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 impacts 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 descending serotonergic RVM–spinal dorsal horn 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 contralateral spinal dorsal horn. Repetitive neonatal noxious procedures resulted in an increased area covered with 5-HT staining in the adult ipsilateral but not 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 5-HT 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 system within the RVM and spinal dorsal horn. Future studies should investigate whether these anatomical changes translate to functional differences in descending serotonergic modulation after neonatal adverse experiences.

Keywords
descending modulation, neonatal pain, rostral ventromedial medulla, serotonin (5-HT), spinal cord, tactile stimulation
The developing somatosensory system of both humans and rodents is highly responsive to both tactile and nociceptive stimulation, and painful stimuli experienced in early life affect the normal maturation and fine-tuning of this system (Fitzgerald, 2005; Schwalzer & Fitzgerald, 2014; van den Hoogen et al., 2017; Williams & Lascelles, 2020). More specifically, repetitive neonatal procedural pain causes long-lasting structural changes, including hyperinnervation of the injured skin by peripheral nociceptive and non-nociceptive fibres and increased central terminal innervation of the injured skin by peripheral nociceptive fibres (Beggs et al., 2012; de Lima et al., 1999; Knaepen et al., 2013; Reynolds & Fitzgerald, 1995). In addition, neonatal pain leads to increased firing of spinal somatosensory neurons upon noxious and non-noxious stimulation in adulthood (Li et al., 2015; van den Hoogen et al., 2018). The latter suggests that neonatal triggering of the nociceptive network results in a hypersensitive spinal nociceptive network in the adult. The spinal dorsal horn is an important hub for the integration of nociceptive and non-nociceptive input (Abraira et al., 2017; Millan, 2002; Todd, 2010). 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 [de Kort, Joosten, Patijn, Tibboel, & van den Hoogen, 2021a]). 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 (Fitzgerald, 2021). The major source of descending serotonergic projections to the spinal dorsal horn is the raphe magnus, located in the brainstem rostral ventromedial medulla (RVM) (Bowker et al., 1981; Tanaka et al., 2006). The RVM is part of a spino-bulbo-spinal loop that is activated by ascending nociceptive input and drives serotonergic descending modulation in adulthood (Schwalzer et al., 2016). Descending inhibition from the RVM is functionally immature at birth (Fitzgerald & Koltzenburg, 1986; Hathway et al., 2009; Hathway et al., 2012), and descending serotonergic RVM–spinal dorsal horn projections facilitate rather than inhibit nociceptive and non-nociceptive signalling in the dorsal horn during the first weeks of life (de Kort et al., 2021a; Schwalzer et al., 2017). Moreover, fine-tuning and developmental restriction of the distribution of descending serotonergic RVM–spinal cord projections and spinal innervation patterns occurs postnatally in rats (Bregman, 1987; Rajaofetra, Sandillon, Geffard, & Privat, 1989; Xia et al., 2017), 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 (Walker et al., 2015) or inflammation (Zhang et al., 2010), 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 RVM–dorsal horn network. With the use of quantitative immunohistochemical analysis, we studied the effect of neonatal repetitive needle pricking on adult 5-hydroxytryptamine (5-HT) staining intensity in both adult RVM and spinal dorsal horn.

## MATERIALS AND METHODS

### 2.1 Animals

All animal experiments are performed in accordance with the European Directive for Protection of Vertebrate Animal Use for Experimental and Other Scientific Purposes (2010/66/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 \( N = 10 \). 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 males and/or female pups were taken from each litter for each condition to control for possible litter effects.

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Abbreviations: f, female; m, male; NP, needle prick; TC, tactile control; UD, undisturbed control.
(Chapman & Stern, 1979). 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.

2.2 | Repetitive neonatal procedural pain stimulation

To model repetitive procedural pain exposure in the neonatal intensive care unit (NICU), rat pups were noxiously stimulated four times a day via unilateral 2 mm calibrated needle pricks (NPs) in the mid-plantar surface of the left hind paw from P0 to P7 (NP; N = 7) as described previously (Knaepen et al., 2013; van den Hoogen et al., 2020). Control littersmates 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.

2.3 | Tissue isolation and preparation

When animals reached adulthood (aged 8 weeks), all animals were terminally anaesthetized 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-buffered 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 24 h, 25% sucrose solution for 72 h 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.

2.4 | Immunohistochemical detection of 5-HT

Spinal and RVM sections were stained for 5-HT immunoreactivity as described previously (Strackx et al., 2008). Slides were thawed at room temperature for 2 h 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 h (1:20,000 RVM, 1:10,000 spinal cord, 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 h 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 tetrahydrochloride (DAB)/nickel-chloride solution to visualize the immune complex of horseradish peroxidase reaction product (10 min for spinal cord, 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).

2.5 | 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 Provix 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 (ROIs) were made of Rexed lamina I–III of the dorsal horn as descending serotonergic fibres terminate here (de Kort et al., 2021a). Grayscale values were obtained for these laminae, based on atlas coordinates and delineation as described previously (Janssen et al., 2011). In addition, mean intensity was measured in 20 × 30 μm boxes in laminae I, II, III, and IV–V of the dorsal horn to obtain grayscale values per laminae (Schwaller et al., 2017). 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 1000 μm, width 800 μm). Six sections per lumbar level (L4 and L5) and per RVM were averaged to create one data point per animal.
2.6 | 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 (Gu & Wessendorf, 2007). 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 (Paxinos & Watson, 1998). ROIs were delineated at 12.5× magnification on live microscopic images displayed on the monitor. The number of cells was 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 150 by 150 μm 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 400× magnification with the optical fractionator method (MBF Bioscience, Williston, USA).

2.7 | Statistical analysis

All data are 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 sex, 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.

3 | RESULTS

3.1 | 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–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. No differences between sex in the intensity of 5-HT staining were observed in the ipsilateral (F (1, 10) = 1.139; p = 0.2775) or contralateral spinal dorsal horn (F (1, 10) = 0.4074; p = 0.5377), and data from both sexes were pooled. 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 with 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 with 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 with UC, evident in all laminae of the ipsilateral spinal dorsal horn (Figure 1c). No significant differences between NP and TC animals were observed in ipsilateral 5-HT immunostaining (p = 0.8640). 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). No sex differences were observed in the intensity of spinal 5-HT immunostaining in the different laminae of the dorsal horn (F (1, 56) = 0.9044; p = 0.3457).

3.2 | 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 with UC, evident in both the ipsilateral \((p = 0.0169)\) and contralateral RVM \((p = 0.0144)\). 5-HT staining intensity in the RVM was not significantly different between NP and TC animals (ipsilateral \(p = 0.4649\); contralateral \(p = 0.6480\)) or between NP and UC animals (ipsilateral \(p = 0.1727\); contralateral \(p = 0.1038\)). Pooled data of ipsi- and contralateral RVM 5-HT immunostaining showed a significant difference between conditions \((F(2, 12) = 4.8333; p = 0.0289; \text{Figure 2c})\), with higher 5-HT staining intensity in the RVM of TC animals as compared with UC \((p = 0.0229)\). No differences were observed between NP animals and TC animals \((p = 0.2405)\) or UC controls \((p = 0.3645)\).

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 \((F(1, 24) = 4.723; p = 0.0178)\) 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 with 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. *\(p < 0.05\) # \(p < 0.10\)
The number of 5-HT immunostained cells in the RVM did not differ between the ipsilateral and contralateral RVM or neonatal condition \( (F(1, 40) = 0.001; p = 0.9698) \) 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 were observed between neonatal conditions \( (F(2, 20) = 2.033; p = 0.1571); \) Figure 2b). No effects of sex were observed \( (F(1, 17) = 1.125; p = 0.3037) \).

### DISCUSSION

The present study is the first to analyse serotonergic alterations within the RVM–spinal dorsal horn network 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 NPs, whereas neonatal tactile stimulation increases 5-HT intensity and percentage of stained area in both the ipsi- and contralateral RVM. These long-lasting serotonergic changes in the RVM–spinal dorsal horn network 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 (Millan, 2002). Serotonergic projections from the RVM are the major source of serotonergic input in the spinal cord (Bowker et al., 1981). 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 (Bregman, 1987; Marlier, Poulat, Rajaofetra, Sandillon, & Privat, 1992). In adulthood, local release in the spinal dorsal horn results in predominant inhibition...
of nociceptive signalling (Ali et al., 1994; Bardin et al., 1997; de Kort et al., 2021a; Liu et al., 2007; Xie et al., 2012; Xu et al., 1994). 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 (Schwaller & Fitzgerald, 2014; van den Hoogen et al., 2017; Williams & Lascelles, 2020). In fact, nociceptive C-fibre terminal innervation and firing of somatosensory second-order neur- ons in the spinal dorsal horn are increased after neonatal noxious procedures, suggesting a hypersensitive spinal nociceptive network in adulthood (Knaepen et al., 2013; van den Hoogen et al., 2018). Serotonergic receptors, including the 5-HT1a and 5-HT3, are expressed on both C-fibre terminals and second-order projection neurons and have the ability to regulate nociceptive processing the release of 5-HT (Bardin, 2011; de Kort et al., 2021a). The decrease in spinal 5-HT staining intensity may contribute to the sensitized spinal nociceptive network after neonatal procedural pain due to lower levels of descending serotonergic projections and serotonin levels in the dorsal horn. 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 (de Kort, Joosten, Patijn, Tibboel, & van den Hoogen, 2021b; Knaepen et al., 2013; van den Hoogen et al., 2016; van den Hoogen, van Reij, Patijn, Tibboel, & Joosten, 2018). In line with this, depletion of descending serotonergic projections does not alter mechanical or thermal sensitivity (Minor et al., 1988; Palm et al., 2008; Wei et al., 2010). 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 (Knaepen et al., 2013; van den Hoogen et al., 2020; van den Hoogen et al., 2016).

An important observation of our results is that repet- itive tactile stimulation also decreases 5-HT staining intensity in the ipsilateral but not the contralateral dorsal horn at a level comparable with neonatal painful pro- cedures. No effects of tactile (or painful) stimulation were observed in the contralateral dorsal horn. Hence, these unilateral effects of early life tactile stimulation are due to the stimulation of the left hind paw and not due to early life separation, or effects would be observed bilaterally (Bravo et al., 2014). Tactile input has the abil- ity to guide nociceptive synaptic organization during early development, even in the absence of noxious input (Waldenström et al., 2003). More importantly, noxious procedures also activate low-threshold touch receptors and play an important role in acute nociceptive signalling and the development of mechanical paw withdrawals (Arcourt et al., 2017). Anatomical localization of 5-HT positive fibres suggests that there may be a relationship between sensory modality and the type of 5-HT innervation it receives (Wu & Wessendorf, 1992). 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 3 weeks of life in rats (Schwaller et al., 2017). This early-life facilitation of descending RVM projections is selective for sensory inputs from A-fibres but not C-fibres (Koch & Fitzgerald, 2014). 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 behavioural and functional effect, repetitive tactile procedures also increase firing of somatosensory projec- tion neurons upon noxious and non-noxious stimulation in adulthood at baseline, although to a lesser extend (van den Hoogen et al., 2018).

Descending serotonergic modulation of spinal somatosensory and nociceptive processing is mediated via seven 5-HT receptor families (Hannon & Hoyer, 2008). 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 (de Kort et al., 2021a; Heijmans et al., 2021). Therefore, their respective balance in the RVM and spinal dorsal may be altered differently after repetitive noxious as compared with 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 and in the periaqueductal gray, the latter an important regulator of RVM serotoner- gic modulation (Anseloni et al., 2005; Ren et al., 2005). In addition, the 5-HT3 and 5-HT7 receptors play a promi- nent role in facilitating nociception in chronic pain states (Heijmans et al., 2021; Huang et al., 2018) and may also contribute to a sensitized spinal nociceptive network after neonatal pain. Future studies should aim to link the observed anatomical changes in the descending seroto- nergic system within the RVM and spinal dorsal horn to the role and modulation of specific serotonin receptors to explain the long-term effects in following neonatal nox- ious 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 stimula- tion, without differences in the number 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 fibres in the RVM. To what extent 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 (Walker et al., 2015) and neonatal inflammation (Zhang et al., 2010), but the role of descending serotonergic fibres in these functionality changes is yet to be investigated. Neonatal injury-induced alterations in RVM descending control were prevented by perioperative anaesthetic 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 (Walker et al., 2015). 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-HT staining intensity in the RVM appears to be more general as both the ipsilateral and contralateral sides are affected. Moreover, both 5-HT grayscale values and 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 (Imbe et al., 2004; Shimizu et al., 2020).

5 | CONCLUSION

This detailed anatomical study demonstrates that neonatal noxious and tactile procedures result in long-lasting anatomical changes in the descending serotonergic RVM–spinal dorsal horn network. These changes may underlie the long-term effects following painful stimulation in early life.

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CONFLICT OF INTEREST

The authors have no conflict of interests to declare.

AUTHOR CONTRIBUTIONS

A.R. de Kort, E.A.J. Joosten, J. Patijn, D. Tibboel and N.J. van den Hoogen conceived the research. A.R. de Kort, E.A.J. Joosten and N.J. van den Hoogen designed and conceptualized the study and contributed to its structure and content. A.R. de Kort performed the experimental research, tissue processing and collection and immunohistochemical staining. H.E. Steinbusch provided technical support during immunohistochemical staining. A.R. de Kort analysed the RVM data. E. Versantvoort analysed the spinal dorsal horn data. A.R. de Kort drafted the manuscript and figures. All authors commented on previous versions of the manuscript, critically revised and quality assessed the manuscript. All authors have read and approved the final version of the manuscript.

ETHICAL APPROVAL STATEMENT

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).

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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The Development of Central Nociceptive


