory or inhibitory function via binding to 5-HT receptors throughout the central nervous system. So far, seven families of 5-HT receptors (5-HT_1 through $_7$) that are further subdivided into 15 distinct receptor

subtypes are currently identified [79]. Using the present knowledge on 5-HT receptor functioning at different developmental periods (see review in Chapter 3), selective 5-HT receptors important for inhibition or facilitation can be targeted to investigate the functional effects of descending serotonergic projections on pain processing in the absence or presence of neonatal procedural pain (see RQ4 in Chapter 5).

RQ4: Does serotonin-mediated analgesia prevent the acute and long-term effects of repetitive neonatal procedural pain on mechanical sensitivity and anxiety?

In **Chapter 5** *"Selective targeting of serotonin 5-HT_{1a} and 5-HT_3 receptors attenuates acute and long-term hypersensitivity associated with neonatal procedural pain"* serotonin-mediated analgesia was studied in the context of the acute and long-term effects of repetitive neonatal procedural pain on pain and anxiety in later life. In this experimental study the focus was on ondansetron for antagonizing the 5-HT_3 receptor to block enhanced facilitation and buspirone as a 5-HT_{1a} receptor agonist to inhibit excessive input. The pharmacological studies presented in Chapter 5 demonstrate that agonizing the 5-HT_{1a} receptor (using buspirone) during neonatal procedural pain prevented the acute hypersensitivity whereas antagonizing the 5-HT_3 receptor (using ondansetron) prevented the longer duration of hypersensitivity after re-injury in adulthood normally observed after repetitive neonatal procedural pain. Postnatal developmental milestones were unaffected and state anxiety in adulthood was decreased with use of neonatal ondansetron or buspirone.

The presence of repetitive neonatal procedural pain in the clinic [80-82] as well alterations in pain sensitivity later in later life [33,83-85] suggests a strong need for treatment which might be either pharmacological or non-pharmacological. The effects of neonatal procedural pain are not solely limited to somatosensory long-term effects but also influence later-life cognition, stress response, anxiety behavior [29,31,35,45,48,86-90] (see Chapter 2). Effective management of pain in neonates is necessary to minimize acute physiological and behavioral distress, but also mitigate potential long-term effects [91]. Pharmacological treatment in neonates should not only be focused on pain-related symptoms but also take cognition, anxiety and stress effects into account by focusing on an underlying system such as serotonin that modulates all these behaviors. Using a mechanism-based approach, 5-HT_3 and 5-HT_{1a} receptors in neonates can be selectively targeted and this results in the

prevention of neonatal procedural pain-induced effects. Our data forms a fundament for the use of 5-HT receptor mediated therapy in the treatment of procedural pain in human neonates. Several steps remain to be taken before the introduction of either buspirone and/or ondansetron in a clinical setting in the NICU. Buspirone may be a new therapeutic venue for the prevention of neonatal procedural pain-induced effects in the NICU. Based on our pre-clinical findings the anti-nociceptive role of the 5-HT_{1a} receptor in neonates previously described in *in vitro* studies [61,62,92,93] was confirmed *in vivo* after repetitive needle pricking in rat pups in Chapter 5. Buspirone administration not only during the neonatal period, but also during gestation or adolescence mitigates the adverse behavioral effects of inflammation in early life [94,95]. Of note, buspirone alters mechanical sensitivity throughout development and does not abolish the long-term mechanical hypersensitivity effects of neonatal procedural pain after re-injury (see Chapter 5). The latter indicates that buspirone may be of interest as an acute analgesic at the time of neonatal procedures but not for the prevention of its long-term effects. Although buspirone is tolerated among pediatric patients [96], neonatal data on the safety and pharmacodynamics are essential before buspirone is introduced in a clinical setting of neonatal procedural pain.

The 5-HT_3 antagonist ondansetron is a possible treatment option targeted towards the prevention of injury-induced plasticity in developing serotonergic projections in newborn neonates. Although antagonizing the 5-HT_3 receptor with ondansetron did not abolish acute mechanical hypersensitivity it does reverse the long-term effects of repetitive neonatal procedural pain in our preclinical model. The latter suggests that the 5-HT_3 receptor plays a role in the mechanism(s) underlying these effects. This is in line with the known involvement of 5-HT_3 receptors in descending serotonergic facilitation of touch and nociception at an early age [55]. After (chronic) injury, 5-HT_3 receptors may become facilitatory [78,97]. The increased 5-HT levels in the ipsilateral RVM observed in Chapter 4 may mediate descending serotonergic facilitation via the 5-HT_3 receptor. This facilitation can be prevented using the 5-HT_3 receptor antagonist at the time of neonatal injury. Currently ondansetron is FDA approved in pediatric patients >1 year related to chemotherapy use, radiation therapy and postoperative anesthesia [98]. Ondansetron has also been proven effective to treat nausea and vomiting during pregnancy, and is often prescribed [99]. Systematic reviews suggests that the use of ondansetron during pregnancy is associated with an overall low risk of birth defects in neonates [100,101]. Pharmacokinetic data of ondansetron in neonates is available [102] or could be adequately captured using mechanistic modelling [103]. However, as ondansetron operates peripherally at the vagal nerve terminals as well as central in chemoreceptor trigger zone of the area postrema, side-effects related to nausea should be closely monitored in any future studies using this drug. In summary,

the pre-clinical results presented in Chapter 5 may form the fundament of a new mechanism-based therapeutic venue in treatment of procedural pain in human neonates using ondansetron or buspirone.

The 5-HT_3 and 5-HT_{1a} may not be the only suitable candidates for the prevention of the acute and long-term effects of neonatal procedural pain. For example, also the 5-HT_2, 5-HT_4 and 5-HT_5 receptors were shown to be upregulated after neonatal injury in other areas in the nociceptive network [104,105]. All upregulated receptors have been reported to reduce both postsynaptic glutamate and GABAergic neurotransmission in the periaqueductal gray, the latter known to be an important regulator of serotonergic descending control via its connection with the RVM [106-108]. Moreover, an inhibitory role of the 5-HT_2, 5-HT_4 and 5-HT_5 receptors in the spinal dorsal horn is also described in adulthood [97]. When targeting any of these 5-HT receptors in neonates, especially using systematic administration, side effects may occur that need to be monitored. For example, 5-HT and its receptors play an important role in network formations during perinatal development [42]. Interfering with serotonin signaling during this perinatal time-frame alters 5-HT levels, transcription factors, neurotropic factors in the amygdala and prefrontal cortex, that consequently may impact later-life nociception, anxiety and depression [109]. Therefore, when modulating the (descending) serotonergic system during this vulnerable time in neonatal development caution should be taken, as timing and specificity are important factors contributing to the balance between adverse and/or beneficial effects. It needs to be stressed that pain management in neonates remains a complex problem where the variety in NICU patients regarding postnatal and post conceptual age, the use of validated assessment instruments, liver and kidney function related to pharmacodynamics, and developmental neurobiology have to be taken into account to reach a next step of therapy in this vulnerable population [110].

Future perspectives in management of acute and long-term effects of neonatal procedural pain.

Based on the data presented in this thesis, several lines of research are recommended to move the field forward. As mentioned, the findings presented in this thesis have focused on anatomical alterations in descending serotonergic projections from the RVM to the spinal nociceptive network as well as a functional examination of the role of the 5-HT_{1a} and 5-HT_3 receptors during repetitive neonatal procedural pain. This is the first time that studies have investigated the role of descending serotonergic projections after neonatal

procedural pain. It is important to place our current findings in context of other neurotransmitter systems projecting from the RVM to the spinal dorsal horn and link them to functionally distinct (ON, OFF or neutral) cells within the RVM [111]. Next to the RVM, the periaqueductal gray (PAG) also plays a pivotal role in descending modulation through opioid projections. The PAG is an important regulator of serotonergic projections through its connections with the RVM and central opioid signaling is necessary for early life RVM-mediated facilitation [112]. Changes in opioid receptor expression and opioid tone after neonatal procedural pain [18] or neonatal inflammation have previously been reported [113]. Individual differences in response to analgesics as well as pain processing may be explained by alterations in descending pain systems after neonatal procedural pain. So far, the majority of studies investigating the descending (serotonergic) modulatory projections are performed in rodents. Although major steps have been made related to the understanding of the normal development of descending pathways and their alterations after neonatal pain in animal models, clinical studies investigating descending modulation in children are still at its infancy. An important first step has been made by Goksan and colleagues using functional MRI. They were able to show that functional connectivity between areas involved in descending pain modulation regulates the magnitude of noxious-evoked brain activity in infants [114]. Evidence for the emergence of descending inhibition in neonates has been suggested by the observed increase in noxious-evoked brain activity that coincides with a decrease in reflex withdrawal with postnatal age in human infants [115]. In a more clinical setting conditioned pain modulation (CPM) is referred to as the measure of the net effect of descending pain pathways. CPM has the future potential to test pain modulation in patients and also guide mechanism-based treatments [116]. The use of CPM paradigms may provide an added bed-side assessment for sensory profiles that are indicative of altered pain mechanisms, and aid the unraveling of the long-term effects of neonatal pain-related stress in the clinic [117]. More importantly, pharmacological targeting of serotonergic projections could affect CPM, thereby restoring the normal balance of inhibition and facilitation of nociception [118]. Testing of CPM in children should be based on a developmental framework as older children (12-17 years) exhibit a greater CPM as compared to younger children (8-11 years) [119]. The age of the child and other contributing factors should be included to guide age-appropriate interventions for pain using CPM as a read-out of descending pain pathways.

Epigenetics are also likely to play a key role in later life pain sensitivity after repetitive neonatal procedural pain. Epigenetics are defined as a plethora of processes involved in translating exposure to environmental stimuli to changes in gene expression patterns. At a molecular level, epigenetics consists of a complex and dynamic modifications

in DNA methylation, histone acetylation and RNA interference [120]. Specific DNA methylation patterns regulate genome function, where increased methylation inhibits gene expression and decreased methylation increases gene expression. As such, epigenetic processes are responsive to the environment in a more dynamic fashion. Neonatal procedural pain-induced changes are observed in synaptic transmission [6] and structural architecture [3,18], that may be induced through epigenetic processes. In 2015, Montirosso and Provenzi have provided a model referred to as preterm behavioral epigenetics, by applying epigenetic research to the study of prematurity and effects of NICU stay [121]. In this model, prenatal exposure to adverse events, NICU-related stress exposure and developmental care contribute to developmental trajectories through epigenetic modifications. Since then several systematic reviews have identified epigenetic modifications observed in preterm infants that are associated with prenatal (i.e. maternal stress) and postnatal adverse conditions (i.e. painful procedures) [122-125]. Epigenetically induced changes may help us unveil potential mechanisms related repetitive procedural pain and its acute and long-term consequences [124]. Methylation of the 5-HT transporter gene (SLC6A4) increased from birth to discharge in the NICU and shorter telomere length often dysregulated by early life adversity and NICU care [123,124]. These biomarkers identified based on epigenetic profiling can be useful to identify neonates more at risk to develop later life behavioral adversity. Modifications in epigenetic regulation also represent a promising new area of research into understanding the transition from acute to chronic pain [126] and the role of previous painful experiences in early life on this transition.

Throughout this thesis the role of repetitive tactile stimulation in neonates, originally included as a control procedure for the repetitive neonatal needle pricking, cannot be unnoticed. Neonatal repetitive tactile stimulation of the hind paw resulted in a marginal decrease in state anxiety measured in the EZM which was almost similar to that observed after neonatal repetitive procedural pain (see Chapters 2 and 5). Tactile stimulation-specific effects can also be observed in the spinal dorsal horn where 5-HT staining intensity was decreased ipsilateral to the side of innocuous stimulation. Descending serotonergic projections facilitate tactile input to a similar extend as noxious input and may therefore have the potential to induce similar effects [55,58]. This indicates that the microcircuits that regulate noxious and non-noxious input in the spinal dorsal horn are highly overlapping in the neonate. Subpopulations of interneurons are crucial in the integration of both nociceptive and tactile inputs in the neonate and wide dynamic range neurons in the deep dorsal horn fire in response to tactile stimulation [49,127,128]. Tactile input has the ability to guide nociceptive synaptic organization during early development even in the absence of noxious input [129] and low-threshold touch receptors are playing an

important role in acute nociceptive signaling and the development of mechanical paw withdrawals [130]. Human neonates are more sensitive to tactile and noxious stimulation in early life as compared to adults [128,131]. Functional tactile and nociceptive systems rely on activity-dependent maturation that can be influenced by excessive input. Future research should continue to include tactile stimulated groups, next to repetitive noxious procedures, to detect not only similarities but also to study possible differences between the two modalities of stimulation in early life.

Although it remains vital to investigate neonatal procedural pain-induced plasticity of the nociceptive system as well as optimal treatment strategies, the overall experience in the NICU encompasses more than painful procedures alone. As such, even in absence of medical complications and painful procedures, the NICU is a source of enormous distress for preterm infants. NICU-related stress includes physical and sensorial stimulations and maternal separation [34]. Recent systematic reviews have highlighted the efficacy of non-pharmacological strategies, skin-to-skin contact, sucrose administration and multisensory stimulation in exerting beneficial effects on the neonate or infant [132-135]. Preclinical studies may aid in unraveling possible underlying mechanisms by which these interventions may be effective. Another important line of future research is related to the importance of caregivers in the context of neonatal pain and management. Clinical studies show that neonatal procedural pain is not only related to changes in anxiety of the child itself, but also influences anxiety of the parent. During early stay in the NICU, maternal state as well as trait anxiety is increased [136]. Increased levels of stress in the neonate results in increased maternal anxiety levels. Pain sensitivity of former preterm children at school-age can be predicted by cumulative painful procedures as well as parent trait anxiety [84]. The latter points to an important modulatory role of parental behavior linking neonatal procedural pain and later life behavioral adversity. Future research should include examination of maternal state and trait anxiety or maternal behavior at the time of early life injury in order to assess the reciprocal effect here. On the other hand, sensitive and responsive caregiving appears to ameliorate some negative effects of neonatal pain-related stress on brain, stress, and behavioral outcomes [137,138]. The above findings point to the importance of caregiver inclusion in the implementation of pain management and/or prevention of pain in hospital settings. Prevention of pain is just one step ahead of balanced analgesia towards the growing evidence in favor of developmental care programs that include caregiver involvement [139].

Concluding remarks

The studies compiled in this thesis offer a deeper insight into the role of descending serotonergic projections as an important component in the mechanism underlying both acute and long-term consequences of neonatal procedural pain. The effects of neonatal procedural pain are not limited to somatosensation but also affect anxiety. Our results show that anatomical changes in the descending serotonergic projections from the RVM to the spinal cord may underlie the observed acute and long lasting effects on pain sensitivity and anxiety after repetitive neonatal procedural pain. Using a mechanism-based approach, selective targeting of identified serotonin receptors during the time window of neonatal procedural pain has shown to be promising in the prevention of its acute and long-term effects in an animal model of neonatal repetitive needle pricking. This may form the fundament of new mechanism-based therapeutic venues that may be effective in the treatment of procedural pain in human neonates.

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7

Summary

The primary aim of this academic thesis was to investigate the acute and long-term effects of repetitive neonatal procedural pain and the role of the descending serotonergic projections to the spinal dorsal horn nociceptive network. Next, it aimed to assess whether modulation of these serotonergic projections via pharmacological intervention could aid in the prevention of long-term consequences of neonatal repetitive procedural pain. In order to meet these aims we formulated various research questions (see **Chapter 1** General Introduction) and answered these in the various chapters.

In order to understand if neonatal procedural pain also influences anxiety behavior in later life, next to its effects on pain sensitivity, **Chapter 2** (RQ1) describes an experimental study. This study describes the impact of repetitive neonatal procedural pain on trait anxiety, evaluated in the open field test (OFT), and state anxiety evaluated in the Elevated Zero Maze (EZM) in adulthood. We replicated earlier findings by showing that repetitive neonatal needle pricking leads to robust hypersensitivity during the neonatal period, without altering baseline sensitivity. Adult animals previously exposed to neonatal procedural pain exhibit lower state anxiety behavior as compared to controls. No differences in trait anxiety in adulthood were noted in our study. Treatment of neonatal procedural pain should therefore also consider these differential effects on trait or state anxiety.

Before experiments focused on descending serotonergic projections and their role in modulation of the spinal nociceptive network after neonatal procedural pain were performed, a complete and detailed understanding of the physiological development of descending serotonergic projections and their modulation of the spinal nociceptive network in neonatal, pre-weaning and adult phase is described in **Chapter 3** (RQ2). This review highlights a rapid postnatal development, with increased sprouting of serotonergeric projections from the rostral ventromedial medulla (RVM) to the spinal dorsal horn that continues up to postnatal day 21. Functionally, descending serotonergic modulation switch from facilitation in early life to bimodal control in adulthood. Descending serotonergic facilitation in early life up to weaning is mediated via the 5-HT_3 and 5-HT_7 receptors, and masks an already functional inhibitory serotonergic system mainly via the 5-HT_{1a}, 5-HT_{1b} and 5-HT_{2a}. All 5-HT receptors, with the exception of the 5-HT_7 receptor, play a role in the modulation of acute spinal nociceptive signaling in adulthood. This review highlights the potential for serotonin-mediated treatment based on the developmental phase to the prevention of neonatal pain and its long-term effects.

To understand how repetitive neonatal procedural pain affects the anatomical plasticity of the descending serotonergic projections (RQ 3), we investigated intracellular serotonin (5-hydroxytryptamin; 5-HT) in the spinal dorsal horn and RVM in **Chapter 4.** Using quantitative immunohistochemistry, we show that repetitive neonatal painful procedures decreased serotonin staining levels in the ipsilateral spinal dorsal horn in adulthood. Within the RVM neonatal procedural pain results in an increase in serotonin staining levels ipsilateral to needle pricking. Moreover, repetitive tactile procedures also affect serotonin levels in the spinal dorsal horn and RVM in adulthood. Our findings highlight, for the first time, the importance of descending serotonergic projections from the RVM to the spinal cord in the long-term effects of neonatal procedural pain.

From the narrative review described in chapter 3, it became evident that several 5-HT receptors play an important role in descending serotonergic modulation in neonates. The 5-HT_3 seems distinctly important for facilitation, whereas the 5-HT_{1a} is involved in inhibition of the spinal nociceptive network. **Chapter 5** describes the use of subcutaneously administered ondansetron, a 5-HT_3 antagonist, and buspirone, a 5-HT_{1a} agonist as analgesia during repetitive neonatal procedural pain. Neonatal activation of the 5-HT_{1a} effectively attenuates acute hypersensitivity, but decreases baseline sensitivity throughout development and has no effect on the long-term consequences of repetitive neonatal procedural pain. On the other hand, neonatal 5-HT_3 inactivation does not affect neonatal or baseline sensitivity but attenuates the longer hypersensitivity following re-injury in adulthood. Neither treatment affects early postnatal development or adult anxiety behavior. This study guides way to use mechanism-based treatment that targets descending serotonergic projections for the prevention of neonatal pain and its long-term effects in a clinical setting.

In conclusion, this thesis shows that neonatal procedural pain leads to anatomical alterations in serotonergic projections regulating spinal nociception, as well as changes in anxiety and nociception in an animal model of neonatal repetitive needle pricking. Targeting descending serotonergic projections using mechanism-based pharmacology are promising in the prevention of neonatal procedural pain induced effects in the clinic.

Nederlandse samenvatting

In de neonatale intensive care worden pasgeborenen of te vroeggeboren kindjes (voor 37 weken zwangerschap) opgenomen die intensieve zorg nodig hebben. Tijdens deze opname ondergaan pasgeborenen tien tot vijftien pijnlijke procedures per dag, als onderdeel van hun medische zorg. Deze procedures variëren van injecties tot hielprikken tot luchtwegintubatie. Wetenschappelijk onderzoek heeft aangetoond dat deze neonatale procedurele pijn niet alleen acuut leidt tot pijn, maar ook lange-termijn gevolgen heeft voor het brein en pijngevoeligheid. Het doel van deze academische thesis was om de acute en lange-termijn effecten van neonatale procedurele pijn te onderzoeken, gefocust op de rol van descenderende (dalende) serotonine projecties die lopen vanuit de Rostral Ventral Medulla (RVM) in de hersenstam naar het nociceptieve (pijn) netwerk in het ruggenmerg. Daarnaast werd er gekeken of modulatie van deze serotonine projecties door middel farmacologische behandeling zou kunnen helpen bij het voorkomen van lange-termijn gevolgen van neonatale procedurele pijn. Om aan deze doelstellingen te voldoen hebben we verschillende onderzoeksvragen geformuleerd (in **hoofdstuk 1**), die in verschillende hoofdstukken van deze thesis werden beantwoord.

In **hoofdstuk 2** (RQ1) werd een experimentele dierenstudie beschreven, waar de impact van neonatale procedurele pijn op pijngevoeligheid en angstgedrag op latere leeftijd werd bekeken. Hierbij werd gebruik gemaakt van een rat model waarin pups tijdens de eerste week van hun leven 4 keer per dag naaldenprikken ondergingen. Nadat deze ratten volwassen werden, werd er gekeken naar pijngevoeligheid, basale angstgevoelens en toestandsangst (een staat van voorbijgaande angstgevoelens). In dit hoofdstuk repliceerden we eerdere wetenschappelijke bevindingen door aan te tonen dat repetitieve naaldenprikken in pasgeboren ratten pups leidt tot acute overgevoeligheid rondom deze naaldenprikken, zonder de pijngevoeligheid te veranderen tijdens de verdere ontwikkeling. Volwassen dieren die als pups waren blootgesteld aan neonatale procedurele pijn vertoonden een lager staat van voorbijgaande angst, zonder dat er verschillen werden gevonden in basale angstgevoelens. Bij de behandeling van procedurele pijn bij pasgeborenen moet rekening worden gehouden met mogelijke gevolgen op angstgedrag bij volwassenen.

Voordat verdere studies focussen op descenderende serotonine projecties en hun rol in de modulatie van het pijn netwerk na neonatale procedurele pijn, was een samenvatting van de huidige literatuur over de ontwikkeling van deze descenderende serotonine projecties

nodig. In **hoofdstuk 3** (RQ2) kwam naar voren dat er een snelle ontwikkeling plaatsvindt in de eerste drie weken na de geboorte van een rat, waarin het aantal vertakkingen van deze serotonine projecties vanuit de hersenstam (RVM) naar de het ruggenmerg toenemen. Functioneel vindt er een switch plaats waarin serotonine in de eerste drie weken pijnsignalen versterkt, terwijl er in volwassenen zowel versterking als remming van het pijnsignaal kan plaatsvinden door middel van serotonine. Tijdens de verschillende fasen van de ontwikkeling zijn verschillende serotonine receptoren betrokken bij remming ofwel versterking van het pijnsignaal in het ruggenmerg. In volwassenen kunnen serotonine receptoren beide effecten teweegbrengen. Dit review benadrukt dat serotonine neurotransmissie in het ruggenmerg een uitstekend farmacologisch doelwit in de preventie van neonatale procedurele pijn-geïnduceerde effecten, rekening houdend met de ontwikkelingsfase van het nociceptieve (pijn) systeem.

Om te begrijpen hoe neonatale procedurele pijn de anatomie van de descenderende serotonine projecties (RQ 3) beïnvloedt in de hersenstam en het ruggenmerg, hebben we intracellulair serotonine (5-hydroxytryptamine; 5-HT) in het ruggenmerg en de hersenstam onderzocht in **hoofdstuk 4**. Met behulp van immunohistochemie hebben we laten zien dat neonatale procedurele pijn de serotonine levels in het ruggenmerg en de hersenstam van volwassen dieren verlaagden, alleen aan de zijde corresponderend met de kant waar de prikken gegeven zijn. Daarnaast hebben we laten zien dat niet alleen neonatale pijn, maar ook herhaaldelijke tast ook de serotonine levels in het ruggenmerg en hersenstam op volwassen leeftijd beïnvloed. Onze bevindingen benadrukken een potentiële rol van descenderende serotonine projecties van de RVM naar het ruggenmerg in de lange-termijn effecten van neonatale procedurele pijn.

Uit het literatuur overzicht beschreven in hoofdstuk 3 werd duidelijk dat verschillende 5-HT receptoren een belangrijke rol spelen bij het moduleren van inkomende nociceptieve (pijn) signalen door serotonine. In pasgeborenen speelt de 5-HT3 speelt een belangrijke rol in de versterking, terwijl de 5-HT1a betrokken is bij de remming van het pijnsignalen in het ruggenmerg. **Hoofdstuk 5** beschrijft het gebruik van ondansetron, een 5-HT3-antagonist, en buspirone, een 5-HT1a-agonist, als behandeling voor repetitieve neonatale procedurele pijn. Activatie van de 5-HT1a receptor in het vroege leven vermindert acute overgevoeligheid in ratten pups, maar verhoogt de basisgevoeligheid gedurende de ontwikkeling en heeft geen effect op de lange-termijn gevolgen van neonatale procedurele pijn na nieuw letsel in volwassenheid. Neonatale 5-HT3-inactivatie geen invloed op de gevoeligheid in het vroege level of tijdens de ontwikkeling, maar vermindert de langere overgevoeligheid na nieuw letsel op volwassen leeftijd. Geen van beide behandelingen

heeft een negatieve invloed op de ontwikkeling in ratten pups of het angstgedrag in volwassen dieren. Deze studie is een eerste stap tot het gebruik van behandeling die gericht is op descenderende serotonine projecties voor de preventie van neonatale pijn en de lange-termijn effecten ervan in een klinische setting zoals de neonatale intensive care.

Dit proefschrift laat zien dat procedurele pijn bij pasgeborenen leidt tot anatomische veranderingen in serotonine projecties die het pijnsignaal in het ruggenmerg reguleren, evenals veranderingen in angst en nociceptie (pijngevoeligheid) op latere leeftijd. Ontwikkeling-adequate farmacologische behandeling gericht op descenderende serotonine projecties is veelbelovend in de preventie van neonatale procedurele pijn-geïnduceerde effecten in een klinische context.


7

Impact Paragraph

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In this section the scientific and societal impact of the research described in this thesis will be discussed.

Scientific impact of experimental neonatal pain research

Significant advances have been made in the understanding, recognition and management of neonatal pain over the last three decades. The scientific field of neonatal pain research has shifted from a general believe that human neonates did not consciously experience, remember, or respond to painful stimuli, to the present understanding that nociception and pain are already apparent in neonates from 20-24 weeks of gestation. Today, the importance and optimization of pain management for neonates is widely acknowledged. Effective management of pain in neonates is necessary to minimize acute physiological and behavioural distress, but also to improve the potential long-term and negative effects. However, at this stage many questions remain that cannot easily be answered in humans. In this context preclinical research has provided tremendous information regarding the development of the nociceptive system in neonates based on clinical relevant juvenile animal models [1]. Common painful procedures such as heel sticks in the neonatal intensive care unit (NICU) are mimicked by repetitive needle pricking as described in **Chapters 2, 4 and** 5 of this thesis. A growing body of evidence reports long-term consequences of painful experiences during a critical window of development, where changes in somatosensory and brain development, mood/anxiety, pain responses and cognitive functioning are observed [1,2]. So far, research has not yet focussed on alterations in descending inhibitory pathways that control the balance of excitation and inhibition at the level of the spinal dorsal horn, the most important level of central nociceptive and somatosensory processing [3]. Serotonergic projections originating in the rostral ventromedial medulla play an important role in this descending modulation of spinal nociception [4].

The studies described in this thesis aim to unravel the involvement of descending serotonergic projections from the brainstem rostral ventromedial medulla in the mechanisms underlying the long-term effects of neonatal procedural pain. In doing so, we provide a first read-out of injury-induced plasticity in supraspinal areas involved in processing of both pain and anxiety. In this thesis, we have provided clarity in the contradicting evidence for long-term effects of neonatal procedural pain on anxiety. In **Chapter 2** we point to a distinct profile of (state) anxiety affected by neonatal procedural pain when investigating these in separate and validated behavioural constructs in rodents. This may provide the foundation for future clinical studies to integrate tests measuring

different anxiety profiles in former NICU patients, an area that is hardly investigated in a structured manner.

Although descending modulation forms an important aspect of the nociceptive network there is an unmet scientific need for a better understanding of the descending serotonergic projections that regulate pain processing throughout postnatal development. The review in **Chapter 3** describes the postnatal development of such serotonergic projections, and highlights important developmental differences in anatomy and function at different developmental phases in rodents. This review contributes to the scientific field by providing an extensive overview of the anatomy and functionality of descending serotonergic projections throughout the life span, not only focussing on the adult system. The latter also demonstrates that the descending serotonergic projections show high plasticity in neonates, and excessive stimulation that intervenes with this normal development may result in long-lasting changes. Evidence for structural anatomical changes in this descending serotonergic system after painful stimulation in neonates is described in **Chapter 4.** The results point to a potential role of descending serotonergic projections in the modulation of injury-induced long-term effects. This is the first time that anatomical alterations in serotonin projections are described after pain in early life. This information can be placed in the context of other injury-induced changes in the nociceptive network in both a pre-clinical and clinical setting. Pain management may also be targeted to restore these anatomical alterations in descending serotonergic projections using the concept of pre-emptive analgesia targeted at the serotonergic system in future research. When doing so, the presence or absence as well as the functionality of specific serotonergic receptors should be taken into account using knowledge of the developing neonatal nociceptive system. This may provide new therapeutic venues for pain management in neonates and changes the focus of therapy from opioids presently considered a first choice drug following major tissue injury in the NICU.

Adequate pre-emptive pharmacological therapy to manage the acute pain associated with skin-breaking procedures in neonates may play an important role in preventing the long-term negative effects observed in both clinical and pre-clinical studies. Moreover, long-term effects of neonatal procedural pain are not limited to somatosensory processing, but also affect anxiety behaviour as described in **Chapter 2.** Going forward, greater emphasis should be placed on pain management that also mitigates these adverse effects of pain exposure such as the serotonergic system. The pharmacological studies in **Chapter 5** use developmentally identified serotonin receptor targets within the descending serotonergic system selected based on the review in **Chapter 3**. Studies like these

contribute to establishing the scientific foundation the clinical field needs to provide adequate analgesia in neonates, along with increased knowledge of neonatal pharmacodynamics and pharmacokinetic data.

Anticipated societal impact

Worldwide, one of every ten babies are born prematurely (<37 weeks of gestation). The increased survival rates after preterm birth present a public health concern and economic burden due to high costs of hospitalization and long term sequelae even in adulthood, especially in extreme low birthweight infants. While hospitalized in the neonatal intensive care unit (NICU) preterm babies undergo up to 14 painful procedures daily [5]. Using a clinically relevant rodent model that mimics common painful procedures in the NICU, as described in this thesis, can deepen our in-depth knowledge of the effect of interventions like repetitive neonatal procedural pain on the neurobiology and plasticity of the nociceptive system. The neurobiology of neonatal pain processing differs significantly from more mature infants, children, adolescents and adults. This thesis shows that within the descending serotonergic projections that regulate pain sensitivity, developmental differences in function occur and plasticity changes are observed after neonatal interventions such as repetitive procedural pain, depending on the developmental phase. Moreover, in contrast to underlying diseases as rationale for admission to the NICU that can usually not be prevented, the number of painful preventions can be decreased by an active attitude of treatment teams based on more knowledge on the neurobiology provided by this thesis.

Individual pain sensation is naturally variable and depends on subjective experiences. Descending modulatory systems have been associated with individual differences in pain perception and response to analgesic therapy. Moreover, impaired descending inhibition may contribute to individual risk factors of the development of chronic post-operative pain [6]. Early life adversity such as repetitive neonatal pain exposure may also increase the risk of the development of (chronic) pain syndromes in later life [7]. This thesis shows that descending serotonergic projections play a role in the mechanisms of neonatal pain-induced long-term consequences, including longer post-operative recovery after surgery in adulthood. Permanent alterations in descending serotonin signaling may contribute to impaired descending inhibition in later life leading to a higher risk of developing chronic postoperative pain. A better understanding of descending serotonergic modulation and its role in later-life pain perception contributes to identifying individuals most at risk for long-term effects after NICU admittance. For obvious reasons

other factors have to be taken into account, such as major operative procedures in selected infants, cumulative dose of analgesics, and whether opioids or other classes of drugs were used. Apart from structured follow up, quantitative sensory testing in ex-preterm neonates can be used to identify impaired descending inhibition as an essential tool for pre-operative risk assessment.

Inadequate pain management in neonates impairs neurodevelopmental outcomes (including sensory, cognitive, motor and affective behaviors) [2]. Despite increased knowledge of the developing pain system and a long list of non-pharmacological and pharmacological strategies, pain management remains suboptimal in the neonatal intensive care unit. Results from this thesis provide a new therapeutic venue targeted towards the descending serotonergic system. Using a mechanism-based approach the serotonergic system can be modulated in rodents to prevent neonatal injury-induced acute and long-term effects. Currently serotonin-mediated analgesia is not used in the NICU. At this stage, the findings presented in this thesis are still too preliminary to result in any clinically used pharmacological therapies for the NICU. The wide variability of patients regarding post conceptual and postnatal age with inherent differences in response to pain, the use of validated assessment instruments, evolving liver and kidney function, receptor density and maturation have to be taken into account to reach the next step of therapy. Certainly, one size fits all is an oversimplification of this complex problem. Nevertheless our preclinical studies do provide a foundation for serotonin-mediated therapies that may be effective in the management of procedural pain in the human neonates in the future.

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Curriculum Vitae

Rose de Kort was born on september 22nd 1995, in Zetten, The Netherlands. She attended secondary school at het Peelland College in Deurne, where she optained her Atheneum degree in 2013. That same year, she started her Bachelor studies in Psychology at Tilburg University, with specialisation in Psychology and Health. During her Bachelor internship, she studied tumor related predictors of cognitive functioning in patients with brain metastases under supervision of Dr. Verhaak and Dr. Schimmel at the department of Cognitive Neuropsychology at Tilburg University. As part of an international exchange program, she spend a spring semester at University of Arizona in Tucson, USA in 2016 (Minors: Cogntive Psychology, Human Memory, Introduction to Biopsychology and Drugs and the Brain). After obtaining her bachelor degree in 2016 *cum laude*, she was accepted to the Fundamental Neuroscience track of the Research Master Cognitive and Clinical Neuroscience at Maastricht University. For her Master internship, Rose joined the Department of Aneasthesiology and Pain Management in Maastricht supervised by Prof. Dr. Bert Joosten to study the long-term effects of neonatal repetitive procedural pain. Ongoing collaborations with the hospital for Sick Children (SickKids) in Toronto led her to complete part of her internship at the Prescott Lab under the supervision of Dr. Steve Prescott. Here, she studied the physiological properties of pain by combining both optogenetics and electrophysiology. She graduated *cum laude* in 2018, and continued this research as a PhD student under supervision of Prof. Dr. Bert Joosten and Dr. Nynke van den Hoogen, in collaboration with Prof. Dr. Dick Tibboel from the Intensive Care and Department of Pediatric Surgery of Erasmus MC-Sophia Children's hospital in Rotterdam. During her three year PhD project, Rose studied the role of the descending serotonergic projections on the acute and long-term effects of experimental neonatal procedural pain. The results of her studies are presented in this academic thesis. Following her PhD, Rose is now working as a behavioral scientist at the William Schrikker Jeugdbescherming & Jeugdreclassering (youth protection and youth rehabilitation).

List of Publications

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Published

2019 van den Hoogen, N. J., **de Kort, A. R.**, Allegaert, K. M., Joosten, E. A., Simons, S. H., Tibboel, D., & van den Bosch, G. E. Developmental neurobiology as a guide for pharmacological management of pain in neonates. Seminars in Fetal and Neonatal Medicine. 2019; 24(4): 1-7. https://doi.org/10.1016/j.siny.2019.05.004. **Published.**

2021 **de Kort, A. R.,** Joosten, E. A., Patijn, J., Tibboel, D., & van den Hoogen, N. J. The development of descending serotonergic modulation of the spinal nociceptive network: a life span perspective. Pediatric Research. 2021: 1-9. https://doi.org/10.1038/s41390-021-01638-9. **Published**.

2021 **de Kort, A. R.,** Joosten, E. A., Patijn, J., Tibboel, D., & van den Hoogen, N. J. Neonatal procedural pain affects state, but not trait anxiety behavior in adult rats. Developmental Psychobiology. 2021; 63(8); 1-8. https://doi.org/10.1002/dev.22210. **Published**.

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Under review

2021 **de Kort, A. R.,** Joosten, E. A., Patijn, J., Tibboel, D., & van den Hoogen, N. J. Selective targeting of serotonin 5-HT_{1a} and 5-HT_3 receptors attenuates acute and long-term hypersensitivity associated with neonatal procedural pain. **Under review.**

2021 **de Kort, A. R.,** Joosten, E. A., Patijn, J., Tibboel, D., & van den Hoogen, N. J. Anatomical changes in descending serotonergic projections from the rostral ventromedial medulla to the spinal dorsal horn following repetitive neonatal painful procedures. **Under review.**

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“Showing gratitude is one of the simplest yet most powerful things humans can do for each other “
– Randy Pausch

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