RESEARCH ARTICLE

Hypoxia-ischemia in the immature rodent brain impairs serotonergic neuronal function in certain dorsal raphé nuclei

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Abstract

Neonatal hypoxia-ischemia (HI) results in losses of serotonergic neurons in specific dorsal raphé nuclei. However, not all serotonergic raphé neurons are lost and it is therefore important to assess the function of remaining neurons in order to understand their potential to contribute to neurological disorders in the HI-affected neonate. The main objective of this study was to determine how serotonergic neurons, remaining in the dorsal raphé nuclei after neonatal HI, respond to an external stimulus (restraint stress). On postnatal day 3 (P3), male rat pups were randomly allocated to one of the following groups: (i) control + no restraint (n = 5), (ii) control + restraint (n = 6), (iii) P3 HI + no restraint (n = 5) or (iv) P3 HI + restraint (n = 7). In the two HI groups, rat pups underwent surgery to ligate the common carotid artery and were then exposed to 6% O_2 for 30 minutes. Six weeks after P3 HI, on P45, rats were subjected to restraint stress for 30 minutes. Using dual immunolabeling for Fos protein, a marker for neuronal activity, and serotonin (5-hydroxytrypamine; 5-HT), numbers of Fos-positive 5-HT neurons were determined in five dorsal raphé nuclei. We found that restraint stress alone increased numbers of Fos-positive 5-HT neurons in all five dorsal raphé nuclei compared to control animals. However, following P3 HI, the number of stress-induced Fos-positive 5-HT neurons was decreased significantly in the dorsal raphé ventrolateral, interfascicular and ventral nuclei compared with control animals exposed to restraint stress. In contrast, numbers of stress-induced Fos-positive 5-HT neurons in the dorsal raphé dorsal and caudal nuclei were not affected by P3 HI. These data indicate that not only are dorsal raphé serotonergic neurons lost after neonatal HI, but also remaining dorsal raphé serotonergic neurons have reduced differential functional viability in response to an external stimulus. Procedures were approved by the University of Queensland Animal Ethics Committee (UQCCR958/08/NHMRC) on February 27, 2009.

Key Words: *dorsal raphé nuclei; Fos; hypoxia-ischemia; neonate; newborn brain injury; preterm; restraint stress; serotonin*

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Introduction

Hypoxic-ischemic (HI) brain injury is a major contributor to mortality and long-term morbidity in preterm newborns (Volpe, 1997; Gopagondanahalli et al., 2016). White matter and neuronal damage are key features of preterm HI injury (Leviton and Gressens, 2007; Pierson et al., 2007; Volpe, 2009) but relatively few studies have examined, and thus characterized, the extensive neuronal injury in the immature brain. Reports from human and animal studies show the serotonergic neural network is an important system disrupted after neonatal HI or asphyxia (Leech and Alvord, 1977; Peters et al., 2000; Logitharajah et al., 2009; Reinebrant et al., 2010; Wixey et al., 2011b). Major neurological impairments are seen in HI-affected neonates and include life-long disorders such as learning disabilities, cerebral palsy, mental retardation, hyperactivity and depression (Anderson et al., 2003; Peterson et al., 2003; Hack et al., 2004; Logitharajah et al., 2009); many of which match those reported following early disruption to the serotonergic network (Hornung, 2003; Murphy et al., 2004; Popa et al., 2008; Dayan and Huys, 2009).

Serotonergic neurons are distributed in an array of raphé nuclei in the brainstem and constitute the sources of a pervasive serotonergic network in the brain (Kosofsky and Molliver, 1987; Baker et al., 1990; Hornung, 2003). Different raphé sub-division represent differential brain connectivity patterns. Axons project from the dorsal raphé dorsal, caudal, and ventrolateral predominantly to the cerebral cortex, basal ganglia, thalamus, hypothalamus, hippocampus and amygdala. Due to the high innovation network through the brain, these raphé subdivisions are able to regulate or influence numerous functions. Animal models show significant losses of dorsal raphé serotonergic nuclei following a neonatal HI insult (Reinebrant et al., 2010; Wixey et al., 2011a, b; Reinebrant et al., 2012, 2013). Furthermore these losses occur in association with disruption of the serotonergic transporter (SERT), reductions in serotonergic fibers, decreased serotonin (5-hydroxytrypamine; 5-HT) levels and loss of 5-HT7 receptors in the brain (Reinebrant et al., 2010; Wixey et al., 2011a, b; 2018). However, not all serotonergic raphé neurons

are lost after a neonatal HI insult and thus the functional viability of remaining serotonergic neurons is likely to be important in determining neurological, physiological and autonomic outcomes in the HI-affected neonate.

Using a well-established preterm HI model in the postnatal day 3 (P3) rat pup (Carty et al., 2008; Wixey et al., 2011a), we tested whether P3 HI affects the responses of serotonergic raphé neurons to an external stimulus. We examined restraint stress-induced activation of serotonergic raphé neurons in five dorsal raphé nuclei with or without P3 HI using dual immunolabeling for Fos (a marker of neuronal activation) and 5-HT (a marker for serotonergic cells). Restraint stress is known to activate raphé neurons and alter 5-HT release in the brain (Lee et al., 1987; Mo et al., 2008; Yoshida et al., 2010; de Andrade et al., 2012). Determining the stress-induced Fos response in serotonergic raphé neurons will help ascertain the functional viability of remaining serotonergic neurons after P3 HI brain injury and will also provide novel characterization of Fos expression in phenotypically identified serotonergic raphé nuclei.

Materials and Methods

Animals

This study utilized Sprague-Dawley rat dams (The University of Queensland) and their pups (10–12 pups per litter). Rats were kept in normal laboratory conditions (22°C and 12-hour light/dark cycle) with free access to standard rat feed and water. To minimize the possible impact of circadian rhythms, experimental procedures were performed between 9:00 am and 11:00 am. Procedures were approved by the University of Queensland Animal Ethics Committee (UQCCR958/08/NHMRC) on February 27, 2009 and efforts were made to minimize the number of animals used and limit suffering.

Hypoxic-ischemic brain insult

On postnatal day 3 (P3), male rat pups were randomly allocated to one of the following groups: (i) control + no restraint (n = 5), (ii) control + restraint (n = 6), (iii) P3 HI + no restraint (n = 5) or (iv) P3 HI + restraint (n = 7). Only male rat pups were studied to eliminate potential sex effects. HI was induced as described previously (Carty et al., 2008). Briefly, under 2% isoflurane anaesthesia (inhalation; Baxter Healthcare, NSW, Australia), the right common carotid artery of the P3 rat pup was carefully dissected from surrounding tissue and permanently ligated. Pups recovered for 10 minutes before exposure to 6% O_2 for 30 minutes at 37°C in a humidified chamber. Control pups were treated the same way without ligation of the carotid artery and no exposure to 6% O_2. All pups were then returned to the dam. On P21, rats were weaned from the dam and housed with 3–4 same-sex littermates.

Induction of HI on P3 in rats produces features of brain pathology typically seen in preterm human neonates. These features include hypomyelination, neuronal losses and encephalopathy (Marin-Padilla, 1999; Back et al., 2001; Hagberg et al., 2002; Peterson et al., 2003; Carty et al., 2008). In addition, the P3 rat brain is considered developmentally similar to the human brain at 24 to 28 gestational weeks (Clancy et al., 2001; Hagberg et al., 2002).

Restraint stress and brain collection

Animals were exposed to restraint stress on P45. We examined restraint stress in the rats exposed to HI at P3, an age equivalent to early adulthood in human terms. Animals were wrapped carefully in individual plied mesh moulds to immobilize them for 30 minutes. Two hours after the initiation of restraint stress, animals were sacrificed by sodium pentobarbitone (80 mg/kg, i.p.; Lethabarb, Virbac, France). This restraint stress procedure has previously been shown to produce a robust and consistent activation pattern of nuclear Fos protein in the rodent brain (Dayas et al., 2001; Crane et al., 2005). Animals were perfused transcardially through the left ventricle with sodium nitrite solution (1%) and formaldehyde (4% in 0.1 M PBS, pH 7.4). The brain was removed and post-fixed for 2 hours in formaldehyde followed by cryoprotection overnight in sucrose (10% in 0.1 M PBS, pH 7.4, 4°C).

Immunohistochemistry

Forebrain (40 µm) and brainstem (50 µm) coronal sections were collected using a sliding microtome (Thermo Fisher Scientific, Victoria, Australia). For forebrain hemisphere size measurements, forebrain sections (1-in-4 series, 160 µm intervals) were mounted on chrome-alum subbed slides, dehydrated in a series of alcohol, cleared in xylene and cover-slipped. For labeling of brainstem sections, one 1-in-5 series (250 µm intervals) was used. Every brain section is collected in a sequential manner, and a 1-in-5 series is the selection of every 5th consecutive section that is 250 µm apart for in the brainstem. The sections were exposed to a dual immunoperoxidase technique to visualise nuclear Fos protein in 5-HT neurons in the dorsal raphé nuclei. Sections were labeled using a rabbit anti-Fos-antibody (1:10,000, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) for 48 hours at room temperature followed by biotinylated anti-rabbit (1:400, Jackson ImmunoResearch, West Grove, PA, USA) for a further 2 hours at room temperature. Sections were then incubated in an avidin-biotin-horseradish peroxidase complex (ABC) (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame, CA, USA) for 1.5 hours. Horseradish peroxidase activity was visualized by nickel diaminobenzidine solution (DAB) to produce black nuclear labeling in Fos-positive neurons. The sections then underwent labeling to identify 5-HT-positive neurons. Sections were incubated with a rabbit anti-5-HT antibody (1:7000, gift from D. Pow, The University of Queensland) for 48 hours at room temperature, followed by incubation in biotinylated anti-rabbit secondary antibody (1:400, Jackson ImmunoResearch, West Grove, PA, USA) for 2 hours at room temperature. Sections were then incubated for 1.5 hours in ABC as above and immunolabeling was visualized using DAB (nickel omitted) producing amber coloured 5-HT-positive neurons. To minimize variations in immunohistochemistry, sections from all experimental groups were incubated simultaneously. Brainstem sections were then mounted on chrome-alum subbed

slides, dehydrated in a series of alcohol, cleared in xylene and cover-slipped.

Data analysis

All cell counts were performed by an observer blind to the experimental condition. The total number of 5-HT-positive neurons and Fos-positive 5-HT neurons were counted in specific brainstem raphé nuclei: (i) dorsal raphé dorsal - DRd (three sections, bregma –7.04 to –7.64 mm), (ii) dorsal raphé ventrolateral - DRvl (three sections, bregma –7.64 to –8.3 mm), (iii) dorsal raphé ventral - DRv (five sections, bregma –7.64 to –8.0 mm), (iv) dorsal raphé interfascicular - DRif (five sections, bregma –8.0 to –9.16 mm) and (v) dorsal raphé caudal - DRc (four sections, bregma –8.72 to –9.3 mm) (Paxinos and Watson, 1997). Serotonergic neuronal morphology is distinctly disparate between each dorsal raphe nucleus, and counts and delineation of these dorsal raphé nuclei were based on cytoarchitecture and morphology as previously described (Baker et al., 1990; Hornung, 2003).

Brain injury severity was measured in P3 HI animals by calculating the cerebral hemisphere size in the ligated hemisphere, relative to the non-ligated hemisphere. Hemispheric outlines of the right and left brainstem (bregma –7.64 to –8.3 mm) and forebrain (bregma 3.7 to 2.7 mm) over three consecutive sections were traced using phase analysis software (Analysis Life Science Research, Victoria, Australia). The percentage change in hemisphere size ipsilateral to the ligation was then calculated.

Data are presented as the mean ± SEM. One-way ANOVA was performed using GraphPad Prism (Version 7.04, GraphPad Sodtware, San Diego, CA, USA) followed by Student's *t*-tests for statistical analyses of results and the level of significance was $P < 0.05$.

Results

Hemisphere size after P3 HI

In the forebrain of P3 HI animals, there was an 11 ± 0.52% reduction in cerebral hemisphere size on the ligated side compared to the non-ligated side. This is consistent with our previous studies using the same P3 HI model on P45 (Reinebrant et al., 2012; Wixey et al., 2011a) and confirms a reproducible severity of injury was produced. In addition, the brainstem size did not change after P3 HI as we have reported previously (Buller et al., 2008; Wixey et al., 2011a; Reinebrant et al., 2012).

Numbers of 5-HT-positive neurons in dorsal raphé nuclei after P3 HI and restraint stress

There were no effects of restraint or P3 HI on numbers of 5-HT-positive neurons on the non-ligated side in all raphé nuclei examined (**Figure 1E**). In P3 HI animals, on the ligated side, there was a significant reduction in the numbers of 5-HT-positive neurons in the DRd, DRvl and DRv nuclei compared to control animals (**Figure 1F**). These P3 HI effects were mirrored in animals subjected to restraint stress and thus restraint stress had no effect on 5-HT-positive neuron counts in control and P3 HI groups (**Figure 1A–D**). These outcomes reaffirm the reliability of the P3 HI model to alter 5-HT neuron counts in specific dorsal raphé nuclei (Reinebrant et al., 2010, 2012; Wixey et al., 2011a).

Effects of restraint stress on numbers of Fos-positive 5-HT neurons in dorsal raphé nuclei

In control animals not exposed to restraint stress, only a paucity of Fos-positive 5-HT neurons was found in the dorsal raphé nuclei (**Figure 2**). Restraint stress (in control animals not subjected to P3 HI) significantly increased the number of Fos-positive 5-HT neurons in all raphé nuclei examined compared with control animals (no restraint) in both hemispheres (**Figure 2**). In addition, the proportion of Fos-positive neurons to 5-HT neurons in each raphé division was highest in the DRd (23%) followed by the DRc (17%), DRif (16%), DRvl (15%) and DRv nuclei (10%).

Effects of P3 HI on numbers of Fos-positive 5-HT neurons in dorsal raphé nuclei after restraint stress

As detailed above, in animals subjected to restraint stress (control + restraint) there were significant increases in numbers of Fos-positive 5-HT neurons in all five raphé nuclei examined compared to controls (no restraint). However, in animals that were subjected to P3 HI and restraint stress, the numbers of Fos-positive 5-HT neurons on the ligated side were reduced significantly in the DRvl (reduced by 37%), DRif (30%) and DRv nuclei (44%; **Figure 2F**). In contrast, P3 HI had no effect on numbers of Fos-positive 5-HT neurons in the DRd and DRc nuclei (**Figure 2F**). There was no effect of P3 HI on numbers of restraint stress-induced Fos-positive 5-HT neurons on the non-ligated side (**Figure 2E**).

Discussion

This study provides novel findings that P3 HI brain injury in rat pups alters serotonergic raphé neuronal responses in the brainstem. P3 HI resulted in losses of serotonergic raphé neurons and also reduced the responsiveness of remaining raphé serotonergic neurons to an external stimulus, restraint stress. These changes were dependent on the dorsal raphé division examined. The differential effects of P3 HI on the raphé serotonergic neurons suggest that each division varies in its susceptibility to P3 HI and that each may also have a different role in the serotonergic response to restraint stress. Defining the function of remaining serotonergic neurons is important to help devise interventions aimed to rescue remaining serotonergic raphé neurons, prompt recovery of these neurons and improve functional outcomes after neonatal HI brain injury.

Distinct raphé nuclei recruited after restraint stress

The serotonergic system has critical roles in generating central stress responses to a variety of stressors. For example, swim stress activates 5-HT neurons in selective dorsal raphé subdivisions (Kelly et al., 2011; Drugan et al., 2013). Although restraint stress alters 5-HT release in the forebrain (Lee et al., 1987; Mo et al., 2008) and recruits raphé neurons (Yoshida et al., 2010; de Andrade et al., 2012), this is the first

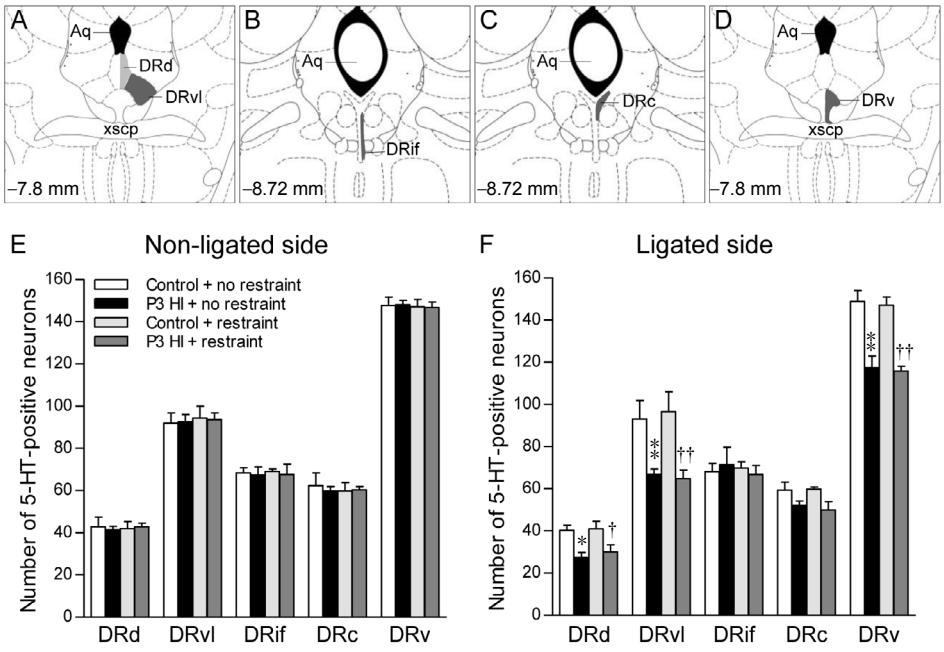

Figure 1 Effects of P3 HI and restraint stress on numbers of 5-HT-positive neurons in dorsal raphé nuclei.
Schematics present the approximate location (mm relative to bregma) of counts in each of the five dorsal raphé nuclei (A–D). The total numbers of 5-HT-positive neurons in each dorsal raphé nucleus on the non-ligated (E) and ligated side (F) are presented. There were no effects of restraint stress or P3 HI on numbers of 5-HT neurons on the non-ligated side (E). On the ligated side in P3 HI animals, there was a significant reduction in the numbers of 5-HT-positive neurons in the DRd, DRvl, and DRv nuclei compared to control animals (F). Values are presented as mean ± SEM (n = 5 and 6 in the control + no restraint and control + restraint groups, respectively). ∗P < 0.05, ∗∗P < 0.01, *vs.* control + no restraint animals; †P < 0.05, ††P < 0.01, *vs.* control + restraint (one-way analysis of variance followed by Student's t-test). 5-HT: 5-Hydroxytryptamine; Aq: aqueduct (Sylvius); DRc: dorsal raphé caudal nucleus; DRd: dorsal raphé dorsal nucleus; DRif: dorsal raphé interfascicular nucleus; DRv: dorsal raphé ventral nucleus; DRvl: dorsal raphé ventrolateral nucleus; P3 HI: postnatal day 3 hypoxia-ischemia; xcsp: decussation of the superior cerebellar peduncle.

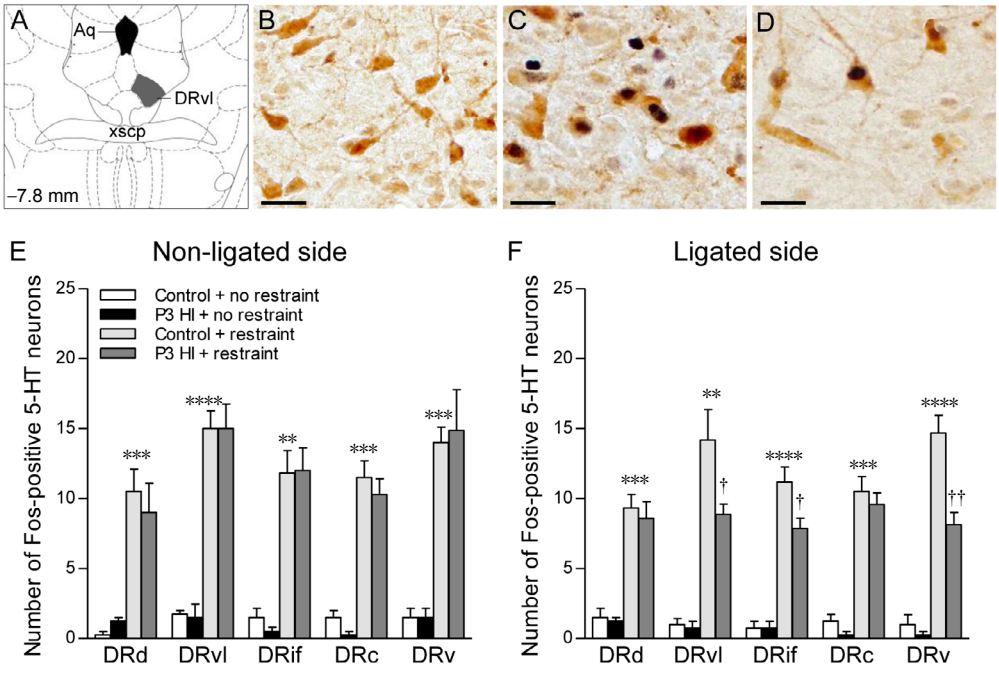

Figure 2 Effects of P3 HI and restraint stress on numbers of Fos-positive 5-HT dorsal raphé neurons.
Photomicrographs of brainstem sections through the dorsal raphé ventrolateral nucleus (A, mm relative to bregma) from a control + no restraint (B), control + restraint (C) and P3 HI + restraint animals (D). Brainstem sections have been immunolabeled for Fos protein (black nucleus) and 5-HT (amber cytoplasm). Scale bars represent 25 µm. The histograms present the total numbers of Fos-positive 5-HT neurons in each dorsal raphé nucleus on the non-ligated (E) and ligated side (F). Restraint stress in control animals significantly increased the number of Fos-positive 5-HT neurons in comparison to control animals with no restraint (E and F). In P3 HI animals subject to restraint stress, the numbers of Fos-positive 5-HT neurons on the ligated side were significantly reduced in the DRvl, Drif, and DRv nuclei (F). Values are presented as mean ± SEM (n = 5 and 6 in the control + no restraint and control + restraint groups, respectively). ∗∗P < 0.01, ∗∗∗P < 0.001, ∗∗∗∗P < 0.0001, *vs.* control + no restraint animals; †P < 0.05, ††P < 0.01, *vs.* control + restraint (one-way analysis of variance followed by Student's t-test). 5-HT: 5-Hydroxytryptamine; Aq: aqueduct (Sylvius); DRc: dorsal raphé caudal nucleus; DRd: dorsal raphé dorsal nucleus; DRif: dorsal raphé interfascicular nucleus; DRv: dorsal raphé ventral nucleus; DRvl: dorsal raphé ventrolateral nucleus; P3 HI: postnatal day 3 hypoxia-ischemia; xcsp: decussation of the superior cerebellar peduncle.

study to show phenotypically identified serotonergic neurons are activated in multiple dorsal raphé nuclei following restraint stress in neonatal HI rats. All dorsal raphé nuclei examined responded robustly to restraint stress with a significant increase in numbers of activated serotonergic neurons. However, different degrees of recruitment were observed. For example the greatest numbers of activated neurons were found in the DRv and DRvl nuclei, and the least in the DRd, suggesting that serotonergic raphé nuclei are differentially recruited following restraint stress. These findings are consistent with reports that different 5-HT release patterns are apparent in raphé and forebrain nuclei after restraint stress (Lee et al., 1987; Mo et al., 2008). Other stressors also have selective actions in terms of Fos responses within different serotonergic dorsal raphé nuclei and can modulate stress responses (Grahn et al., 1999; Abrams et al., 2005; Gardner et al., 2005; Staub et al., 2005). Furthermore, it is plausible that serotonergic neurons in each dorsal raphé nuclei may subserve different functions in response to restraint stress. Each dorsal raphé nucleus has a distinct innervation pattern in the forebrain and, as shown for a variety of other stressors, may influence stress responses depending on the recruitment of specific neural pathways (Lowry et al., 2005). For example, neurons in the DRd and DRvl modulate anxiety behaviours via neural projections to the basolateral amygdala (Abrams et al., 2005). Future studies that identify dorsal raphé neural pathways recruited following restraint stress, in concert with central stress responses, could be key to characterizing critical functional pathways.

Differential effects of P3 HI on serotonergic raphé neuronal responses to restraint stress

In animals subjected to P3 HI, we observed major effects on the responsiveness of remaining serotonergic raphé neurons following restraint stress. The DRvl, DRif and DRv nuclei were particularly affected by P3 HI with significant reductions in numbers of restraint stress-induced activated serotonergic raphé neurons. Furthermore changes were only evident ipsilateral to the carotid ligation. In contrast, there was no effect of P3 HI on the Fos response to restraint of serotonergic neurons in the DRd nor DRc. Thus, after a P3 HI insult, remaining serotonergic neurons elicited reduced responsiveness to an external stimulus and the functional viability of serotonergic neurons appears to depend on their localisation in dorsal raphé nuclei.

The reduced recruitment of serotonergic raphé neurons in P3 HI animals may reflect changes in P3 HI-induced numbers of actual serotonergic raphé neurons. Consistent with our previous studies using the same model, P3 HI significantly reduced counts of serotonergic neurons in the DRd, DRvl and DRv nuclei ipsilateral to the carotid ligation (Reinebrant et al., 2010, 2012; Wixey et al., 2011a, b). However, although a reduced stress response was observed in the DRvl, the proportion of activated serotonergic neurons after restraint stress in the DRvl remained unchanged after P3 HI compared with control animals. Thus the reduced responsiveness may simply reflect the overall loss of serotonergic neurons in the DRvl. Conversely, the DRd incurred significant losses of serotonergic neurons and yet the Fos response to restraint in the DRd was seemingly not affected after P3 HI. On the other hand, serotonergic neurons were not lost in the DRif after P3 HI but there was a significant reduction in numbers of restraint-induced activated neurons in the DRif after P3 HI. Thus after P3 HI, remaining neurons in the DRif appeared to be anatomically sound, at least in terms of expressing antigenic sites for 5-HT and serotonin transporter antibodies (Reinebrant et al., 2010, 2012), and yet had a reduced functional response to restraint stress. Taken together, P3 HI significantly affected the stress response in specific serotonergic dorsal raphé nuclei and that the overall loss of serotonergic neurons only partly accounts for the reduced functional response.

Mechanisms of reduced responsiveness of remaining raphé serotonergic neurons after P3 HI

Multiple factors may contribute to the differential responsiveness to restraint stress of remaining serotonergic raphé neurons in P3 HI animals. The brainstem does not endure local ischemic changes, interruption of blood flow nor significant neuroinflammatory changes after neonatal HI (Vannucci et al., 1988; Wixey et al., 2011b). It is therefore considered that factors external to the brainstem raphé nuclei might contribute to the disrupted function of serotonergic raphé neurons.

Each dorsal raphé nucleus has a distinct innervation pattern in the forebrain and also receives distinct descending neural inputs from various forebrain regions (Imai et al., 1986; Vertes, 1991; Peyron et al., 1998; Kanno et al., 2008). These neural connections with the dorsal raphé nuclei may not only influence the effects of restraint stress on the recruitment of serotonergic raphé neurons in the intact brain but also after P3 HI brain injury. Forebrain regions innervated by dorsal raphé nuclei, such as the frontal cortex and thalamus, are damaged after neonatal HI (Towfighi et al., 1997; Stadlin et al., 2003). In addition, we have recently shown that P3 HI disrupts serotonergic fibers in the forebrain (Wixey et al., 2011b) as well as afferent and efferent dorsal raphé neural connections with forebrain regions (Reinebrant et al., 2012, 2013). We propose that the detrimental effects of P3 HI on these neural connections may influence the survival and the ability of remaining neurons to function normally. This may occur as a result of damage to axonal transport systems, changes to survival and death signals relayed between damaged forebrain and dorsal raphé nuclei and interruption of neurochemical storage and release (Northington et al., 2007; Perlson et al., 2010). In addition, P3 HI may disrupt the heterogeneous population of serotonergic receptor subtypes in dorsal raphé nuclei (Commons et al., 2003; Anju et al., 2011) and consequently influence the responsiveness of serotonergic neurons to restraint stress. Nevertheless it remains to be determined whether these various factors might impact on the survival and/or modulate the function of remaining serotonergic dorsal raphé nuclei after neonatal HI.

Conclusions

We present novel findings that neonatal HI affects the restraint stress-induced activation of select dorsal raphé nuclei. Neonatal HI reduced the responsiveness of serotonergic raphé neurons in the DRvl, DRif and DRv, but not in the DRd or DRc nuclei. These outcomes suggest that there are differing susceptibilities of dorsal raphé nuclei to P3 HI and that sub-populations of remaining neurons have decreased function. We also demonstrated that there is a topographical distribution of restraint-stress activated serotonergic neurons in dorsal raphé nuclei. This work provides a foundation to examine the possible mechanisms contributing to the observed reduced function of select serotonergic dorsal raphé neurons after neonatal HI. However, further studies are required to examine whether changes in serotonergic-dependent behaviours are associated with the observed effects on the raphé neurons in this rat model. In conclusion, these findings demonstrate that P3 HI leads to long-lasting changes in the central serotonergic system and that these changes to serotonergic neuronal function may help elucidate the mechanisms underpinning life-long functional deficits associated with neonatal HI brain injury.

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