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Cesarean birth elicits long-term effects on vasopressin and oxytocin neurons in the hypothalamic paraventricular nucleus of mice

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ABSTRACT

Birth is an extraordinary event for placental mammals and occurs at a time when key developmental processes are shaping the brain. Remarkably, little is known about the contributions of birth to brain development and whether birth mode (vaginal vs. Cesarean) alters neurodevelopmental trajectories. We previously reported that Cesarean birth reduces vasopressin (VP) neuron number in the hypothalamic paraventricular nucleus (PVN) of mice at weaning. In this study, we investigated whether this effect extends to adulthood and whether birth mode affects oxytocin (OT) neurons, which are another prominent population in the PVN. We found that Cesarean-born adults had fewer VP neurons in the PVN, specifically in magnocellular regions. Interestingly, these regions also had more dying cells following a Cesarean birth, suggesting that cell death may be the underlying mechanism. The PVN of Cesarean-born adults also had smaller VP neuron somas and reduced VP efferent projections. Additionally, Cesarean-born mice showed fewer and smaller OT neurons in the PVN, but these effects were less robust than for VP neurons. We also examined VP and OT neuron number in the supraoptic and suprachiasmatic nuclei but found no effect of birth mode in these regions. Thus, Cesarean birth causes long-term effects on the VP and, to a lesser extent, OT systems in the PVN, suggesting that this region is particularly sensitive to the effects of birth mode. Our findings may help explain the social deficits reported for Cesarean-born mice, and are also of clinical significance given the widespread practice of Cesarean births across the world.

1. Introduction

Cesarean deliveries now account for about one-third of all births in the United States, with rates even higher, and rising, in other countries (Hehir et al., 2018; Martin et al., 2021). Although C-section can be life-saving for the mother and/or infant, rates of the procedure far surpass what is deemed medically necessary by the World Health Organization (Betran et al., 2016; Sandall et al., 2018). Cesarean delivery has been associated with negative health consequences for the offspring, including higher rates of asthma, allergies, type-2 diabetes, and obesity (reviewed in Tribe et al., 2018). There are also associations of Cesarean birth with behavioral and cognitive consequences in humans (Curran et al., 2015; Polidano et al., 2017; Yip et al., 2017; Zhang et al., 2019), suggesting effects on neurodevelopment. However, these latter findings are more controversial, and it is difficult to determine causation from human epidemiological studies. For example, effects of elective Cesarean births are confounded with changes in birth timing, and emergency Cesarean deliveries are often confounded with birth complications. In addition, Cesarean birth is associated with higher rates of maternal obesity and changes in breast feeding (Stinson et al., 2018), and is accompanied by antibiotic treatment of the mother, which alters the microbiome of both mother and infant (Azad et al., 2016; Dierikx et al., 2020; Stearns et al., 2017).

Carefully controlled animal studies may be able to address whether birth mode, per se, affects neural development, and some of the earliest studies found alterations in neurotransmitter systems in Cesarean-born adult rats (Boksa and El-Khodor, 2003; El-Khodor and Boksa, 2003; El-Khodor et al., 2004). Recently, social deficits have been found during development and in adulthood in Cesarean-born mice (Morais et al., 2021; Morais et al., 2020; Nagano et al., 2021). Even many of the rodent studies, however, have introduced confounds into their designs, such as earlier delivery for Cesarean births versus vaginal deliveries, or cross-fostering only for Cesarean-delivered pups. Indeed, Chiesa et al. (2019) concluded that the effects of Cesarean birth they found on brain development were due not to birth mode, but to subtle changes in birth timing between Cesarean- and vaginally-born groups.

In a carefully controlled study in mice, in which we matched subjects for foster rearing, gestation length, and time of day of birth (to control

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for circadian effects), we reported greater developmental neuronal cell death in Cesarean-delivered newborns compared to those born vaginally, and this was associated with decreased vasopressin (VP) neuron numbers in the paraventricular nucleus of the hypothalamus (PVN) at weaning (Castillo-Ruiz et al., 2018). It is unknown whether this phenotype persists into adulthood, or if VP in other hypothalamic regions is affected by birth mode. Cesarean birth could also affect VP system parameters other than cell number (e.g., soma size, efferent projections), but this has not been examined. The current study was designed to address these questions. In addition, we examined oxytocin (OT) neuron number and morphology in Cesarean-born animals. VP and OT are closely related neuropeptides that bind to each other's receptors (Caldwell and Albers, 2016; Hicks et al., 2016; Manning et al., 2008; Ragnauth et al., 2004; Song and Albers, 2018). Besides their peripheral roles (VP: vasoconstriction, osmolality, stress response; OT: labor and milk letdown), VP and OT also play important roles in sociality and social recognition memory (Caldwell, 2017; Johnson and Young, 2017).

As in our previous study (Castillo-Ruiz et al., 2018), Cesarean births were yoked to vaginal births to precisely match total gestation length and time of day, and pups of both birth modes were cross-fostered and raised in mixed-group litters to minimize maternal effects. VP and OT immunoreactive cells were then examined in the PVN, supraoptic nucleus (SON) and suprachiasmatic nucleus (SCN) in adulthood. We also examined cell death in the neonatal PVN after a vaginal or Cesarean birth to explore a potential developmental mechanism for the long-term effects of birth mode.

2. Methods

2.1. Animals

Adult female and male C57BL/6J mice were bred in-house or obtained from The Jackson Laboratory (Bar Harbor, ME, USA). Animals were maintained on a 12 h:12 h light dark cycle with food and water available ad libitum. All procedures were approved by the Georgia State University Institutional Animal Care and Use Committee and were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Timed pregnancies and birth delivery mode

Timed pregnancies were generated by pairing animals ($n = 6$ pairs) 1 h before lights off, and separating them on the following morning, within 2 h after lights on. This was considered embryonic day (E) 0. Starting on the eve of E19, births were monitored hourly (under red light illumination during the dark phase), and when a female had vaginally delivered one pup, another female not in labor was randomly chosen for a Cesarean delivery. In this way, birth mode groups were matched for gestation length and time of day of parturition. For Cesarean deliveries, dams were exposed to 2% CO_2 and, upon anesthesia onset, were rapidly decapitated ($n = 3$). An incision was made first through the skin and abdominal wall, and then through the uterine horns to remove the pups one by one. Cesarean-delivered pups were stimulated to breath by cleaning membranes from their muzzles and gently prodding them with a cotton tip applicator. Vaginally-delivered pups were removed from cages within an hour of birth and, together with Cesarean-born pups, placed on a heating pad kept at ±32 °C. Pups were marked with tattoo ink, and 7–9 pups were randomly assigned to foster dams ($n = 6$), with each dam receiving a mixture of Cesarean- and vaginally-delivered pups. A subset of the animals was collected postnatally to participate in another research study. The remaining offspring (8 vaginally born and 6 Cesarean delivered) were weaned into same-sex groups and sacrificed at P60.

2.3. Brain collection

Adult mice were exposed to 2% CO_2 and rapidly decapitated upon anesthesia onset. Their brains were removed, dropped-fixed in 5% acrolein in 0.1 M phosphate buffer, transferred to 30% sucrose 24 h later, and, after several days, stored in cryoprotectant solution at −20 °C until sectioning. Brains were frozen-sectioned coronally into three series at 30 μm in a sliding microtome. One series was used for the immunohistochemical detection of VP, another for OT, and the third for thionin staining.

2.4. Immunohistochemistry

Unless otherwise stated, tissue was washed between steps in 1× tris buffered saline (TBS) and all steps were carried out at room temperature. For VP and OT detection, sections were subjected to antigen retrieval via incubation in 0.05 M sodium citrate for 1 h, with the first 30 min at 70 °C. Tissue was then incubated in a blocking solution (10% normal goat serum (NGS), 1% H_2O_2, 0.4% Triton X in TBS), followed by an overnight incubation in a solution (2% NGS, 0.4% Triton X in TBS) containing the primary antibody: rabbit anti-VP (T-4563, lot A09319; Bachem, Torrance, CA, USA; 1:400,000) or rabbit anti-OT (T-4084, lot A16207; Bachem; 1:160,000). Sections were washed in a dilute blocking solution (2% NGS, 0.4% Triton X in TBS), incubated for 1 h in a goat anti-rabbit secondary antibody (BA-1000, lot ZF0430; Vector Laboratories, Burlingame, CA, USA; 1:500; 0.4% Triton X in TBS), washed in 1× TBS-0.4% Triton X, and incubated for 1 h in an avidin-biotin solution (Vector, 1:500; 0.4% Triton X in TBS). Tissue was washed in acetate buffer and reacted for 30 min in a solution containing 0.02% diaminobenzidine tetrahydrochloride, 2% nickel sulfate, and 0.0025% H_2O_2 made in the same buffer. Sections were mounted onto gelatin-coated slides, dehydrated, and coverslipped.

2.5. Quantification of VP and OT neuron number and soma size

All analyses were performed using Stereo Investigator software (MBF Biosciences, Williston, VT, USA) by an investigator blind to experimental condition. Numbers of VP and OT neurons were quantified bilaterally throughout each region of interest and multiplied by three to account for sampling ratio. We counted VP and OT neurons in the PVN, VP neurons in the SCN, and OT neurons in the SON. Due to the intensity of the immunoreactivity and the overlap of cell bodies, VP neurons in the SON could not be individually counted. Instead, we used area covered by VP-immunoreactivity as a way to assess differences between birth modes. The SON was outlined in all available sections, and the two sections with the greatest area covered by label in each animal were summed.

To determine whether birth mode affected magnocellular and parvocellular VP neurons differentially, we analyzed the rostro-caudal distribution of VP neurons within the PVN. In contrast to the rat, the mouse PVN does not show a clear distinction between magnocellular and parvocellular populations, but magnocellular neurons predominate in the rostral PVN (Plates 59–61 of the Allen Mouse Brain Atlas, 2008), parvocellular VP neurons are concentrated in the caudal PVN (Plates 63–66 of the Allen Mouse Brain Atlas, 2008), and the mid PVN contains a mixture of both cell types, as reported by Biag et al. (2012). We used the thionin stained series from each animal to confirm region selection.

Soma sizes of VP and OT neurons in the PVN were quantified by centering the PVN in the microscope field of view at low power, and then switching to high power (40× objective) and tracing around all somas in the field of view. This included an average of 33 VP and 50 OT cells per animal.

2.6. Quantification of VP and OT projections

Photomicrographs of the PVN were captured in all sections

containing well-defined efferent projection streams. A counting box (400 μm (W) × 500 μm (L)) was placed just lateral to the most lateral immunoreactive cell in the PVN and parallel to the third ventricle, as depicted in Fig. 2C. A threshold for labeling was determined using the pre-set "moments" algorithm in Image J software (National Institutes of Health, Bethesda, MD, USA) and the area covered by VP or OT immunoreactive pixels above threshold was recorded for each section. If VP or OT cell bodies from the anterior hypothalamic area fell within the sampling area, their areas were removed from the calculation. Then, the two sections with the greatest staining per animal were summed. To estimate VP and OT output per neuron, the total number of VP or OT immunoreactive neurons in the PVN was divided by the number of pixels in the sections selected to quantify projections.

2.7. Stereological analysis of the PVN

Volume and total neuron number of the PVN were determined in thionin-stained sections. The PVN was traced in one hemisphere and counts of cells with a neuronal morphology were made using the optical fractionator function of Stereo Investigator (counting frame: 18 μm × 18 μm; sampling grid: 85 μm × 85 μm). The coefficient of error (Gundersen, m = 1) was ≤0.09. Volume and neuronal counts were multiplied by two to estimate bilateral PVN volume and total neuron number, respectively.

2.8. Quantification of neuronal cell death and VP neuron number in newborns

To examine neuronal cell death in the PVN, brains were collected 3 h after a vaginal ($n = 9$) or Cesarean ($n = 10$) birth and every other section was processed for the immunohistochemical detection of the cell death marker activated caspase-3 (9661L, lot 45; Cell Signaling, Beverly, MA, USA; 1:20,000) and counterstained with thionin as part of our previous work (see Castillo-Ruiz et al., 2018 for additional details of tissue processing). For the current report, numbers of dying cells were quantified in the rostral, mid, and caudal PVN, using the same criteria as above, and multiplied by two to account for sampling ratio.

We also examined effects of birth mode on VP neuron number in newborns. Timed pregnancies were generated and Cesarean deliveries were yoked to vaginal births ($n = 3$ dams per birth mode) as described above. Upon birth, pups of both birth modes were placed on a heating pad kept at ±32 °C, and 3 h later their brains were collected (vaginal birth: $n = 5$; Cesarean birth: $n = 7$) and fixed in 4% paraformaldehyde for 24 h, followed by immersion in 30% sucrose for several days. Brains were sectioned at 40 μm and every other section was processed for the immunohistochemical detection of VP, following the protocol described above, with the following exceptions: a 0.01 M glycine incubation was added after the antigen retrieval step, and the concentration of the blocking solution (20% NGS, 3% H_2O_2, 0.4% Triton X in TBS) and VP antibody (1:75,000) were increased. These steps optimized VP staining in the neonatal brains. Numbers of VP+ neurons were quantified throughout the rostral PVN, and the two sections with the greatest cell counts in each animal were summed and multiplied by two to account for sampling ratio.

2.9. Data analyses

We pooled females and males for all analyses because the low number of females in the Cesarean group prevented us from testing sex as a factor, and we did not find an effect of sex on VP number or total cell numbers in the PVN at weaning in our previous study (Castillo-Ruiz et al., 2018). All dependent measures were analyzed following two approaches: (1) using individual offspring in each group as the "n", and (2) by averaging the values of individuals per biological litter, and using this value as the "n" to account for possible litter effects (Jimenez and Zylka, 2021; Lazic and Essioux, 2013). Because all animals were cross-fostered into mixed-group litters, litter of rearing was controlled for in this study. Independent samples *t*-tests (two-tailed) were used to evaluate birth mode differences in VP and OT neuron number, soma size, efferent projections, and the ratio of projections to neuron number, as well as volume and total neuron number in the PVN. For examining rostro-caudal differences in VP neuron number and perinatal cell death in the PVN, we used a mixed-effects ANOVA, with birth mode as the between-subjects factor and rostro-caudal level (rostral, mid, or caudal) as the within-subjects factor. Post-hoc comparisons were performed only after significant interactions using Fisher's LSD. GraphPad Prism for Windows (GraphPad software LLC, San Diego, CA, USA) was used for all analyses. Partial eta squared (η^2) and Cohen's d (d_s) were used to calculate effect sizes for overall ANOVAs and pair-wise comparisons, respectively.

3. Results

Results using individual offspring or litter as the "n" gave the same statistical outcome in most cases. We present the data for individual offspring below, and indicate the few cases where results differed for litter analyses (see Supplementary Tables 1 and 2 for all analyses by biological litter).

3.1. Cesarean birth reduces VP neuron number, soma size, and fiber output in the PVN

We previously found an effect of birth mode on VP neuron number in the PVN at weaning, with 20% fewer neurons in Cesarean-born weanlings than in those delivered vaginally (Castillo-Ruiz et al., 2018). Here, we find that this deficit extends to adulthood (Fig. 1A-C), with Cesarean-born adults having 19% fewer VP neurons in the PVN at P60 than those born vaginally ($p = 0.003$, $d_s = 2.06$) (Fig. 1A). The effect was localized to the rostral PVN (Fig. 1B, C). Specifically, we found a birth mode-by-region interaction in the two-way ANOVA ($F_{2,24} = 10.08$, $p = 0.0007$, $\eta^2 = 0.46$) (Fig. 1B), due to the fact that VP number was significantly reduced in the rostral ($p = 0.004$, $d_s = 1.85$) but not in the mid or caudal PVN ($p = 0.09$, $d_s = 0.96$; $p = 0.14$, $d_s = 0.83$, respectively). An identical pattern of effects on VP neurons in the PVN was found when using biological litter as unit of analysis (Supplementary Tables 1 and 2).

We also examined the number of VP neurons in the SON and SCN and found no effect of birth mode in these regions using either method of analysis (SON: $p = 0.74$, $d_s = 0.19$; SCN: $p = 0.83$, $d_s = 0.12$) (Fig. 1D, E and Supplementary Table 1).

To further characterize the effect of birth mode in the PVN, we measured soma size and output fibers of VP neurons in this region, and again found the identical pattern of effects whether individual offspring or litter was used as the unit of analysis. The soma size of VP neurons in the PVN of Cesarean-born mice was slightly (9%), but significantly, reduced relative to that of their vaginally-born counterparts ($p = 0.02$, $d_s = 1.45$) (Fig. 2A and Supplementary Table 1). Moreover, Cesarean birth caused a 15% reduction in VP projections ($p = 0.003$, $d_s = 1.99$) (Fig. 2B, C and Supplementary Table 1). To obtain a measure of output per cell, we divided fiber density by the number of VP immunoreactive neurons. This analysis showed no difference between birth modes (VP: $p = 0.31$, $d_s = 0.57$) (Fig. 2D and Supplementary Table 1), suggesting that while Cesarean section reduced the number of VP neurons, fiber output *per cell* was not affected.

3.2. Cesarean birth effects on OT neurons in the PVN

A quantification of OT neuron number in the PVN revealed a reduction in Cesarean-born mice that did not reach statistical significance when individual offspring was the experimental unit ($p = 0.09$, $d_s = 1.01$) (Fig. 3A), but did so when average per litter was used instead ($p = 0.049$, $d_s = 2.28$) (Supplementary Table 1). A birth mode-by-region interaction was found for the distribution of OT neurons in the PVN

when individual was used as experimental unit ($F_{2,24} = 3.67$, $p = 0.04$, $\eta^2 = 0.23$); however, post-hoc comparisons were not significant for any PVN region (rostral, mid, or caudal), and the litter analysis did not find a significant birth mode-by-region interaction ($F_{2,6} = 2.34$, $p = 0.16$, $\eta^2 = 0.37$) (Supplementary Table 2).

There also was a small (8%) reduction in OT soma size in the PVN of Cesarean-born mice when individual offspring was the experimental unit ($p = 0.02$, $d_s = 1.53$) (Fig. 3C), but not in the litter analysis ($p = 0.17$, $d_s = 1.37$) (Supplementary Table 1). In addition, there was no effect of birth mode on the density of OT efferent projections using either analysis ($p = 0.56$, $d_s = 0.33$) (Fig. 3D; Supplementary Table 1). Thus, while there may be subtle effects of birth mode on OT neurons in the PVN, effects are less robust than for VP neurons.

Finally, OT neuron number in the SON was not affected by birth mode (SON: $p = 0.83$, $d_s = 0.13$) (Fig. 3B and Supplementary Table 1), again suggesting that the PVN may be especially sensitive to birth mode.

3.3. Cesarean birth does not affect PVN volume or overall cell numbers

The results above suggested that the PVN as a whole could be affected by birth mode. However, we did not find support for this as PVN volume ($p = 0.73$, $d_s = 0.26$) and total cell number ($p = 0.06$, $d_s = 1.18$) did not differ by birth mode using either method of analysis (Fig. 4A, B and Supplementary Table 1). If anything, cell number was slightly (non-significantly) *higher* in Cesarean-born adults. This is in accord with our previous finding that birth mode did not alter total cell number in the PVN at weaning (Castillo-Ruiz et al., 2018).

3.4. Cesarean birth is associated with greater cell death in the PVN after birth

On the day of birth, vaginally born mice show a suppression of cell death in the PVN that is not seen in Cesarean-born mice (Castillo-Ruiz et al., 2018). Whether this effect is localized to particular regions of the nucleus was not evaluated previously. This is of interest as cell death could be the mechanism underlying the reduced numbers of VP neurons we see in the rostral PVN of adult Cesarean-born mice. To test this possibility, we used sections stained for activated caspase-3 to examine the rostro-caudal distribution of dying cells 3 h after a vaginal or Cesarean birth. The total number of dying cells in the PVN was small. However, as predicted, we found regional differences in cell death as revealed by a significant birth mode-by-region interaction in the two-way ANOVA ($F_{2,34} = 5.10$, $p = 0.01$, $\eta^2 = 0.23$; Supplementary Table 2). Post-hoc comparisons showed that cell death was significantly increased in the rostral and mid PVN of Cesarean-born mice ($p = 0.01$, $d_s = 1.24$; $p = 0.003$, $d_s = 1.67$; respectively) but not in the caudal PVN ($p = 0.11$, $d_s = 0.75$) (Fig. 5A) when individual offspring were used as the unit of analysis. However, the effect was significant only for the mid-PVN when litter was used as the unit of analysis (Supplementary Table 2).

These findings hinted that Cesarean-born mice might already have fewer VP neurons in the rostral PVN at 3 h postnatal. We tested this, and found a reduction in VP neurons in Cesarean-born mice that did not reach significance when individual offspring was the experimental unit ($p = 0.10$, $d_s = 1.05$) (Fig. 5B), but did when litter was used ($p = 0.006$, $d_s = 4.37$; Supplementary Table 2). We note that the percent reductions in VP neurons (22% and 27% in the individual and litter analyses, respectively) were similar to what we found in weanlings (Castillo-Ruiz et al., 2018) and adults (current study).

4. Discussion

Using a carefully controlled study in which subjects were matched for gestation length, time of birth and foster rearing, we find that Cesarean birth has long-term effects on the VP system of the PVN and that

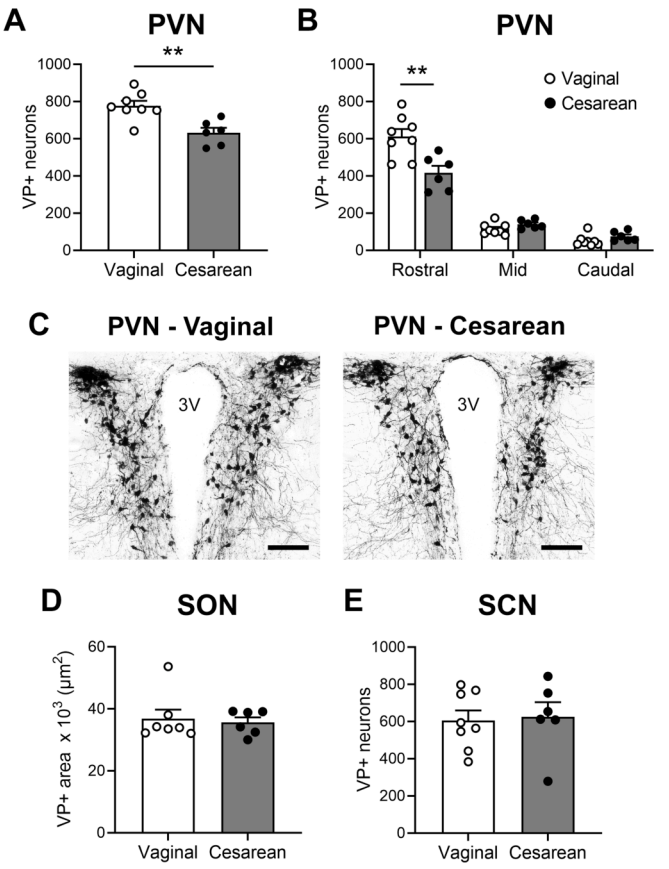

Fig. 1. Cesarean birth reduced VP neuron number in the rostral PVN. (A, B) Quantification of overall (A) and regional distribution (B) of VP neurons in the PVN. (C) Photomicrographs of VP immunoreactivity in the rostral PVN of a representative vaginally- and Cesarean-born animal. Scale bar = 100 μm. (D, E) No effect of birth mode was observed on VP neuron number in the SON (D) or SCN (E). Abbreviations: 3 V, third ventricle. Means + SEM and individual offspring data points are depicted $^{**}p \leq 0.003$. $N = 6$–8 per group.

the mechanism underlying this effect may be perinatal neuronal cell death. Our finding of reduced VP cell numbers in Cesarean-born mice replicates and extends to adulthood our previous finding in weanling mice (Castillo-Ruiz et al., 2018). In fact, the magnitude of the reduction in VP neurons in the previous and current study is remarkably similar (i.e., ~20%). We also found that the PVN of Cesarean-born mice had smaller VP neurons and reduced VP efferent projections in adulthood. There were also possible effects of birth mode on OT neuron number and soma size in the PVN. These effects, however, appear to be less robust than effects on VP neurons, as the statistical significance depended on the method of analysis. In addition, there was no effect of birth mode on VP or OT cell number in the SON or SCN, suggesting that the PVN is particularly sensitive to the effects of birth mode.

A fine-grained analysis revealed that the effect of birth mode on VP neuron number was localized to the rostral PVN, which houses primarily magnocellular VP neurons (Biag et al., 2012). Magnocellular VP neurons mainly project to the posterior pituitary where they release VP into the bloodstream to control osmolality and vasoconstriction (Swanson and Sawchenko, 1980), but can also have central effects via somato-dendritic release of VP (Brown et al., 2020). In contrast, parvocellular VP neurons mainly project to the median eminence to control the release of stress hormones by the anterior pituitary (Swanson and Sawchenko, 1980). Both magnocellular and parvocellular VP neurons also project within the brain, where they influence aspects of social behavior (Johnson and Young, 2017; Kelly and Goodson, 2014) and other functions. While our results suggest that functions orchestrated by

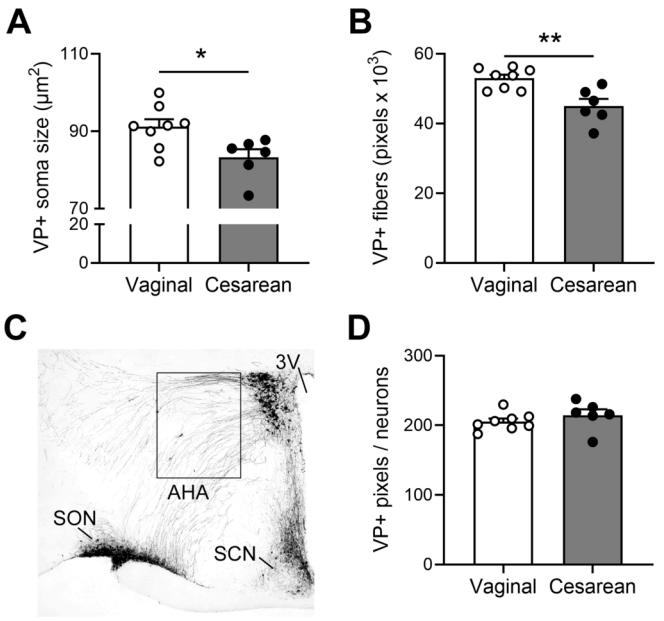

Fig. 2. Cesarean birth reduced VP soma size (A) and efferent output (B) in the PVN. (C) Photomicrograph showing the area sampled for VP projections. Sampling box = 400 μm × 500 μm. (D) VP projections per neuron did not differ by birth mode. Abbreviations: 3 V, third ventricle; AHA, anterior hypothalamic area. Means + SEM and individual offspring data points are provided. *p = 0.02; **p = 0.003. N = 6–8 per group.

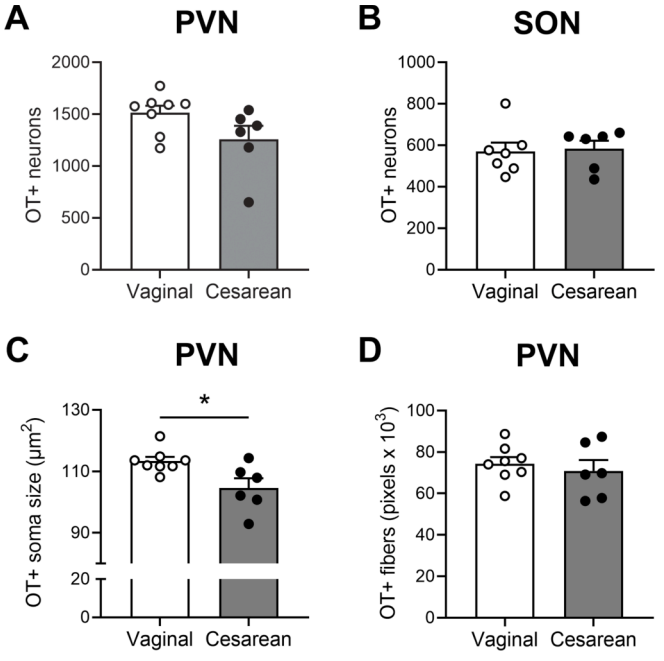

Fig. 3. Cesarean birth did not affect OT neuron number in the PVN (A) or SON (B). (C, D) Cesarean birth did, however, reduce OT soma size in the PVN (C), without altering OT+ efferent projections (D). Means + SEM and individual offspring data points are provided. *p = 0.02. N = 6–8 per group.

magnocellular VP neurons would primarily be affected by birth mode, magnocellular axons can release VP *en passant* as they traverse the median eminence (Engelmann et al., 2004; Holmes et al., 1986) and a vascular pathway connects the posterior to the anterior pituitary (Baertschi et al., 1980; Engelmann et al., 2004), suggesting that effects on classically "parvocellular" functions are possible. Thus, a reduction in magnocellular VP neurons could affect both peripheral and central VP

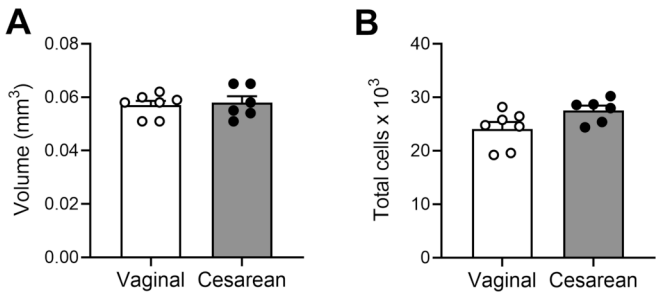

Fig. 4. Cesarean birth did not alter the volume (A) or overall number of cells (B) in the PVN. Means + SEM and individual offspring data points are provided. N = 6–7 per group.

functions.

Birth mode also affected the soma size of VP neurons in the PVN. VP neurons are remarkably plastic and increase in size to accommodate enhanced peptide production. For example, physiological challenges (sodium loading, water deprivation) are characterized by increased production of VP with concomitant increases in soma size (Hyodo et al., 1988; Hyodo et al., 1989). Although our study was not designed to challenge the mice in any way, it is possible that the acute exposure to CO_2 for euthanasia may have affected neuropeptide release (Reed et al., 2009). We note, however, that it is unlikely that CO_2 exposure would impact VP soma size so rapidly because previous work suggests that VP neuron soma size does not change until several days following a physiological challenge (Hyodo et al., 1989).

We also observed a reduction in VP efferent projections in the PVN of Cesarean-born mice. Taken together with reduced VP neuron numbers and soma size, our results suggest that Cesarean-born mice produce lower amounts of VP. However, we cannot rule out the converse hypothesis, namely that reduced immunohistochemical detection of VP indicates enhanced peptide release and, hence, less peptide storage (e.g., Wang et al., 1994). It would be interesting to follow up our findings by comparing vasopressin release – peripherally and/or centrally - between vaginally- and Cesarean-born adults at baseline and following an acute homeostatic challenge, such as sodium loading.

A pressing question is how a Cesarean birth, which constitutes an acute event, can exert long-lasting effects on the VP and, to a lesser extent, OT systems. Birth occurs at a time when the VP and OT systems are developing (Aulino and Caldwell, 2020; Hammock, 2015; Madrigal and Jurado, 2021; Tamborski et al., 2016) and may be susceptible to environmental factors. The stimuli accompanying birth (e.g., hypoxia, mechanical pressure, microbiota colonization) trigger an adaptive stress response that aids the newborn's transition to postnatal life and that differs between birth modes (Evers and Wellmann, 2016; Kenkel, 2021; Lagercrantz, 2016). One possibility is that Cesarean birth elicits epigenetic changes that have lasting effects on VP expression. For example, perinatal exposure to stress causes a permanent increase in VP production via epigenetic modifications of its gene (Murgatroyd et al., 2009). Our current data, however, suggest that developmental neuronal cell death may underlie the birth mode effect on VP neuron number.

Cell death is greater in the PVN of Cesarean-born mice than in vaginally-born mice during the first day of postnatal life (Castillo-Ruiz et al., 2018). This is not an artifact of the CO_2 exposure used for Cesarean delivery, because newborn mice are refractory to CO_2 (Pritchett et al., 2005), and nearly identical increases in cell death in the PVN are seen after Cesarean delivery with or without CO_2 exposure (Castillo-Ruiz et al., 2018). Here, we localized this effect to the mid and perhaps rostral PVN, where we also saw the effect of birth mode on VP cell number. Moreover, our data suggest that VP cell number may already be reduced within hours of a Cesarean birth, which is consistent with a cell death mechanism. We did not see an effect of birth mode on PVN volume or total cell number, which would be expected if differences in cell death between birth modes are cell-type specific. The 20% reduction in VP

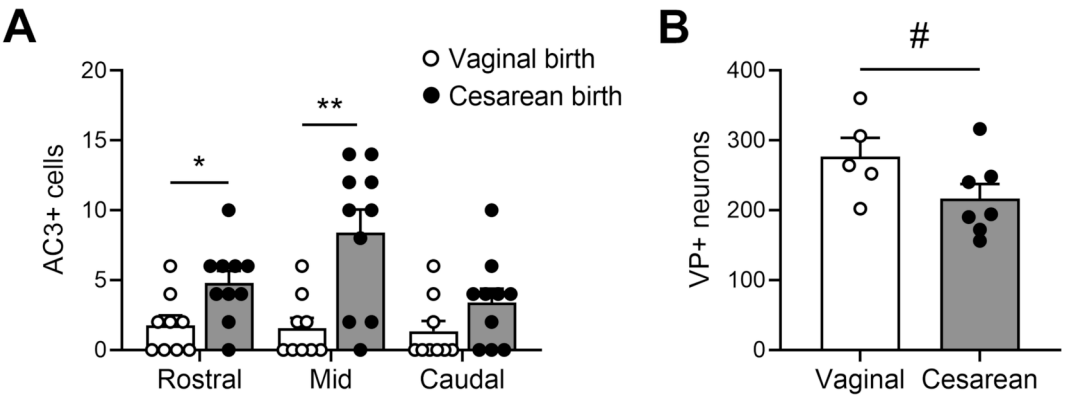

Fig. 5. Cesarean birth was associated with greater cell death in the rostral and mid PVN (A) as well as with a trend for a reduction in VP neurons in the rostral PVN (B) 3 h after birth that was significant when litter was used as experimental unit (see text). Abbreviation: AC3, activated caspase-3. $^{*}p = 0.01$; $^{**}p = 0.003$; $^{\#}p = 0.10$ (two-tailed). Means + SEM and individual offspring data points are provided. N = 9–10 per group (A); 5–7 per group (B).

neuron number in Cesarean-born mice was probably not captured in our gross PVN analyses because it would be difficult to detect such a reduction when VP neurons make up only a small percentage of all cells in the PVN.

Although speculative at this point, VP itself could mediate the effect of birth mode on VP cell number. Both humans and mice have a massive surge of VP at birth, which is muted in Cesarean-born offspring (Hoffiz et al., 2021; Polin et al., 1977; Rees et al., 1980). VP neurons in the PVN are activated at birth (Hoffiz et al., 2021) and, in adult rodents, neuronal activity causes dendritic release of VP within the PVN (Ludwig and Stern, 2015). VP released via this mechanism may act in an autocrine and paracrine manner by binding to VP receptors (Brown et al., 2020), which are preferentially localized to magnocellular VP neurons in the PVN (Berlove and Piekut, 1990; Hurbin et al., 1998). We propose that the outcome of this cascade of events could be neuroprotection. Indeed, VP is neuroprotective in vitro (Chen and Aguilera, 2010) and we previously demonstrated that intracerebroventricular injections of VP reduce cell death in the PVN of Cesarean-delivered mice (Hoffiz et al., 2021). Thus, the VP surge that is especially prominent following a vaginal birth may spare particular populations of VP neurons in the PVN. Similarly, the number of OT neurons in the PVN is altered by peripheral OT treatment at birth in prairie voles (Yamamoto et al., 2004). Whether this effect is cell death-related is unknown.

The specific VP and OT functions affected by Cesarean birth remain to be elucidated. VP and OT participate in many functions, including social behavior. Several recent reports indicate that Cesarean-born mice show social deficits as neonates and in adulthood (Morais et al., 2021; Morais et al., 2020; Nagano et al., 2021). In addition, in humans, some epidemiological studies report an increased risk of autism associated with Cesarean birth (Curran et al., 2015; Yip et al., 2017; Zhang et al., 2019). Our results therefore may provide a neural explanation for these observations. Therapeutic interventions in Cesarean-born mice have involved the administration of OT during the neonatal period, which rescues the social deficits in adulthood (Morais et al., 2021; Morais et al., 2020; Nagano et al., 2021). It is worth noting, however, that the OT doses administered in these studies are supra-physiological, and because VP and OT can bind to each other's receptors (especially when at high levels), it is possible that the VP system mediates the rescuing effects.

In sum, we find that birth mode has multiple long-lasting effects on the VP system of the PVN. OT neurons of the PVN may also be affected by birth mode, although the data are less clear. Cesarean delivery is an increasingly common practice around the world and experimental work in rodents and epidemiological studies in humans indicate that Cesarean-born offspring are at increased risk for immune, metabolic, and social deficits. As these are all functions influenced by the VP and OT systems of the PVN, our current findings may help to explain these effects.

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Declaration of competing interest

The authors have nothing to disclose.

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