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THE EFFECT OF BIRTH ON NEURAL ACTIVATION IN THE RODENT BRAIN

by

YARELY CHISELLE HOFFIZ

Under the Direction of Nancy G. Forger, PhD

ABSTRACT

A vaginal birth is a dramatic event for the mammalian fetus. Birth is accompanied by hormonal surges, mechanical forces from uterine contractions, and activation of the hypothalamic-pituitary axis to prepare the fetus to survive *ex utero*. For example, respiration, thermoregulation, and mature circulation are triggered by birth. However, virtually nothing is known about how, or if, the brain responds to the challenges of birth. Moreover, some important neurodevelopmental events, including developmental neuronal cell death, coincide with the timing of birth, but it is unclear whether birth influences these processes. Here, using a variety of histological, molecular, and pharmacological approaches, we investigate the response of the brain to birth. First, we find that a vaginal birth triggers neural activation in specific brain areas, including discrete nuclei within the hypothalamus. Based on c-Fos immunoreactivity, activation in the paraventricular,

suprachiasmatic, and supraoptic nuclei of the hypothalamus increases up to 500% 3h after birth compared to one day before or one day after birth. Second, using carefully controlled experiments to isolate the events associated with labor and delivery, we demonstrate that neural activation occurs as a result of the exposure of the fetus to the extrauterine environment regardless of gestational length or birth mode, and that a large portion of the activated neurons are vasopressinergic. Given that vasopressin is elevated in plasma samples of human neonates on the day of birth, we next measured perinatal levels of vasopressin using the more stable surrogate marker, copeptin, and found that copeptin is elevated perinatally compared to adult samples. To determine possible functions of vasopressin at birth, we examined plasma osmolality and neuronal cell death. We show that plasma osmolality is acutely decreased following a vaginal birth, but not a Cesarean birth, and that vasopressin decreases cell death in specific hypothalamic areas of Cesarean-delivered pups, likely acting via oxytocin receptors. Collectively, these studies provide support for the hypothesis that a vaginal birth triggers a neuroendocrine response that provides neuroprotection to the newborn via vasopressinergic signaling. Furthermore, our results suggest that modifications in the normal progression of birth (i.e. birth mode) may alter brain development.

INDEX WORDS: Birth, Neural activation, Paraventricular nucleus of the hypothalamus, Vasopressin, Cell death, Neuroprotection

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by

YARELY CHISELLE HOFFIZ

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2020

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2020

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May 2020

DEDICATION

To my beloved husband-- Simon J. Hoffiz; your unconditional love, selfless care, and incredible patience never cease to amaze me, and have carried me through the toughest times of my life.

To my dear son-- Eren A. Hoffiz; your life changed my life forever in innumerable good ways, and I hope that the years we share together will be enough to show you how much I love you.

To my loving father, mother-- Eric F. Davila-Lozada and Nancy E. Vazquez-Gomez; your love, confidence, endless support, and perseverance have shaped me into the woman I am today. I am forever indebted to you for all you have done and continue to do for me.

I thank you and love you with all my heart.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank the person who primarily made this dream a reality: my mentor, Dr. Nancy G. Forger. Nancy, I do not think you will ever understand how grateful I am for having you in my life. We met when my life had hit rock bottom, yet you believed in me and encouraged me to continue on this journey even when I thought I had zero potential. On a professional level, I thank you for your dedication, for celebrating every research victory (no matter how small) and for helping me brainstorm and re-focus after each failure, for the innumerable hours you spent crafting my scientific mind, and for being truly committed to guiding me to achieve a successful career in science. On a personal level, I thank you for nurturing my personality and independence, for always showing me the right way of doing things with infinite patience and without ever demanding anything. You are a true inspiration in all aspects of your life, and I only hope to move forward from my doctoral training having absorbed at least one ounce of your scientific integrity and wisdom, and to impact at least one person's life the way you impacted my life and the life of everyone who meets you.

I will also be forever grateful to past and current members of the Forger lab that I had the privilege to work with, especially Dr. Alexandra Castillo-Ruiz. Alex, I cannot begin to express how grateful I am for your significant contribution to the development of this dissertation and my scientific career. Thank you for the countless hours of training, the sleepless nights you spent helping with my experiments, and for always providing feedback to help me develop my experimental design skills and performance. Thank you for being my friend, for truly caring about my personal wellbeing and professional growth, and for providing a shoulder to cry on when life gets hard. Finally, thank you for teaching me that science could be the means by which we cultivate relationships that last a lifetime. I would also like to especially thank Laura R. Cortes, Dr. Carla

D. Cisternas, Andrew Jacobs, Megan Hall, Jennifer Gray, and Taylor Hite for your invaluable help with my research experiments, your friendship, and for gifting me a lab culture that feels a lot like home. Words cannot express how much I appreciate you and the time and effort you have dedicated in helping me complete my PhD. I could not have done this without you. All of you are my family.

I would like to acknowledge the substantial contributions of my dissertation committee in the development of my dissertation project. Thanks, Dr. Geert De Vries, for sharing your expertise in neuroendocrinology, and for always providing unique and innovative perspectives to approach problems, answer questions, and interpret data. Your passion for science has been a source of inspiration and continuous encouragement over the years, and I hope to motivate others and instill a unique enthusiasm for the scientific enterprise the same way you do. I would also like to thank you for generously sharing lab resources that have been instrumental for the successful completion of my dissertation experiments. Thank you, Dr. Javier E. Stern, for also sharing your expertise in neuroendocrinology, and for providing thoughtful insights to improve my research methodology. Thank you for opening the doors of your lab for me to perform experiments, and for generously offering your time to mentoring and teaching me new techniques. Thanks for always asking questions that I do not have the answer to, because they have forced me to think much more critically about my experimental procedures and results; your guidance has been vital for developing a question-oriented approach to science. Finally, thank you, Dr. Aaron Roseberry, for all the time you invested learning about my research topic, and for providing invaluable advice for all my dissertation experiments. Thanks for also showing a genuine interest in my future goals, and for advising me accordingly.

I would like to acknowledge the considerable contribution of Dr. Sarah Pallas to my scientific career. Thank you so much for believing in my potential, for opening the doors of your lab without hesitation, for the time and effort you dedicated to mentoring me, and for reaffirming my passion for developmental neuroscience. Thanks for promoting my independence since the early stages of my PhD, and for helping me improve my scientific writing. I will forever be grateful to you for gifting me the opportunity to be part of the incredible family of the Neuroscience Institute at GSU, and hope that we cross paths again in the future. I would not be here without you.

I would like to thank past and present members of the Neuroscience Institute who have trained me and/or provided much-appreciated technical assistance, including Mary Karom, Greg Suess, Anna Rosenhauer David Mudd, Deanna Ross, Precious Corinthus, Morgan Moseley, Mary Holder, Nicole Peters, Jack Whylings, Christopher Fields, Joe Terranova, Johnathan Borland, Alyssa Norvelle, Atit Patel, Meng Zhang, Hildebrando Ferreira-Neto, Quentin Richardson, Stephen Estes, Lilliana Artinian, Samara Rivers, Shian McLeish, Dina Yakout, Arelene George, and members of Dr. Didier Merlin's lab. I would also like to thank faculty members Dr. Vincent Rehder and Dr. Angela Mabb for allowing me to rotate in their labs and for dedicating many hours to pouring decades of research experience and scientific knowledge into me in an 8-week period, and Dr. Elliott Albers for kindly providing feedback that helped improve my pharmacological experiments. Special thanks to Dr. Dan Cox for his invaluable advice in fluorescence microscopy. Your selfless commitment to helping others excel in science (or anything!) is truly admirable; we are very fortunate to have you in our department.

I would like to acknowledge the incredible work of our past and present staff members, including Tenia Wright, Emily Hardy, Elizabeth Weaver, Rob Poh, Ryan Sleeth, and Racquel Lowe. Thank you so much for making our lives so much easier, and our graduate student

experience considerably more fun! Finally, I am grateful to all the members of our Division of Animal Resources, for their exceptional work and endless effort to work hand to hand with researchers.

I would like to thank my friend Katie Partrick, and all members of the Neuroscience Institute who helped me through a physically- and mentally-debilitating illness. I will never find the words to express my gratitude for the overwhelming support that I received from all of you. Katie, I am forever indebted to you, and I thank you for your unconditional love and for lifting me up over the years. I would also like to thank Chuck Derby for being my sounding board, for believing in me, and for pushing me to continue on this journey during my time of uncertainty. I would not be here without you.

Lastly, I would like to thank the hidden heroes of this story: my family! First of all, thanks to my parents, Eric F. Davila-Lozada and Nancy Vazquez-Gomez, my sister, Sugeily Davila-Vazquez, and my parents in-law, Simon A. Hoffiz and Mary Hernandez, for their unceasing love, for celebrating every accomplishment, for pushing me forward through my failures, and for being there every time I needed you. I love all of you very much, and thank you for helping and supporting me throughout this process. I would also like to thank the members of my extended family, Julio Crespo, Jamilye Crespo, Miriam Porras, Hernan Porras for providing emotional support through the hardest times of my life. Also, special thanks to Mayra Hernandez for taking care of my son while I wrapped up my dissertation experiments, and for loving him as her own. Finally, I am exceptionally grateful to the person that I am lucky enough to call my husband. Simon J. Hoffiz, you never cease to amaze me. Every day you find new ways to show me your love, and having you by my side has been my driving force, what kept me sane and grounded over the years. Thanks for keeping my life organized, and for everything you have sacrificed to help me achieve

this goal. You are my safe place, and I love you with all my heart. Each and every one of you helped me in unique ways throughout my PhD, and made the completion of this dissertation possible. Therefore, a piece of this work belongs to you, too. Thank you!

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LIST OF ABBREVIATIONS

ABC – Avidin-Biotin Complex	LHb – Lateral habenula
ACSF – artificial cerebrospinal fluid	NKCC1 – Sodium-potassium-chloride cotransporter 1
AC3 – Activated caspase 3	OTA – Oxytocin receptor antagonist
ANOVA – Analysis of variance	OXT – Oxytocin
AVP – Arginine vasopressin	PBS – Phosphate buffered saline
CNS – Central nervous system	PG - Prostaglandin
COX – Cyclooxygenase synthase	PVN – Paraventricular nucleus of the hypothalamus
CO ₂ – Carbon dioxide	PVT – Paraventricular nucleus of the thalamus
CPu – Caudate putamen	P – Postnatal day
CRH – Corticotropin releasing hormone	RVLM – rostral ventrolateral medulla
C-section – Cesarean section delivery/birth	SCN – Suprachiasmatic nucleus of the hypothalamus
DAB – Diaminobenzidine	SEM – Standard error of the mean
E – embryonic day	SFO – Subfornical organ
fMRI – Functional magnetic resonance imaging	SON – Supraoptic nucleus of the hypothalamus
GABA – γ -aminobutyric acid	TBS – Tris buffered saline
HPA – Hypothalamic-pituitary-adrenal axis	TRH – Thyrotropin releasing hormone
H ₂ O ₂ – Hydrogen peroxide	V1aR – V1a vasopressin receptor
ICV – intracerebroventricular	WT – Wild type
IL - Interleukin	2-DG – 2-Deoxy-Glucose
IEG – Immediate early gene	
IHC – Immunohistochemical	

1 CHAPTER ONE: GENERAL INTRODUCTION

1.1 Overview

A vaginal birth is a dramatic experience for the mammalian fetus. The process of birth initiates with marked changes in the levels of several hormones during labor, which are sustained by a combination of maternal, placental, and fetal endocrine signals (Chard 1989, Ng 2000). The onset of labor triggers a tightly regulated cascade of events that progressively intensifies uterine contractions until the expulsion of the fetus through the birth canal (Chard 1989, Hirst, Chibbar et al. 1993, Alotaibi, Arrowsmith et al. 2015). Upon delivery, additional extraordinary changes need to occur to ensure the independent survival of the newborn in the extrauterine environment. Air breathing, mature circulation, thermoregulation, and changes in energy expenditure and metabolism, are examples of the many physiological changes that are required following the abrupt loss of the placenta and life outside of the womb (Gluckman, Sizonenko et al. 1999, Morton and Brodsky 2016). While the effects of birth on the development of the fetal/neonatal lungs, heart, liver, gut and other peripheral organs are well established, one deceptively simple question remains poorly understood: what are the consequences of birth for brain development? Thus, for this dissertation I examined this question.

Previously, neural activation of corticotropin releasing hormone (CRH) neurons in the paraventricular nucleus of the hypothalamus (PVN) of fetal lambs was reported during labor, but not prior to labor or shortly after birth (Hoffman, McDonald et al. 1991). Given that birth marks the initiation of many peripheral functions in the newborn, and that the brain houses the respective control centers, it is surprising that so few studies have addressed the brain's normal response to birth, especially in rodent laboratory species. Therefore, in the first set of experiments (Chapter 2), we assess what brain areas respond to birth in mice, using immunohistochemistry for the

immediate-early gene product, c-Fos, as a marker of neural activation. Contrary to our prediction that c-Fos expression might be widespread, we find that neural activation is localized to specific brain areas, including discrete hypothalamic areas shortly after birth, and a large portion of the activated neurons are vasopressinergic. Analysis of the rat brain showed that similar hypothalamic areas are also acutely activated shortly after birth.

In the second set of experiments (Chapter 3), we manipulate the mode of birth [i.e., vaginal versus Cesarean section (C-section)] to parse out the aspect(s) of birth that trigger neural activation in the newborn brain. We find that neural activation at birth, including activation of arginine vasopressin (AVP) neurons, occurs after exposure of the newborn to the extrauterine environment regardless of manipulations in birth mode and gestational length. Finally, in a third set of experiments (Chapter 4), we quantify the levels of peripheral AVP using copeptin as a surrogate marker, and identify possible peripheral and central roles of AVP in the newborn. Collectively, the findings of this dissertation provide support to the overarching hypothesis that birth plays an important role in neural development. In the following sections of this introduction, I review concepts and previous research related to my experiments.

1.2 Background

1.2.1 Neuroendocrinology of pregnancy and birth

Pregnancy is governed by endocrine signals. From conception to delivery, circulating levels of numerous maternal, placental, and fetal hormones change dynamically to ensure a successful gestation. An important step in the final stage of pregnancy is the surge in circulating levels of stress hormones in the mother and the fetus near/during labor and parturition that occurs as a result of the activation of the hypothalamic-pituitary-adrenal (HPA) axis (Atkinson and Waddell 1995, Challis, Sloboda et al. 2001, Russell, Douglas et al. 2001). Activation of the HPA

axis in both the mother and the fetus is regulated at the level of the hypothalamus, largely by the paraventricular nucleus (PVN) (Makara, Stark et al. 1981). The PVN is a heterogeneous nucleus consisting of a magnocellular and a parvocellular division, each playing an essential role in neuroendocrine and autonomic regulation (Swanson and Sawchenko 1980). Large neurons in the magnocellular division project to the posterior pituitary and primarily synthesize the neuropeptides AVP or oxytocin (OXT). The parvocellular division contains smaller and a more diverse range of neural subtypes including corticotrophin releasing hormone (CRH), AVP, OXT, neurotensin and thyrotropin releasing hormone neurons projecting to the median eminence as well as caudal pre-autonomic or spinal autonomic cells (Lennard, Eckert et al. 1993). Parvocellular AVP and OXT neurons in the PVN also project centrally to numerous targets, as will be discussed in a subsequent subsection of this introduction (section 1.2.2.1). Within the dorsomedial portion of the parvocellular division of the PVN in rats, a discrete subset of CRH neurons project to the median eminence to release the CRH peptide into the hypophysial portal plexus of veins; AVP is also released into the portal circulation and plays a major role in the stress response (Aguilera 1994, Herman, McKlveen et al. 2016). CRH signals corticotropes in the anterior pituitary and stimulates the release of the adrenocorticotrophic releasing hormone (ACTH) from the proopiomelanocortin (POMC) precursor into the systemic circulation (Muglia, Jacobson et al. 2001, Jenks 2009). ACTH then binds to melanocortin 2 receptors (MC2R) in the zona fasciculata of the adrenal gland to stimulate corticosteroid synthesis and secretion, which ultimately acts in other regions to allow for cellular resources or regulate the HPA axis via negative feedback (Gallo-Payet 2016).

Notably, the endocrine cascade that results from perinatal activation of the HPA in the fetus is important in facilitating the adaptation to extrauterine life (Lagercrantz and Slotkin 1986, Liggins 1994, Kota, Gayatri et al. 2013, Herman, McKlveen et al. 2016). During gestation, most

physiological processes that sustain the fetus are carried out by the mother, via the placenta. At birth, fetal organs need to take over the functions of the placenta and stress hormones, namely glucocorticoids, norepinephrine and AVP, trigger maturation of major fetal organs, and/or initiate physiological functions that are necessary for fetal independent survival (Hillman, Kallapur et al. 2012).

1.2.2 Peripheral adaptations for birth

1.2.2.1 Role of glucocorticoids

Levels of glucocorticoids (cortisol in humans or corticosterone in rodents) rise substantially in the fetus near parturition (Talbert, Easterling et al. 1973, Wood and Keller-Wood 1991, Liggins 1994, Atkinson and Waddell 1995, Bagnoli, Mori et al. 2013). While the timing, magnitude and mechanism responsible for the surge in glucocorticoids near birth varies among species, the result is changes in the structure and functionality of fetal organs regardless of whether the species is altricial or precocial (Fowden, Li et al. 1998). Glucocorticoids prepare fetal lungs for oxygen-carbon dioxide (CO₂) gas exchange by promoting structural maturation (Crone, Davies et al. 1983), production of pulmonary surfactant (Kitterman, Liggins et al. 1981), and lung liquid reabsorption (Wallace, Hooper et al. 1995). In the liver, glucocorticoids increase glycogen stores (Klepac 1985), increase the activity of important enzymes for gluconeogenesis (Fowden, Mijovic et al. 1993), and can regulate gene expression of specific insulin-like growth factors and their binding proteins (Price, Stiles et al. 1992). Glucocorticoid signaling in the fetal heart is necessary to achieve structural maturation, and deficiency of glucocorticoid receptors leads to impaired heart contractibility (Rog-Zielinska, Thomson et al. 2013). Glucocorticoid-induced maturational changes in the kidneys include increased glomerular filtration, tubular reabsorption, and total renal blood flow (Stonestreet, Hansen et al. 1983). In the gastrointestinal tract, glucocorticoids promote

structural maturation of the small intestine (Trahair, Perry et al. 1987, Trahair, Perry et al. 1987), and stimulate digestive activity evidenced by increased gastric acidity and gastrin levels (Sangild, Hilsted et al. 1994). During the immediate postpartum period, glucocorticoids also play an important role in providing the fetus with nutrients via gluconeogenesis until suckling is established (Fowden, Mundy et al. 1991, Fowden, Mijovic et al. 1993, Fowden, Apatu et al. 1995).

1.2.2.2 Role of corticotropin releasing hormone

CRH neurons develop in the fetal rat PVN around mid-late gestation (Bugnon, Fellmann et al. 1982, Grino, Young et al. 1989, Baram and Lerner 1991, Keegan, Herman et al. 1994) and, as discussed previously, serve as an important regulator of the HPA axis (McLean and Smith 1999, McLean and Smith 2001, Power and Schulkin 2006) – some studies argue that AVP is just as important as CRH in the stress response, therefore, its role in other areas (such as development) has been under-appreciated and will be discussed in a subsequent section within this introduction (section 1.2.2.1). In mice and rats, a discrete subset of CRH neurons co-release AVP under basal conditions (Kiss, Mezey et al. 1984, Piekut and Joseph 1986, Tanoue, Ito et al. 2004), and the proportion of CRH neurons co-expressing AVP increases substantially under stressful conditions to potentiate CRH-mediated activation of the HPA axis (Whitnall 1989, Antoni 1993, Aguilera and Rabadan-Diehl 2000, Muglia, Jacobson et al. 2001, Aguilera, Subburaju et al. 2008). Furthermore, within the magnocellular division of the PVN, a few CRH neurons also co-express OXT (Sawchenko, Swanson et al. 1984).

CRH also plays an important role in multiple events during pregnancy (McLean and Smith 1999). For instance, embryonic, fetal, and/or maternal CRH suppresses the maternal immune system in early pregnancy to avoid the rejection of the embryo (Makriganakis, Zoumakis et al. 2001), influences fetal-placental blood flow (Clifton, Read et al. 1995), and is involved in the

maturation of fetal organs (CRH is upstream in the glucocorticoid production pathway) (Kotas and Avery 1980, Challis, Sloboda et al. 2001). CRH might also be involved in regulating the timing of birth, as acute peripheral administration of CRH to ovine fetuses triggers premature delivery (Wintour, Bell et al. 1986). Moreover, bilateral ablation of the fetal sheep PVN results in delayed birth (McDonald and Nathanielsz 1991).

1.2.2.1 Role of vasopressin and oxytocin

1.2.2.1.1 Central projections

Magnocellular OXT and AVP neurons project to the posterior pituitary and are involved in multiple peripheral functions (Cunningham and Sawchenko 1991), but each are mainly known for their specific roles in milk ejection, uterine contractions, and labor (OXT), and in the stress response, and osmotic and electrolyte regulation (AVP) (Robertson, Shelton et al. 1976, Antoni 1993). OXT can also complement AVP in maintaining plasma osmolality (Verbalis, Mangione et al. 1991). In addition, AVP and OXT neurons also send projections to central targets (i.e. other brain areas) where they can act as neuromodulators/neurotransmitters (de Wied, Diamant et al. 1993, Landgraf 1995) and influence behavior (Kompier, Keyzers et al. 2019). For example, OXT neurons from the PVN and the supraoptic nucleus of the hypothalamus (SON) project to the hippocampus, periaqueductal gray, dorsal raphe, locus coeruleus, bed nucleus of the stria terminalis, caudate putamen, cingulate cortex, nucleus accumbens, prefrontal cortex, amygdala and ventral tegmental area (Sofroniew 1980, Sofroniew 1983, Whitman and Albers 1998, Cui, Gerfen et al. 2013, Song and Albers 2018). AVP neurons from the PVN, SON, suprachiasmatic nucleus of the hypothalamus (SCN) and extended amygdala send projections to the bed nucleus of the stria terminalis, lateral septum, habenula, hippocampus, periaqueductal grey, dorsal raphe, locus coeruleus, periventricular nucleus of the hypothalamus, dorsomedial hypothalamus,

organum vasculosum laminae terminalis, olfactory tubercle and the ventral pallidum (Sofroniew 1980, de Vries, Buijs et al. 1981, Sofroniew 1983, DeVries, Buijs et al. 1985). Importantly, emerging evidence suggests dynamic cross-communication of AVP and OXT neurons with other neural sub-populations to coordinate neuroendocrine responses to stress (Neumann, Douglas et al. 1996, Ferguson, Latchford et al. 2008, Son, Filosa et al. 2013, Jiang, Rajamanickam et al. 2018), rendering the PVN an important integration center for stress signaling.

1.2.2.1.2 Vasopressin and oxytocin are important birth signals

It has been established for over four decades that a vaginal birth in humans is accompanied by a massive surge of AVP (Chard, Hudson et al. 1971, Polin, Husain et al. 1977, Hoppenstein 1980, Leung, McArthur et al. 1980, Rees, Forsling et al. 1980). Specifically, vaginally-delivered human neonates have higher circulating AVP levels than those seen at any other time in life, including during pathological states (Polin, Husain et al. 1977, Rees, Forsling et al. 1980, Acher and Chauvet 1995). C-section-delivered human neonates also show elevated AVP levels at birth, but the increase is muted compared to vaginally-delivered newborns – studies report a 100-fold difference between vaginally- and C-section-delivered human neonates suggesting that specific signals of labor and/or parturition are necessary to trigger the surge of AVP in the human newborn (Leung, McArthur et al. 1980, Rees, Forsling et al. 1980, Parboosingh, Lederis et al. 1982, Evers and Wellmann 2016). Interestingly, peripheral levels of AVP strongly predict central levels of vasopressin in CSF of human newborns (Carson peptides, and Bartrons et al., *Perinat Med* 1993), suggesting that AVP is also released centrally at birth. Animal studies using chronic catheterization of lamb fetuses to repeatedly sample AVP levels during the last stage of pregnancy and postpartum, find that AVP levels rise markedly in the last few days before birth, suggesting that AVP release may peak prior to birth (not tested in humans). Much more recently, a study in rodents showed

that copeptin levels are also elevated in vaginally-delivered pups immediately after birth (Summanen, Back et al. 2018), and that copeptin levels drop rapidly within 5h similar to human newborns (Leung, McArthur et al. 1980, Rees, Forsling et al. 1980), suggesting an evolutionarily conserved endocrine response to birth across species.

Studies in AVP deficient Brattleboro rats suggest that the release of perinatal AVP could play an important role in neural development in an area-specific manner. The absence of AVP in Brattleboro rats impairs brain development with specific brain areas showing greater developmental deficiencies than others, but prenatal AVP supplementation is sufficient to restore normal development in AVP-deficient Brattleboro rats (Boer 1985).

In contrast to AVP, fetal OXT levels are not affected by birth-associated events (Thornton, Charlton et al. 1993), although levels of circulating OXT in the mother increase markedly at birth (Fuchs, Goeschen et al. 1983, Thornton, Davison et al. 1992). Recent studies argue that maternal OXT can cross the placenta, and is responsible for regulating a transient switch in the polarity of γ -aminobutyric acid (GABA) from excitatory to inhibitory at birth to reduce neural activity (Tyzio, Cossart et al. 2006). Perturbations in maternal OXT signaling have been linked to the development of autism spectrum-like disorders in the offspring (Tyzio, Nardou et al. 2014). Furthermore, maternal OXT signaling at birth could influence neural morphology and synapse transmission in excitatory hippocampal neurons (Ripamonti, Ambrozkiewicz et al. 2017). Despite these findings, whether maternal OXT crosses the placenta and reaches the fetal brain remains controversial (Brown and Grattan 2007, Carbillon 2007). Moreover, a recent study showed that AVP, not OXT, is responsible for regulating GABA-mediated transmission and reducing neural activity at birth (Spoljaric, Seja et al. 2017). These seemingly contradictory findings suggest the interesting possibility that both neuropeptides act by different mechanisms to influence neural activity at birth,

potentially via modulation of GABA signaling, or may simply reflect differences in experimental methodologies (Ben-Ari 2018).

1.2.2.1.3 Vasopressin and oxytocin receptors in newborn analgesia

The AVP and OXT peptides are remarkably similar, differing only in two amino acids (Acher and Chauvet 1995). The structures of AVP and OXT receptors also show some sequence homology (Gimpl and Fahrenholz 2001) and the two peptides can therefore cross-communicate (Schorscher-Petcu, Sotocinal et al. 2010, Song and Albers 2018). AVP and OXT receptors are members of the G-protein coupled receptor superfamily with seven putative transmembrane domains (Song and Albers 2018). Only one OXT receptor (OTR) has been identified in mammals so far, whereas three mammalian AVP receptors have been identified and classified according to their pharmacological and G-coupled properties: V1a, V1b, and V2 receptors (V1aR, V1bR, and V2R, respectively) (Koshimizu, Nakamura et al. 2012). OTRs and V1aR are widely expressed in the brain and their activation affects social behavior (Veinante and Freund-Mercier 1995, Paul, Terranova et al. 2014, Dumais and Veenema 2016, Paul, Peters et al. 2016, Song and Albers 2018). V1bRs are also expressed in the brain, specifically in the hippocampus, hypothalamus, and the amygdala (Young, Li et al. 2006, Stevenson and Caldwell 2012). V1bRs are also expressed in the anterior pituitary where AVP acts to stimulate the release of ACTH from corticotropes (Antoni 1993). Peripheral actions of AVP occur via V1aRs and V2Rs and include control of vasoconstriction and blood pressure regulation via V1aRs, and water reabsorption in the kidneys via V2Rs. While V2Rs have also been reported to be expressed in the newborn rat brain (Kato, Igarashi et al. 1995), no function has yet been identified.

AVP and/or OXT signaling mediate newborn analgesia via V1aR (Wellmann and Buhrer 2012). A recent study showed that rats are less sensitive to pain immediately after birth compared

to two days after birth, and that the analgesic effect is abolished with administration of OXT antagonists (OTA), but rescued with exogenous oxytocin treatment (Mazzuca, Minlebaev et al. 2011). However, one of the OTAs used in this study has better affinity to V1aR than OTRs (Akerlund, Bossmar et al. 1999). Moreover, OXT administration to adult OTR null mice caused analgesia that was identical to that in wild type (WT) controls, and the analgesic effects were prevented by V1aR antagonist (V1aR-A), but not OTA (Schorscher-Petcu, Sotocinal et al. 2010). OXT-induced analgesia was also completely absent in V1aR null mice, suggesting that the analgesic effects of oxytocin occur via the V1aR (Schorscher-Petcu, Sotocinal et al. 2010). While the involvement of AVP signaling via V1aRs in newborn analgesia is not clear, its involvement is complex given its gene-sex-environment interaction (Mogil, Sorge et al. 2011), and likely stronger than OXT signaling; some of the reasons have been outlined by Wellmann and Buhner (2012): 1) neither AVP or OXT have been shown to cross the human placental barrier in vivo (Chard, Hudson et al. 1971, Patient, Davison et al. 1999, Shi, Guerra et al. 2004) suggesting fetal origin; 2) the ratio of fetal autonomous AVP/OXT increases strikingly in the pituitary towards the end of gestation (Schubert, George et al. 1981); 3) a vaginal birth triggers a massive release of AVP (Chard, Hudson et al. 1971, Polin, Husain et al. 1977, Wellmann, Benzing et al. 2010), whereas OXT levels remain relatively low (Thornton et al. 1993); 4) while both AVP- and OXT-induced analgesia seem to be primarily mediated by V1aRs, AVP has greater affinity than OXT (Song and Albers 2018).

Analgesic effects of AVP have been widely reported in adult animals. For example, ICV injections, but not intrathecal or intravenous injections of AVP, increase pain threshold (Yang, Song et al. 2006). Similarly, ICV injections of AVP but not AVP anti-serum into the AVP-deficient Brattleboro rat, results in increased pain threshold (Bodnar, Wallace et al. 1982). Several

studies report increased levels of the opioid beta-endorphin, in human babies following vaginal delivery but not a C-section delivery, suggesting that a vaginal delivery is “less painful” for the newborn than a C-section delivery (Facchinetti, Bagnoli et al. 1986, Raisanen, Paatero et al. 1986, Bacigalupo, Langner et al. 1987). Moreover, a recent study also showed that human newborns display decreased physiological and behavioral responses to high- and low-intensity pain stimulation compared to C-sectioned babies (Bergqvist, Katz-Salamon et al. 2009), further suggesting that a specific aspect of a vaginal birth provides analgesia to the newborn. Given the reported difference in AVP/copeptin levels on the day of birth in several species (Stark, Daniel et al. 1979, Parboosingh, Lederis et al. 1982, Evers and Wellmann 2016, Wellmann, Koslowski et al. 2016, Summanen, Back et al. 2018), there is a strong possibility that AVP mediates newborn analgesia.

1.2.2.2 Role of the paraventricular nucleus of the hypothalamus

As described above, birth triggers major developmental switches in key peripheral organs (Liggins 1976, Liggins 1994, Murphy, Smith et al. 2006), and may play a similar role in brain development. In fact, proper functioning of peripheral organs at birth likely requires neurodevelopmental processes and/or maturation of networks that control the autonomic nervous system. The PVN, for example, plays essential roles in neuroendocrine and autonomic regulation, and is mature (at least in part) near parturition. Studies in sheep show that the fetal HPA axis is activated during the final three weeks of gestation, suggesting that CRH neurons project to the median eminence at this time in development (Magyar, Fridshal et al. 1980) in order to produce the observed gradual increase in fetal plasma cortisol levels that is critical for the maturation of fetal organs (Liggins 1976). The PVN is also an important integrator of the changes in the cardiovascular system that occur at birth, including the increase in neonatal cardiac output that

compensates for the rise in pulmonary blood flow, metabolic demands and thermoregulation (Behrman and Lees 1971, Heymann, Iwamoto et al. 1981). Neurons in the PVN projecting to the rostral ventrolateral medulla (RVLM) can indirectly influence sympathetic nerve activity and thereby affect cardiovascular function, such as blood pressure, blood flow and heart rate (Kannan, Hayashida et al. 1989, Haselton and Vari 1998, Deering and Coote 2000). Similarly, PVN neurons projecting to the RVLM or spinal autonomic control centers play a critical role in cardiovascular responses to changes in blood volume and osmolality (Badoer, McKinley et al. 1993, Kantzides and Badoer 2003, Stocker, Keith et al. 2004, Kantzides, Owens et al. 2005). Additionally, the PVN plays essential roles in regulating body fluid balance via AVP and OXT neurosecretory cells projecting to the median eminence, and in determining the timing of birth (at least, in some species), as bilateral ablation of the fetal sheep PVN results in a prolonged pregnancy (McDonald and Nathanielsz 1991). Taken together, these studies suggest that the PVN plays an important role in the physiological adaptations of birth, and therefore points to it as an area of interest for developmental studies.

1.2.3 Birth and brain development

The early postnatal period is an important time for brain development. For example, sensory maps in the brain including retinotopic and somatotopic maps, are shaped postnatally. After birth, the primary visual cortex forms a complete and precise (retinotopic) map of the visual space, with neurons responding to specific locations within the visual field (Espinosa and Stryker 2012). Similarly, shortly after birth, the primary somatosensory cortex of rodents develops an accurate (somatotopic) map in which individual whiskers are represented in specific cortical areas called “barrels” (Petersen 2007). Given the relationship of the timing of birth with the development of sensory maps, it is plausible that birth serves as an important organizer of brain development.

Indeed, a recent study showed that birth plays a pivotal role in the initiation of barrel formation in the somatosensory cortex (Toda, Homma et al. 2013). Decreased levels of serotonin at birth, likely due to enhanced reuptake by the serotonin transporter protein, are necessary and sufficient to guide somatotopic map formation. This birth-dependent maturational mechanism of the somatotopic map also regulates eye-specific segregation of projections from the retina to the midbrain (Toda et al., 2013). The regulatory action of birth in barrel formation and eye-specific segregation occurs regardless mode of birth, birth hormones, and new sensory inputs experienced at birth. Together, these data suggest that birth may be crucial in orchestrating brain development, but raise the question of whether birth triggers developmental events in the entire brain or in distinct brain areas.

In mice, neuronal cell death is a major neurodevelopmental event that happens around the time of birth. During neural development, approximately 50% of the cells in the brain are eliminated by apoptosis, resulting in a profound pruning and restructuring of neural circuits in the brain (Clarke, Posada et al. 1998). Although potentially counterintuitive, cell death is beneficial to the developing nervous system, and defects in the cell death pathway can be lethal to the embryo (Kuida, Zheng et al. 1996). Despite the importance of cell death in neural development, it is unclear what signals guide the patterning of cell death in the developing nervous system and what triggers the initiation or termination of the cell death period. To begin answering these questions, we previously described the pattern of cell death in the mouse brain at different developmental time points (Ahern, Krug et al. 2013, Mosley, Shah et al. 2017). Using IHC detection of activated caspase 3 (AC3) as a marker of dying cells, our lab recently found that birth is associated with region-specific changes in the patterning of cell death (Ahern, Krug et al. 2013, Mosley, Shah et al. 2017). We showed that cell death increases in most brain areas immediately after birth, but

decreases substantially in others, such as the PVN (Ahern, Krug et al. 2013, Mosley, Shah et al. 2017).

We also recently showed that the initial exposure to microbes that occurs upon birth has region-specific effects on cell death and other neurodevelopmental events (Castillo-Ruiz, Mosley et al. 2018). Accumulating evidence demonstrates a complex bidirectional communication system between the brain and the microbiota residing in the gastrointestinal tract that can influence physiology, behavior and brain development (Collins, Surette et al. 2012, Cryan and Dinan 2012, Sampson and Mazmanian 2015), and the PVN is an important integrator of gut-brain interactions. For example, infection of the gastrointestinal tract of adult rats with pathogenic bacteria activates the PVN via vagus nerve signaling (Wang, Wang et al. 2002). We found that mice born under germ-free conditions have different patterning of developmental cell death and microglial (brain immune cells) labeling in the PVN, with germ-free mice showing higher levels than conventionally colonized mice (Castillo-Ruiz, Mosley et al. 2018).

1.2.3.1 Effects of birth mode on development and pathology

The rates of Cesarean section (C-section) deliveries continue to rise worldwide, and currently exceeds 30% in the USA (Rees, Forsling et al. 1980, Lumbiganon, Laopaiboon et al. 2010, Martin, Hamilton et al. 2018). Multiple effects of C-section on development and pathology have been reported in animal or human studies. For example, birth mode can have direct effects on the activation and development of the immune system, possibly due to alterations in microbiota colonization and composition (Gronlund, Lehtonen et al. 1999, Dominguez-Bello, Costello et al. 2010), and risks of developing intestinal diseases and metabolic and respiratory disorders are greater in babies delivered by C-section (Renz-Polster, David et al. 2005, Cardwell, Stene et al. 2008, Decker, Engelmann et al. 2010). In addition to altering the hormonal signals and mechanical

stimulation associated with a vaginal birth, C-section deliveries may also shift the normal timing of birth. A human fetus is now considered “full-term” at 39-41 weeks of gestation (Fleischman, Oinuma et al. 2010). However, 50% of all C-section deliveries are elective, which, by definition, occur before 39 weeks (Fleischman, Oinuma et al. 2010, Gibbons, Belizan et al. 2012). Therefore, birth mode may also have effects on neural development.

Notably, shortening of gestational length via C-section has detrimental effects on lung development, as newborns delivered before term show insufficient lung clearance and are predisposed to develop pulmonary distress (Janer, Pitkanen et al. 2015). Effects of C-sections are also linked to alterations in gastric development (Sangild, Hilsted et al. 1995). Additionally, our lab recently described effects of birth mode on the patterning of neuronal cell death, with an acute reduction in cell death right after birth in vaginally-delivered but not C-section-delivered mice, suggesting that a specific aspect of a vaginal delivery might be neuroprotective (Castillo-Ruiz, Mosley et al. 2018). The effects of birth mode on cell death may be long-lasting as we find a significant difference in the number of vasopressin immunoreactive cells between vaginally- and C-section delivered mice at weaning (Castillo-Ruiz, Mosley et al. 2018). Moreover, manipulating the timing of birth (i.e. advancing or delaying birth by one day) results in alterations in the timing and magnitude of developmental cell death, thus pointing at birth as an orchestrator of brain development.

Taken together, the data summarized above show that birth triggers a neuroendocrine response that is important for neural development and the initiation of many peripheral functions in the newborn. Thus, in this dissertation we use a systematic approach to describe how the brain responds to the event of birth (Chapter 2), what signal(s) of birth trigger such response (Chapter 3), and what role it plays in neural development and physiology of the newborn (Chapter 4).

2 CHAPTER TWO: VAGINAL BIRTH ACTIVATES HYPOTHALAMIC VASOPRESSIN AND OXYTOCIN NEURONS

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2.1 Abstract

Birth is a dramatic event; the abrupt separation from the placenta and the rapid transition from the uterine environment forces the fetus to make quick physiological adjustments in peripheral organs in order to survive *ex utero*. It is likely that the brain also experiences dynamic changes at birth, but how the brain responds to these challenges has not been explored. Here, we addressed this question by studying the pattern of neural activation in the perinatal mouse forebrain. We established timed-pregnancies in C57BL/6 mice and collected the brains of male and female offspring *in utero* at embryonic day 18.5, and *ex utero* at 1h, 3h and 1 day after birth following vaginal delivery. Brains were processed for the IHC detection of the immediate early gene product, c-Fos, as a marker of neural activation. We found that a vaginal birth triggers activation in discrete hypothalamic areas: the PVN, SON and SCN showed significant changes in c-Fos labeling, with neural activation increasing up to 500% three hours after birth compared to one day before, or one day after birth. These data suggest that specific hypothalamic areas are particularly sensitive to stimuli at birth. We next examined the phenotype of the neurons activated at birth. Immunofluorescent (IF) double labeling of AVP or OXT with c-Fos, or IF labeling of c-Fos in a reporter mouse expressing the tdTomato red fluorescent protein specifically in CRH neurons, showed that a vaginal birth predominantly triggers activation of AVP neurons, compared to OXT or CRH neurons in the newborn hypothalamus at 3h postpartum. AVP is a neuropeptide involved in water balance and blood pressure peripherally, and in the stress response and control of social behaviors when released centrally, and has been shown to be massively released after a

vaginal birth. Therefore, activation of AVP neurons at 3h postpartum may underlie a surge of AVP in the neonate at birth.

2.2 Introduction

Most mammals enter the world in a fairly dramatic fashion. A vaginal birth is accompanied by marked hormonal surges, mechanical forces associated with uterine contractions and delivery through the birth canal, and a transition from the relatively sterile environment of the womb to one teeming with microorganisms. Upon birth, the abrupt separation from the placenta and the rapid transition from the uterine environment forces the fetus to make rapid physiological adjustments in peripheral organs in order to survive *ex utero* (Behrman and Lees 1971, Heymann, Iwamoto et al. 1981, Ward Platt and Deshpande 2005, Jain and Eaton 2006, Hillman, Kallapur et al. 2012). Given that the brain houses the control centers of many of the vital peripheral functions that undergo important changes at birth (i.e. breathing, circulation, thermoregulation), it is likely that the brain also experiences dynamic changes around the time of birth. The sudden exposure to the plethora of novel stimuli that occurs upon birth requires neural integration and processing, but, surprisingly, studies on how the brain responds to birth are limited.

Recent *in vitro* studies show perinatal changes that may prepare the fetal/newborn brain for birth. In the hippocampus, an area particularly sensitive to low oxygen levels (Fujiwara, Higashi et al. 1987, Leblond and Krnjevic 1989), neural network activity is reduced around the time of birth (Tyzio, Cossart et al. 2006, Spoljaric, Seja et al. 2017), and the authors argue that this may protect neurons from the hypoxia associated with labor and a vaginal delivery. In contrast, the newborn piriform cortex and amygdala show spontaneous oscillatory bursting activity that results from coupled respiratory activity in the brainstem (Onimaru and Homma 2007). Together,

these data suggest that the brain may experience dynamic changes in neural activity in the perinatal period that vary depending on the brain area.

We reasoned that to understand how birth affects the brain, a first step is to identify those brain regions activated by birth. Taking a whole-brain approach presents several challenges. For instance, current electrophysiological techniques are limited to the study of a small number of cells or brain areas at a time, and the time required to prepare brain slices for recording make it particularly difficult for birth-related studies. Moreover, whole-brain functional imaging techniques, such as functional magnetic resonance imaging (fMRI), have limited spatial resolution, which becomes even more problematic in studies with the fetal/neonatal brain.

A practical experimental tool to measure neural activity that bypasses these limitations is the analysis of the expression pattern of immediate early genes (IEG). The IEG, *c-fos*, has been widely recognized as an important marker of brain activity after a variety of stimuli. For example, *c-fos* mRNA can be induced in the brain following peripheral noxious stimulation (Bullitt 1989, Redburn and Leah 1997), changes in normal body temperature (Uchida, Onishi et al. 2018), different types of stress (Arnold, De Lucas Bueno et al. 1992, Pezzone, Lee et al. 1992, Beck and Fibiger 1995, Campeau and Watson 1997), and multiple other physiological and pathological stimuli, including seizures, traumatic brain injury, synaptic plasticity, and behavior (Flavell and Greenberg 2008, Kawashima, Okuno et al. 2014).

A few studies have reported induction of *c-fos* mRNA in connection with birth. A burst of *c-fos* mRNA that then quickly subsides is seen in many peripheral organs of the mouse on the day of birth (Kasik et al., 1987; Levi et al., 1989). *C-fos* mRNA is also reported in whole brain homogenates on the day of birth (compared to one day earlier), but in contrast to peripheral organs, *c-fos* expression in whole brain remains elevated at later postnatal ages (postnatal day (P) 3 and

P5) (Kasik, Wan et al. 1987). Moreover, *c-fos* mRNA induction has also been reported in the rat whole brain and mouse neocortex, striatum-diencephalon, and pons-medulla within 30 minutes after a C-section delivery (Ringstedt, Tang et al. 1995, Tang, Ringstedt et al. 2000), and Tang et al. 2000 propose that is due to the surge of catecholamines at birth.

To our knowledge, only one study has looked at the effects of birth-associated events on expression of the c-Fos protein product, or localized c-Fos to a specific cell type in the perinatal brain. Hoffman and colleagues found an increase in c-Fos immunoreactivity in CRH neurons of the fetal ovine PVN during labor that declined rapidly after delivery (Hoffman, McDonald et al. 1991). Here, we examined c-Fos protein at several time points before and after birth throughout the mouse forebrain. We find strong neural activation in discrete hypothalamic areas 3h after birth, and show that many of the activated neurons are vasopressinergic. We also validated this finding in a second species (rats). These findings shed light on how the brain normally responds to birth, and may help us to understand how normal birth, as well as deviations to the normal progression of birth (i.e. via manipulations on birth mode or timing) may affect brain development.

2.3 Methods

2.3.1 Animals

Adult C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) or obtained from our colony. Adult Wistar rats were purchased from The Charles River Laboratory (Wilmington, Massachusetts). Mice and rats were kept in a 12h light and 12h dark cycle, and given ad libitum access to water and food. All procedures were approved by Georgia State University's Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

2.3.1.1 CRH reporter mice (CRH-ires-Cre x Ai14D)

We took advantage of the Cre-LoxP system to generate C57BL/6 mice that express the red fluorescent protein variant, tdTomato, in CRH-producing cells, as previously validated (Smith, Wang et al. 2014). Mice that express Cre recombinase specifically in CRH-producing cells (Jackson Laboratory, #012704) were bred with mice expressing a conditionally activatable red fluorescent construct; specifically, the Gt(ROSA)26Sor locus, which is ubiquitously expressed in mouse embryos (Zambrowicz, Imamoto et al. 1997), was mutated to include transcriptional stop cassettes flanked by loxP sequences that prevents the transcription of the tdTomato gene driven by a CAG promoter (Jackson Laboratory, #012704). Excision of the stop cassette within cells with an active CRH promoter results in expression of the tdTomato reporter in CRH cells.

2.3.2 Timed pregnancies

We established timed-pregnancies by pairing males and females within 2h of lights off. Males were removed from the cages at 1-2h after lights on the next day, which was recorded as embryonic day (E) 0. Overall health and body weight of females was monitored to identify successful pregnancies. Starting at E18.5, expectant females were checked hourly for any signs of labor until vaginal delivery. Brains of male and female mouse pups were collected at 1h, 3h and 1 day after birth (postnatal day 1: P1). Timed-pregnant rats arrived to our facility on E11, and were monitored for the rest of the pregnancy until vaginal delivery as described for mice. Brains of male and female rat pups were collected within 1h of birth, 3h and P1.

We also collected brains during E18 (i.e., one day before the predicted day of birth) via C-section. Timed-pregnant dams were euthanized using 2% CO₂ followed by rapid decapitation. An aseptic abdominal incision was made to expose the uterine horns, fetuses were removed one by one from their gestational sacs, and the brains were immediately collected. Since c-Fos may be

expressed in a circadian rhythm under basal conditions (Earnest and Olschowka 1993, Novak and Nunez 1998, van der Veen, van der Pol-Meijer et al. 2008), C-sections were performed to match the time of the day in which other dams delivered their pups via vaginal birth; for example, if a dam gave birth at 6:00 am, we performed a C-section in a dam at gestation day 18 at 6:00 am as well.

2.3.3 Tissue processing

Collected brains were immersion fixed in 4% paraformaldehyde (PFA) for 24h and then transferred into 30% sucrose solution. Brains were coronally frozen-sectioned into four 40 μ m series using a rotary microtome. Sections were then processed for immunohistochemical (IHC) detection of c-Fos, or immunofluorescent double labeling of c-Fos and arginine vasopressin (AVP) or c-Fos and oxytocin (OXT).

2.3.3.1 c-Fos immunohistochemistry

Free-floating sections were rinsed in 0.01M phosphate-buffered saline (PBS pH: 7.4) and submerged in concentrated blocking solution (0.01M PBS, 20% normal goat serum, 0.4% Triton X-100, 1% H₂O₂) for 1h. Sections were then incubated overnight in primary antibody solution (rabbit anti-c-Fos, Santa Cruz, SC-52; 1:5,000 in 0.01M PBS, 2% normal goat serum, 0.4% Triton X-100) at room temperature. The next day, they were washed in 0.01M PBS, and incubated in secondary antibody solution (goat anti-rabbit, Vector Laboratories, Burlingame, CA; 1:500, 0.01M PBS, 2% normal goat serum, 0.3% Triton X-100) for 1h. Next, sections were washed in 0.01M PBS, and then incubated in an Avidin-Biotin Complex (ABC) solution (Vectastain Elite ABC Kit; Vector Laboratories; 1:500) for 1 hour. Following rinses in sodium acetate buffer, sections were incubated for 30 minutes in diaminobenzidine (DAB) -nickel solution, and then rinsed in

0.01M PBS. Finally, sections were mounted onto microscope slides and dehydrated before counterstaining with neutral red.

2.3.3.2 c-Fos and AVP or OXT immunofluorescent double labeling

Alternate free-floating sections were rinsed in 0.01M PBS, and submerged in 0.05 M sodium citrate in 0.01M PBS at 70°C for 1h. Following rinsing, sections were placed in 0.1M glycine in 0.01M PBS for 30 minutes, rinsed, incubated in a concentrated blocking solution (0.01M PBS, 20% normal goat serum, 0.4% Triton X-100, 3% H₂O₂) for 1h, and then incubated overnight in primary antibody solution against c-Fos (rabbit anti-c-Fos, Santa Cruz, SC-52; 1:100 in 0.01M PBS, 2% normal goat serum, 0.4% Triton X-100) at 4°C. The next day, sections were washed in a dilute blocking solution (0.01M PBS, 2% normal goat serum, 0.4% Triton X-100), and incubated in secondary antibody solution (goat anti-rabbit Alexa 594 (Vector Laboratories, Burlingame, CA; 1:250), 0.01M PBS, 2% normal goat serum, 0.4% Triton X-100) for 2h at 4°C. Sections were washed in dilute blocking solution, and then incubated overnight in primary antibody solution against vasopressin or oxytocin [rabbit anti-oxytocin (Peninsula Labs, T-4084; 1:1,000) or rabbit anti-vasopressin (EMD Millipore, PC234L; 1:1,000), in 0.01M PBS, 2% normal goat serum, 0.4% Triton X-100] at 4°C. The next day, sections were washed in a dilute blocking solution and incubated in secondary antibody solution for 2h at 4°C [goat anti-rabbit Alexa 488 (Vector Laboratories, Burlingame, CA; 1:500) or goat anti-rabbit Alexa 350 (Vector Laboratories, Burlingame, CA; 1:200) for the CRH-tdTomato reporter mouse, in 0.01M PBS, 2% normal goat serum, 0.4% Triton X-100]. To reduce background auto-fluorescence, sections were washed in 0.01M PBS and incubated in 100mM cupric sulfate in 50mM ammonium acetate for 1.5h at room temperature (Schnell, Staines et al. 1999). Finally, sections were rinsed in 1X Tris-Buffered Saline (TBS) and mounted onto microscope slides using Fluoromount medium (Sigma Aldrich).

2.3.4 Image capture and analysis

2.3.4.1 Light microscopy

IHC-processed sections were visualized using a Zeiss AxioImager M.2 microscope (Carl Zeiss, Thornwood, New York). Brain sections were magnified 100X using a 10x objective (Zeiss EC Plan-Neofluar 10x/0.3 Ph1 M27) and imaged using a color camera (Zeiss AxioCam MRc). Brain regions of interest were outlined in both hemispheres with Stereo Investigator software (MBF Bioscience Inc.) using anatomical landmarks (Paxinos 2007) based on neutral red counterstain. The overall cross-sectional area and the number of c-Fos-positive cells within each trace was recorded. The volume of each brain region was determined by summing the areas of all sections and multiplying by the section thickness. Cell density was calculated by dividing the total number of c-Fos-positive cells per animal by the volume of the brain region (c-Fos+ cells/mm³). We used cell density to compare neural activation in the perinatal brain, as it allows us to perform direct comparisons even when the volume of the regions under study differ.

2.3.4.2 Fluorescent microscopy

Fluorescently labeled sections were visualized using a monochrome camera (Hamamatsu ORCA-R2 C10600-10B) attached to an AxioImager M.2 fluorescent microscope (Carl Zeiss, Thornwood, New York) and a LED illumination source (X-Cite 120 LEDBoost, Excelitas Technologies). Brain sections were magnified 100X using a 10x objective (Zeiss EC Plan-Neofluar 10x/0.3 Ph1 M27) and imaged using structured illumination microscopy (Zeiss ApoTome.2) and Stereo Investigator software (MBF Bioscience Inc.). The Apotome system serves as a pseudo-confocal microscope (it eliminates the out-of-focus blur from other focal planes computationally as opposed to physically as in standard confocal microscopy). A Z-stack of images in areas of interest was captured to quantify Fos+AVP or Fos+OXT double labeled cells

in wildtype brain sections. Image size was 1344×1024 pixels and each z-step was $2 \mu\text{m}$ with an average of about 12 optical sections per image. The excitation/emission spectra used in wildtype brain sections to image Fos was 550nm/605nm (Apotome filter 43HE-DsRed), and 470nm/525nm to image AVP and OXT (Apotome filter 38HE-GFP). The excitation/emission spectra used in CRH-tdTomato reporter brain sections to image tdTomato was 550nm/605nm (Apotome filter 43HE-DsRed), 470nm/525nm to image Fos (Apotome filter 38HE-GFP), and 365nm/445nm to image AVP (Apotome filter 39-DAPI). Exposure time was automatically set by Stereo Investigator software and ranged from 900–1200 ms for the channel capturing Fos images, 700-900 ms for the channel capturing tdTomato images, and 700-900 ms for the channel capturing AVP and OXT images.

First, we determined the total number of Fos+, AVP+ or OXT+ neurons in each z-stack using Image J and the Cell Counter plugin to mark each subtype within the areas of interest. Second, we determined the total number of Fos+ cells that are AVP+ or OXT+ by marking co-labeled neurons. Z-stacks were scrolled through and channels were turned on and off as needed to verify that Fos-positive nuclei were within neurons with the peptide of interest. Finally, the percentage of AVP or OXT cells that are Fos+ was determined by dividing total number of Fos+AVP or Fos+OXT co-labeled cells over total number of AVP or OXT cells multiplied by 100. Similarly, the percentage of Fos cells that are AVP+ or OXT was calculated by dividing total number of Fos+AVP or Fos+OXT co-labeled cells over total number of Fos+ cells multiplied by 100. All analyses were performed by researchers blinded to experimental conditions.

Series of optical sections of Fos+CRH-tdTomato+AVP expression in CRH-tdTomato reporter brain slices were captured through the PVN using anatomical landmarks as described above. The excitation/emission spectra used was 550nm/605nm to image tdTomato (Apotome

filter 43HE-DsRed), 470nm/525nm to image Fos (Apotome filter 38HE-GFP), and 365nm/445nm to image AVP (Apotome filter 39-DAPI). Exposure time was automatically set by Stereo Investigator software and ranged from 600–800 ms for the channel capturing Fos images, 700-900 ms for the channel capturing tdTomato images, and 700-900 ms for the channel capturing AVP images. The percentage of Fos+ CRH-tdTomato or Fos+ CRH-tdTomato +AVP co-labeled cells was determined as described above.

2.3.5 Statistical analyses

Two-way ANOVA was used to evaluate effects of age and sex on c-Fos labeling, effects of age and brain area of interest on c-Fos/neuropeptide double labeling, and effects of age and neural phenotype on c-Fos labeling. Data for males and females were combined when no effect of sex was found, and a mixed-effect model (REML) two-way ANOVA was used to evaluate effects of age and brain area. One-way ANOVA was performed to evaluate acute effects of birth on c-Fos labeling in the rat PVN. When applicable, ANOVAs were followed by Fisher's least significant difference post hoc tests. Data transformations and nonparametric tests (Kruskal–Wallis test followed by Dunn's tests) were performed as necessary.

2.4 Results

2.4.1 Discrete hypothalamic areas are activated shortly after birth

To determine how the brain responds to birth, we analyzed the pattern of neural activation in the mouse brain via detection of c-Fos one day before birth at E18.5, and postnatally at 1h, 3h and one day after a vaginal delivery. Given the abrupt changes that occur in the newborn environment upon birth, we predicted a widespread effect of birth on neural activation. Contrary to our prediction, we found that birth triggers neural activation in specific brain areas. We found

strong activation of discrete hypothalamic areas in the mouse at 3h postpartum compared to E18.5, 1h after birth or P1 (**Figure 2.1**). Specifically, c-Fos expression levels were low at E18.5, then increased slightly in most brain areas at 1h postpartum, followed by a significant increase at 3h postpartum; c-Fos expression returned to baseline levels at P1. C-Fos expression was most striking in the PVN, SON and SCN compared to the rest of the brain. We quantified these regions and the neighboring AHA [**Figure 2.2 (A)**].

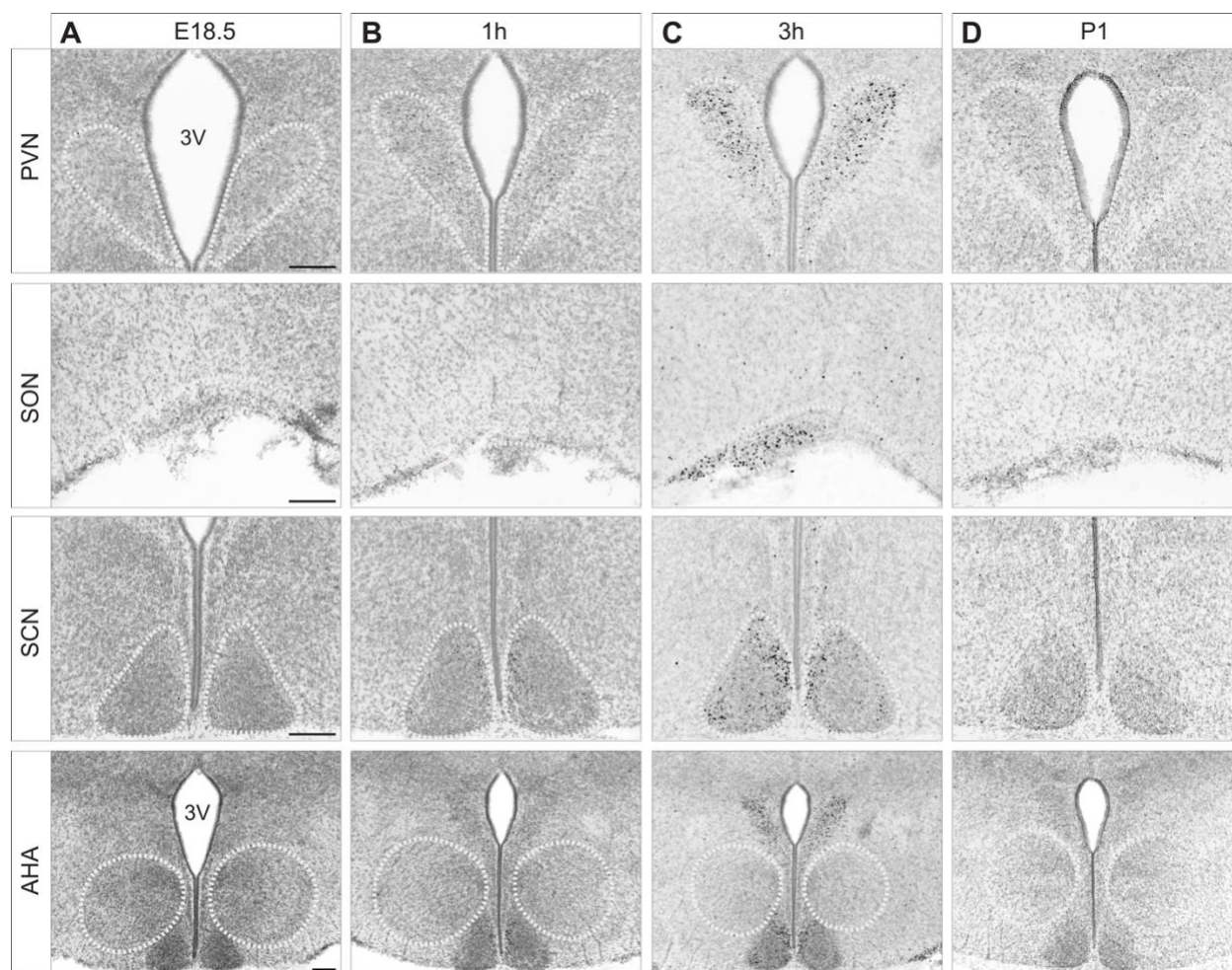


Figure 2.1 *c-Fos* immunoreactivity in the PVN, SON, SCN and AHA one day before birth, and 1h, 3h, or one day after birth.

c-Fos immunoreactivity (dark-stained cell nuclei) in the PVN, SON, SCN and AHA was almost undetectable at E18.5 (A). At 1h postpartum, only a few cells within the PVN, SON, SCN and AHA

showed faint *c-Fos* labeling (**B**). At 3h postpartum, however, *c-Fos* immunoreactivity was stronger in all brain areas (**C**). At P1, *c-Fos* immunoreactivity was greatly reduced, with only a few cells showing scattered *c-Fos* labeling (**D**). White dotted lines delineate brain nuclei of interest. 3V, third ventricle. Scale bar: 100 μm .

Quantification of *c-Fos* immunoreactive (*c-Fos-ir*) cells showed that neural activation peaked at 3h postpartum in the PVN, SON, and SCN; there was a significant main effect of age [$F(1.913, 109) = 18.94$; $P < 0.0001$], a significant main effect of hypothalamic nuclei [$F(3, 171) = 25.06$; $P < 0.0001$] and an age-by-hypothalamic nuclei interaction [$F(9, 171) = 3.72$; $P = 0.0003$], reflecting the fact that the AHA showed significantly lower *c-Fos-ir* than other hypothalamic nuclei at all timepoints ($P < 0.0001$), despite its significant increase at 3h compared to, 1h ($P < 0.02$), E18.5 ($P = 0.0002$) and P1 ($P < 0.0001$) [**Figure 2.2 (A)**]. Specifically, compared to E18.5 or P1, *c-Fos-ir* cell density was higher in each area at 3h postnatal (PVN: E18.5 vs 3h, $P = 0.0002$; 3h vs P1, $P = 0.0001$; SON: E18.5 vs 3h, $P < 0.008$; 3h vs P1, $p < 0.03$; SCN: E18.5 vs 3h, $P = 0.0009$; 3h vs P1, $P < 0.003$). The same pattern was seen for the neighboring AHA, but the number of *c-Fos* cells in this region was extremely low compared to the other three areas (open circles).

We also noticed increased neural activation in several other brain areas at 3h postpartum [ANOVA main effect of age: $F(2.856, 119.9) = 21.78$; $P < 0.0001$; ANOVA main effect of brain area: $F(3, 48) = 11.88$; $P < 0.0001$], including the caudate putamen (CPu) (E18.5 vs 3h, $P < 0.0001$, 3h vs P1, $P < 0.0001$), lateral habenula (LHb) (E18.5 vs 3h, $P = 0.0001$; 3h vs P1, $P = 0.0064$), paraventricular nucleus of the thalamus (PVT) (E18.5 vs 3h, $P < 0.0001$, 3h vs P1, $P < 0.007$), and subfornical organ (SFO) (E18.5 vs 3h, $P = 0.033$; 3h vs P1, n.s.). However, with the exception of the SFO, the effect of birth on neural activation in these areas was not as striking as in the hypothalamus at 3h postpartum [**Figure 2.2 (B)**].

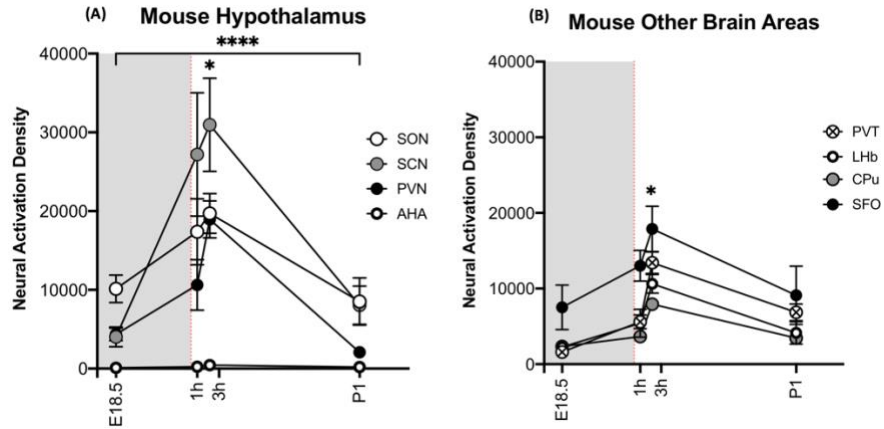


Figure 2.2 Neural activation (number of *c-Fos*+ cells/mm³) peaks at 3h postpartum in specific brain areas of mice.

(A): *c-Fos* immunoreactivity increased shortly after birth in discrete hypothalamic areas. There was a significant main effect of age [$F(1.913, 109) = 18.94; P < 0.0001$], with the density of *c-Fos*-ir cells (number of *c-Fos*+ cells/mm) being highest at 3h compared to E8.5 and P1 in all brain areas ($*P < 0.03$). There was also a significant main effect of hypothalamic nuclei [$F(3, 171) = 25.06; P < 0.0001$], and an age-by-hypothalamic nuclei interaction [$F(9, 171) = 3.72; P = 0.0003$], reflecting the fact that the AHA showed significantly lower *c-Fos*-ir than other hypothalamic nuclei at all timepoints (asterisks over bracket, $****P < 0.0001$), despite its significant increase at 3h. **(B):** *c-Fos* immunoreactivity increased shortly after birth in several other brain areas at 3h postpartum compared to E18.5 and/or P1 ($*P < 0.03$) including the CPu, LHb, PVT, and SFO (3h vs P1, *n.s.*). With the exception of the SFO, the effect of birth on neural activation in these areas was not as striking as in the hypothalamus at 3h postpartum. ANOVA main effect of age: $F(2.856, 119.9) = 21.78; P < 0.0001$; ANOVA main effect of brain area: $F(3, 48) = 11.88; P < 0.0001$, Gray shading indicates in utero timepoints. Red dotted line indicates the timing of birth. Data are mean \pm standard error of the mean (SEM). $n = 10-13$ animals per group.

To verify whether the effect of birth on neural activation generalized to another species, we also analyzed the number of Fos+ cells in the rat brain within 1h of birth (0-1h), at 3h postpartum and on P1. We found that neural activation also peaked at 3h postpartum in the rat PVN compared to 0-1h and P1 ($P < 0.0001$) [Figure 2.3 (A)]. In contrast to the mouse brain, we found a significant main effect of age [$F(1.342, 14.77) = 32.09, P < 0.0001$], and an age-by-sex interaction in the rat SON [$F(2, 22) = 6.19, P = 0.0074$], reflecting that males had higher c-Fos expression than females at P1 ($P = 0.0026$) [Figure 2.3 (B)]. In the rat SON, c-Fos immunoreactivity was higher at 3h postpartum compared to P1 in both males and females ($P < 0.0014$), but not different than 0-1h. We also found a significant main effect of age [$F(1.722, 24.97) = 37.20, P < 0.0001$], and an age-by-sex interaction in the rat SCN [$F(2, 29) = 10.03, P = 0.0005$], reflecting that males had higher c-Fos expression at P1 ($P = 0.0057$), and females had higher c-Fos expression at 0-1h ($P < 0.05$) [Figure 2.3 (C)]. In the rat SCN, c-Fos immunoreactivity was higher at 3h postpartum compared to P1 in both males and females ($P < 0.0071$), but only different than 0-1h in males ($P = 0.004$).

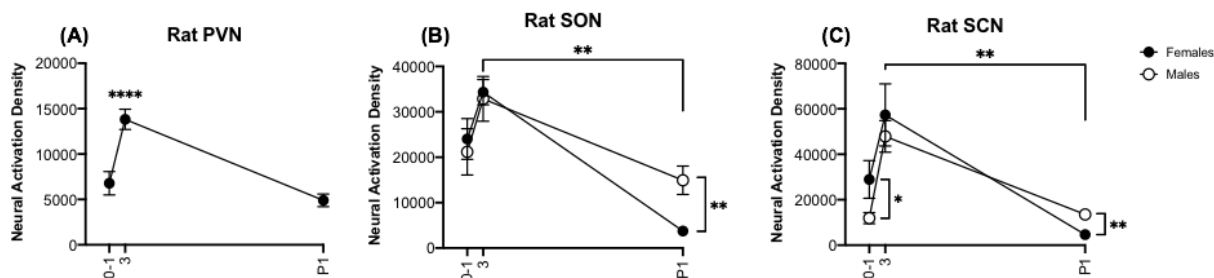


Figure 2.3 Neural activation (number of Fos+ cells per mm³) peaks at 3h postpartum in discrete hypothalamic areas of rats.

(A) c-Fos immunoreactivity was highest at 3h postpartum in the rat PVN (3h vs 1h and P1, **** $P < 0.0001$). (B) In the rat SON, there was a main effect of age [$F(1.342, 14.77) = 32.09, P < 0.0001$], indicating higher c-Fos immunoreactivity at 3h postpartum compared to P1 [asterisks

over bracket, $**P < 0.0014$; 0-1h, n.s.). There also was an age-by-sex interaction [$F(2, 22) = 6.19, P = 0.0074$] reflecting that males had higher c-Fos expression at P1 (asterisks next to vertical bracket, $**P = 0.0026$). (C) In the rat SCN, there was a main effect of age [$F(1.722, 24.97) = 37.2, P < 0.0001$], indicating higher c-Fos immunoreactivity at 3h postpartum compared to P1 (asterisks over bracket at P1, $**P < 0.007$); only males had significantly higher c-Fos-ir cell density at 3h postpartum compared to 0-1h ($P = 0.004$). There also was an age-by-sex interaction [$F(2, 29) = 10.03, P = 0.0005$], reflecting that males had higher c-Fos expression at P1 (asterisks next to vertical bracket, $**P = 0.0057$), whereas females had higher c-Fos expression within 1h of birth (asterisk next to vertical bracket, $*P < 0.05$). Data are mean \pm SEM. $n = 6$ animals for all groups.

2.4.2 Birth activates hypothalamic vasopressin and oxytocin neurons

Essential to understanding the role of neural activation in the perinatal brain is the analysis of the neural subtypes that are c-Fos+. Besides CRH neurons in the sheep PVN (Hoffman, McDonald et al. 1991), no evidence exists of activation of any other neural subtype in the brain around the time of birth. Since our data showed high activation at 3h postpartum in discrete hypothalamic areas (PVN, SCN and SON), we analyzed three prominent cell groups in these regions: AVP-, OXT-, and CRH-expressing neurons. Double labeling of c-Fos with AVP or OXT and triple labeling of c-Fos and AVP in a CRH-tdTomato reporter mouse revealed that many c-Fos+ neurons at 3h postpartum are AVP positive. In the PVN, there was a significant main effect of age [$F(1.455, 37.83) = 33.51, P < 0.0001$], a significant main effect of neural phenotype [$F(2, 29) = 36, P < 0.0001$], and an age-by-neural phenotype interaction [$F(4, 52) = 16.98, P < 0.0001$]. Specifically, 32% of all AVP neurons showed c-Fos labeling at 3h, whereas only 6% of OXT and 6% of CRH neurons were c-Fos+ ($P < 0.0001$) [**Figure 2.4 (A)**]. While the percentage of AVP

neurons that were c-Fos+ was significantly lower at E18.5 and P1 compared to 3h ($P < 0.0001$), it was still significantly higher than OXT neurons at E18.5 ($P < 0.0001$), and significantly higher than CRH neurons at P1 ($P = 0.014$). Only 6% of OXT neurons were c-Fos+ at 3h, but the percentage was significantly higher than E18.5 ($P = 0.0002$), and at P1, we found a sex difference, with females showing greater c-Fos immunoreactivity than males ($P = 0.03$) [Figure 2.4 (B)]. While only 6% of CRH neurons were c-Fos+ at 3h, the percentage was significantly higher than P1 ($P = 0.02$).

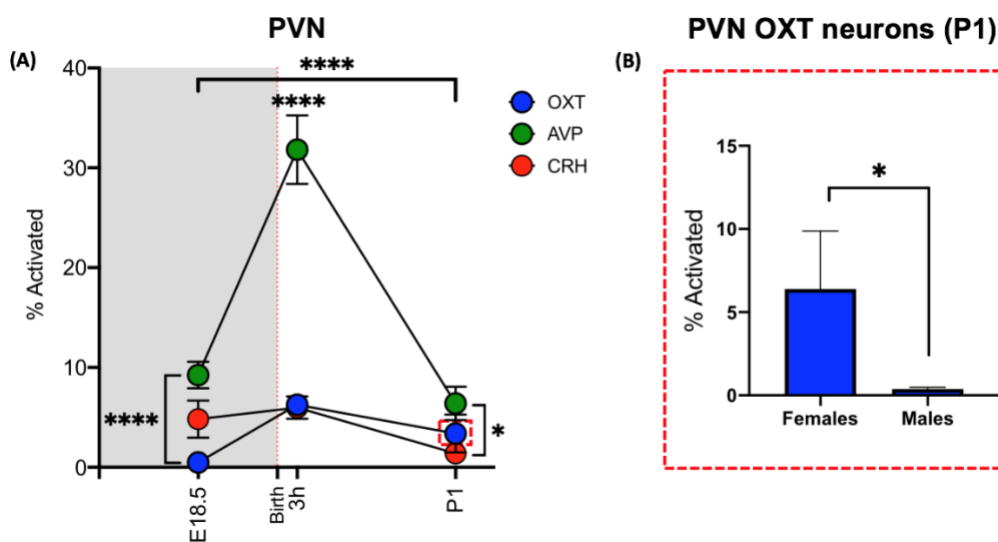


Figure 2.4 Vasopressin neurons show increased c-Fos labeling at 3h in the PVN.

(A) Two-way ANOVA showed a significant main effect of age [$F(1.455, 37.83) = 33.51, P < 0.0001$], a significant main effect of neural phenotype [$F(2, 29) = 36, P < 0.0001$], and an age-by-neural phenotype interaction [$F(4, 52) = 16.98, P < 0.0001$]; repeated measures applied only to AVP and OXT, as CRH labeling was achieved with the CRH reporter mouse. c-Fos immunoreactivity within AVP neurons (green circles) increased significantly at 3h postpartum compared to E18.5 and P1 (asterisks over horizontal bracket, **** $P < 0.0001$). At E18.5, the percent of AVP neurons that were c-Fos+ was significantly higher than OXT neurons (blue circles; asterisks next to vertical bracket, **** $P < 0.0001$). At 3h, 32% of all AVP neurons were c-Fos+,

whereas only 6% of OXT and 6% of CRH neurons were *c-Fos*⁺ (**** $P < 0.0001$). While only 6% of OXT neurons were *c-Fos*⁺ at 3h, the percentage was significantly higher than E18.5 ($P = 0.0002$) and P1 (* $P = 0.02$). At P1, the percentage of AVP neurons that were *c-Fos*⁺ was significantly higher than CRH neurons (red circles) (asterisk next to vertical bracket, * $P = 0.014$). We found a sex difference in *c-Fos* labeling of PVN OXT neurons at P1 (red-dashed square), with females showing greater *c-Fos* immunoreactivity than males (* $P = 0.03$) (B). Gray shading indicates in utero timepoints. Red dotted line indicates the timing of birth. Data are mean \pm SEM. $n = 12$ in the AVP and OXT groups, and $n = 6-8$ animals in the CRH group.

In the SON, we found a main effect of age [F (1.606, 35.32) = 4.204, $P = 0.03$] and a main effect of neural phenotype [F (1, 22) = 5.78, $P < 0.03$], with only OXT neurons showing increased activation at 3h postpartum compared to E18.5 ($P = 0.006$) [Figure 2.5 (A)]. The effect of birth on neural activation of AVP and OXT in the SON was more balanced than the PVN, with activation of 20% and 18% of AVP and OXT neurons, respectively, at 3h postpartum. Moreover, in the SCN, 47% of all AVP neurons showed *c-Fos* labeling at 3h postpartum, which is significantly higher than SCN-AVP neurons showing *c-Fos* labeling at E18.5 or P1 ($P < 0.0001$) [Figure 2.5 (B)].

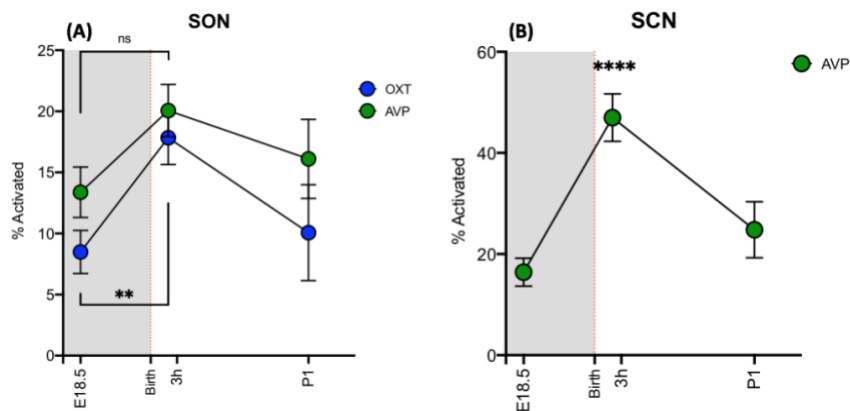


Figure 2.5 Oxytocin neurons in the SON and vasopressin neurons in the SCN show increased *c-Fos* labeling at 3h postpartum.

(A) Two-way ANOVA showed a significant main effect of age [$F(1.606, 35.32) = 4.204, P = 0.03$] and a significant main effect of neural phenotype [$F(1, 22) = 5.78, P < 0.03$], with only OXT+ neurons (blue circles) showing increased activation at 3h postpartum compared to E18.5 (asterisks inside bracket, $**P = 0.006$). (B) Quantification of c-Fos immunoreactivity within AVP neurons (green circles) in the SCN showed 47% of all AVP were c-Fos+ at 3h postpartum, which is significantly higher than SCN-AVP neurons showing c-Fos labeling at E18.5 or P1 ($P < 0.0001$). Gray shading indicates in utero timepoints. Red dotted line indicates the timing of birth. Data are mean \pm SEM. $n = 12$ for all groups.

2.4.3 Percentage of c-Fos immunoreactive cells that are vasopressin-, oxytocin-, or corticotropin-positive

We also determined the percentage of c-Fos-ir cells positive for each peptide analyzed in the PVN, SCN, and SON. For all brain areas of interest, we used two-way ANOVA to evaluate whether c-Fos immunoreactivity varied significantly between neural phenotype groups over the three timepoints. In the PVN, we found a significant main effect of neural phenotype [$F(2, 83) = 57.51, P < 0.0001$], and an age-by-neural phenotype interaction [$F(4, 83) = 3.284, P < 0.02$]. At E18.5, almost all c-Fos-ir cells were AVP+ (39.3%) or CRH+ (48.4%); only 7.8% of c-Fos+ cells were OXT+, which was significantly lower than CRH+ and AVP+ neurons ($P < 0.004$). At 3h postpartum, the percentage of c-Fos+ cells that were AVP+ was higher than OXT+ and CRH+ ($P < 0.0001$); the percentage of c-Fos-ir cells that were CRH+ dropped to 9% (E18.5 vs 3h, $P < 0.001$), and nearly one third of the greater density of c-Fos-ir cells were AVP+ (32.9%). However, only 4.4% were OXT+, leaving a 53.6% of the c-Fos-ir cells unaccounted for [**Figure 2.6 (A)**]. A similar pattern was observed at P1 for c-Fos-ir cells that were AVP+, OXT+, or CRH+ (33.2%,

8.2%, and 15.4%, respectively; c-Fos labeling was greater in AVP neurons, $P < 0.0002$), thus leaving a greater percentage of the c-Fos-ir cells unaccounted for 43.2%.

In the SON, two-way ANOVA analysis showed a significant main effect of neural phenotype [$F(2, 99) = 257.5, P < 0.0001$], and an age-by-neural phenotype interaction [$F(4, 99) = 3.568, P = 0.0092$] [**Figure 2.6 (B)**]. The large majority of c-Fos+ cells were AVP+ or OXT+, and the pattern was similar across all time points. At all timepoints, the percentage of c-Fos-ir cells that were AVP+ was higher than OXT+ or unidentified cells ($P < 0.0001$). The percentage of c-Fos-ir cells that were AVP+ was similar at E18.5 and 3h (75.8% at E18.5, 69.3% at 3h), but increased significantly at P1 compared to 3h (81%, $P < 0.04$). The percentage of c-Fos-ir cells that were OXT+, remained fairly constant across all timepoints (13.2% at E18.5, 15.3% at 3h, and 18% at P1). Few c-Fos-ir cells were unidentified at any timepoint (11% at E18.5, 15.4% at 3h, and 0.97% at P1), and this number was lower at P1 than at E18.5 ($P = 0.023$) or 3h ($P < 0.006$), and significantly lower than c-Fos-ir cells that are OXT+ at P1 ($P = 0.0026$).

In the SCN, two-way ANOVA analysis also showed a significant main effect of neural phenotype [$F(1, 66) = 4.533, P < 0.04$], and an age-by-neural phenotype interaction [$F(2, 66) = 3.809, P < 0.03$] [**Figure 2.6 (C)**]. We found that the total percentage of c-Fos-ir cells that are AVP+ was similar across timepoints (46% at E18.5, 60% at 3h, and 59% at P1), and that a substantial percentage of c-Fos-ir cells were unidentified by our markers (54% before birth and about 40% after birth). The total percentage of c-Fos-ir cells that are AVP+ was greater than the percentage of unidentified c-Fos+ cells at 3h ($P < 0.02$) and P1 ($P < 0.03$).

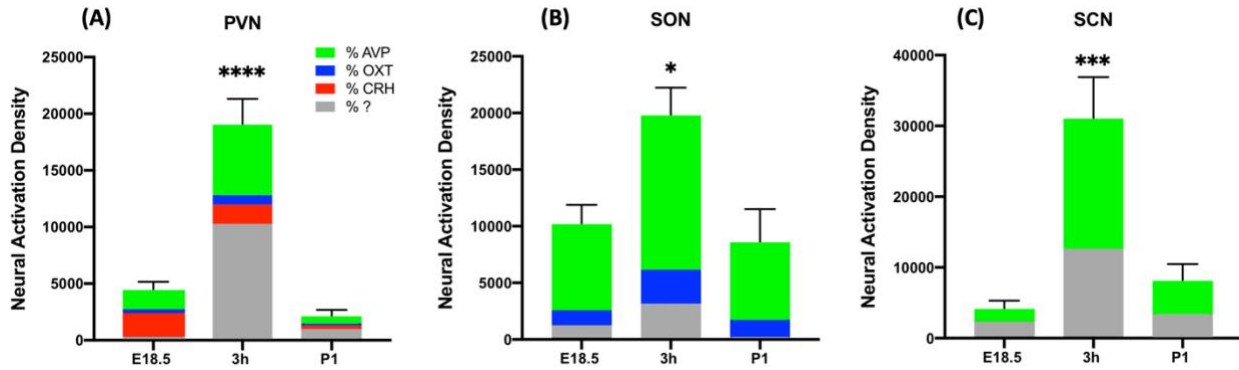


Figure 2.6 Phenotypic distribution of *c-Fos* immunoreactive cells in the PVN, SON, and SCN at E18.5, 3h postpartum, and P1.

Asterisks over bars represent the acute effect of birth on the density of *c-Fos*-ir cells identified previously (**Figure 2.2**): *c-Fos* immunoreactivity was higher at 3h compared to E18.5 or P1 in the PVN (**** $P < 0.0001$), SON (* $P < 0.003$), and SCN (*** $P \leq 0.0001$). (A) Two-way ANOVA showed a significant main effect of neural phenotype [$F(2, 83) = 57.53, P < 0.0001$], and an age-by-neural phenotype interaction [$F(4, 83) = 3.284, P < 0.02$]. At E18.5, 39.3%, 7.8%, and 48.4% of the total *c-Fos*-ir cells were AVP+ (green), OXT+ (blue), or CRH+ (red), respectively. Percentage of *c-Fos* neurons that were CRH+ was higher than OXT+ neurons at E18.5 ($P < 0.004$). At 3h, the percentage of *c-Fos*+ cells that were AVP+ (32.9%) was higher than OXT+ (4.4%) and CRH+(9%) ($P < 0.0001$), but 53.6% of the *c-Fos*-ir cells were unaccounted for. A similar pattern was observed at P1 for *c-Fos*-ir cells that were AVP+, OXT+, or CRH+ (33.2%, 8.2%, and 15.4%, respectively; *c-Fos* labeling was greater in AVP neurons, $P < 0.0002$); 43.2% of the *c-Fos*-ir cells were unaccounted for (gray) (B) Two-way ANOVA analysis showed a significant main effect of neural phenotype [$F(2, 99) = 257.5, P < 0.0001$], and an age-by-neural phenotype interaction [$F(4, 99) = 3.568, P = 0.0092$]. The percentage of *c-Fos*-ir cells that were AVP+ was higher than *c-Fos*-ir cells that were OXT+ or unidentified at all timepoints ($P < 0.0001$). The percentage of *c-Fos*-ir cells that were AVP+ was similar at E18.5 and 3h (75.8% at

E18.5, 69.3% at 3h), but increased significantly at P1 compared to 3h (81%, $P < 0.0001$). The percentage of c-Fos-ir cells that were OXT+, remained fairly constant across all timepoints (13.2% at E18.5, 15.3% at 3h, and 18% at P1). Few c-Fos-ir cells were unidentified at any timepoint (11% at E18.5, 15.4% at 3h, and 0.97% at P1), and this number was lower at P1 than at E18.5 ($P = 0.023$) or 3h ($P < 0.006$), and significantly lower than c-Fos-ir cells that are OXT+ at P1 ($P = 0.0026$). (C) Two-way ANOVA showed a significant main effect of neural phenotype [$F(1, 66) = 4.533, P < 0.04$], and an age-by-neural phenotype interaction [$F(2, 66) = 3.809, P < 0.03$]. Total percentage of c-Fos-ir cells that are AVP+ was similar across timepoints (46% at E18.5, 60% at 3h, and 59% at P1). A substantial percentage of c-Fos-ir cells were unidentified by our markers (54% before birth and about 40% after birth). The total percentage of c-Fos-ir cells that are AVP+ was greater than the percentage of unidentified c-Fos+ cells at 3h ($P < 0.02$) and P1 ($P < 0.03$). Data are mean \pm SEM. $n = 12$ in the AVP and OXT groups, and $n = 6-8$ animals in the CRH group.

2.5 Discussion

Birth is a dramatic event involving extraordinary changes in the newborn's environment. Peripherally, birth triggers developmental switches in the lungs, heart, gut, and liver to kick start autonomous function of vital processes (Liggins 1976, Fowden, Mijovic et al. 1993, Liggins 1994). Centrally, however, it is less clear what changes occur in preparation for the challenges of birth or in response to it. In this study, we generated a detailed profile of the neural activation pattern in the perinatal mouse brain using c-Fos immunoreactivity. We found that discrete hypothalamic areas exhibited up to a 500% increase in neural activation 3h after birth compared to one day before birth, and AVP neurons in the PVN, SCN, and SON were strongly activated at 3h postpartum

compared to OXT or CRH neurons, suggesting that AVP neurons within these hypothalamic regions are particularly sensitive to the stimuli of birth.

C-Fos immunoreactivity provides a powerful anatomical tool for the study of neural activity patterns in response to diverse stimuli given its resolution at the cellular level (c-Fos is located at the nucleus, which makes it practical for identification of the phenotype of the activated cell via IHC double labeling), and its low expression levels in basal conditions (Yamada, Hada et al. 1999). In all of the brain regions examined, neural activation was significantly increased within 3h of birth compared to E18.5 or 1h after birth, and in all cases neural activation returned to baseline levels within 24h of birth. While *c-fos* mRNA can be detected within 20 min of stimulus onset, its protein product takes at least 90 min to accumulate (Sheng and Greenberg 1990, Morgan and Curran 1991). Once induced, c-Fos protein generally peaks 1-3h after stimulus onset and can be observed for several hours before it declines to baseline levels. For example, administration of intraperitoneal hypertonic solution induces c-Fos maximally at 1-2h and Fos increases remain detectible for 4-8h (Sharp, Sagar et al. 1991). Similarly, c-Fos expression peaks at 2h and disappears after 4.5h of continuous noxious stimulation (Bullitt, Lee et al. 1992). Therefore, our results suggest that a stimulus/stimuli related to delivery and the immediate postpartum period triggers perinatal neural activation. However, since birth is a complex, multi-step process, we cannot determine the exact aspect(s) of birth that trigger the perinatal neural activation from this study alone. Further studies dissociating the diverse stimuli accompanying a vaginal delivery (i.e. hormonal signals, mechanical stimuli from uterine contractions and the resulting hypoxia, microbiota exposure, stress, generalized arousal) are required to identify the stimulus/stimuli that triggers neural activation at birth (see Chapter 3).

Our results are in agreement with a recent study reporting increased neural activation in brain nuclei associated with olfaction and movement on the day of birth (Ikeda, Onimaru et al. 2019). Following a vaginal delivery, the piriform cortex, caudate putamen, part of the cerebellum, and facial nucleus are activated; whether neural activation in these areas occurs in connection with birth remains unclear given that 1) the timing between birth and collection for analysis of the neonatal brain was not controlled for across subjects, and 2) no reference baseline levels of neural activation were determined at other timepoints near/on the day of birth (Ikeda, Onimaru et al. 2019). Here, we closely monitored the timing of birth, carefully controlled for the time of collection, and directly show elevated c-Fos expression shortly after vaginal delivery.

A limitation of our study, however, is the fact that c-Fos is not a perfect surrogate for neural activity: c-Fos expression does not always match other techniques measuring neural activity [i.e. 2-Deoxy-Glucose (2-DG) and electrophysiology] and is not induced by all types of stimuli. For example, c-Fos expression but not 2-DG signal intensity increase after water deprivation in the PVN (Sagar, Sharp et al. 1988), and in the CA1 layer of the hippocampus after induced brain ischemia (Jorgensen, Deckert et al. 1989). Moreover, only a few cells express c-Fos in the rat barrel cortex after strong whisker stimulation (Melzer and Steiner 1997). The signal transduction pathways that can robustly and reliably induce *c-fos* in response to diverse stimuli have been well characterized in the central nervous system (CNS), and include interdependent function of at least four regulatory elements in the *c-fos* promoter and combinatorial activation of *c-fos* enhancers (Robertson, Kerppola et al. 1995, Joo, Schaukowitch et al. 2016). Yet, only depolarizations, increases in firing rate, or synaptic activity that result in substantial influx of calcium (Ca^{++}) into the neuron, lead to induction of *c-fos* regardless of the stimulus and signal transduction pathway

(Morgan and Curran 1986, Morgan and Curran 1988, Vendrell, Pujol et al. 1992, Zhu and Herbert 1997) and cause long term adaptive changes in the brain (Jaworski, Kalita et al. 2018).

In contrast, postsynaptic inhibition is generally not associated with c-Fos induction. In fact, inhibiting postsynaptic inputs via administration of an inhibitory neuromodulator analog reduces c-Fos expression in the rat spinal cord (Honore, Buritova et al. 1998). Moreover, inhibitory but not excitatory GABA signaling has been found to decrease c-Fos expression in cultured hippocampal neurons (Berninger, Marty et al. 1995). Previous studies examining functional changes in the fetal/neonatal brain near birth report decreased neural activity in the hippocampus and neocortex of rats and mice via mechanisms that involve GABAergic modulation (Tyzio, Cossart et al. 2006, Tyzio, Nardou et al. 2014, Spoljaric, Seja et al. 2017). Consistent with these findings, we did not find neural activation in the perinatal mouse or rat hippocampus, although lack of c-Fos expression in the hippocampus does not necessarily mean lack of neural activity in the perinatal brain for the reasons explained above. In line with this, decreased c-Fos expression at P1 does not mean lack of neural activity. Induction of c-Fos is normally more effective when associated with novel stimuli or stimulation after sensory deprivation, as c-Fos expression decreases significantly after repeated stress (Melia, Ryabinin et al. 1994). Therefore, in the context of this study, neurons that are continuously activated after the primary stimulus may not show c-Fos labeling at P1. Interestingly, *c-fos* mRNA increases on the day of birth, and continues to increase 5 days after, as measured in whole brain homogenates (Kasik, Wan et al. 1987), but here we report c-Fos levels that return to baseline at P1. In this regard, electrophysiological experiments may be more appropriate for monitoring neural activity after the initial stimulus in specific areas of interest.

Differential c-Fos expression patterns have been reported depending on age, sex and time of day in several brain regions, including the hypothalamus. For example, quantification of c-Fos

in whole brain homogenates via Western blot shows significantly higher levels of c-Fos in male rats compared to females at P1, P5 and P20, but not at P0, or P11 (Olesen and Auger 2005), and further IHC analyses identify the SON as one of the areas that displays a sex difference in c-Fos expression at P1 (Olesen and Auger 2005). Consistent with these results, we also found a sex difference in c-Fos expression in the rat SON, with males showing significantly higher c-Fos expression levels than females at P1. We also found sex differences in the SCN in two of the timepoints analyzed. Within 1h of birth, females showed significantly higher c-Fos expression in the SCN, whereas males showed significantly higher c-Fos expression at P1. Sex differences in the rat SCN, to our knowledge, have not been reported before. Sex differences in c-Fos expression are likely mediated by the testosterone surge at birth acting on estrogen receptors, given that administration of a masculinizing dose of testosterone or estradiol, but not 5 α -dihydrotestosterone to P2 female rats induces *c-fos* mRNA and protein in the hypothalamus and other brain areas, whereas injecting an estrogen receptor blocker along with testosterone abolished *c-fos* mRNA induction (Giannakopoulou, Bozas et al. 2001). Similarly, c-Fos expression also depends on the time of the day. Extensive evidence shows that c-Fos is expressed in a circadian manner in the SCN (Inouye and Shibata 1994, Acher and Chauvet 1995, Novak and Nunez 1998, van der Veen, van der Pol-Meijer et al. 2008), however it is unlikely that the perinatal differences in c-Fos expression that we see in the SCN are due to entrainment or circadian control because we included pups born at different times of the day (subjective day or night) and all showed the same pattern of c-Fos expression.

Birth has been described as an “adaptive stressor” (Lagercrantz and Slotkin 1986, Lagercrantz 2016). Perinatal activation of the HPA axis initiates an endocrine cascade that facilitates the adaptation to the challenges posed by the transition from the uterine environment

(Liggins 1994, Kota, Gayatri et al. 2013), and is largely regulated at the level of the hypothalamus, particularly by CRH neurons in the PVN, and AVP neurons in the PVN and SON. Consistent with this, we find strong activation in discrete hypothalamic areas, including the PVN, SON and SCN. Moreover, we found that at 3h postpartum (when we see the highest levels of c-Fos expression), AVP neurons are significantly more activated than OXT or CRH neurons in the PVN. The only previous study that we are aware of that has evaluated the activation pattern of one of these neuropeptides in the perinatal PVN was done in sheep, and found that CRH neurons are strongly activated during labor, and that c-Fos immunoreactivity declines rapidly after delivery (Hoffman, McDonald et al. 1991). Similarly, we found that approximately half (48.5%) of all the c-Fos-ir neurons in the PVN at E18.5 are CRH positive, but the percentage decreases significantly by 3h of birth, suggesting an important role of CRH neurons in labor, whereas AVP may play an important role at birth.

It will be worth determining whether there is a significant difference in the proportion of AVP neurons that belong to the magnocellular or parvocellular division of the PVN, given that neurons from these subgroups have distinct functional roles (Ferguson, Latchford et al. 2008). We were not able to discern this in the current study, because the distinction between magnocellular and parvocellular AVP subdivisions is less clear in the mouse than in the rat brain. Therefore, an important follow-up analysis will be to perform c-Fos and AVP fluorescent double labeling in the rat brain, where it may be easier to characterize the type of AVP neurons that are activated at 3h postpartum.

Notably, we found regional differences in c-Fos expression levels in AVP and/or OXT neurons between the PVN, the SON and the SCN, and previous studies suggest they may result from distinct functional responses to specific stimuli. For instance, subjecting adult rats to

hemorrhagic (hypovolemic) challenge results in reciprocal induction of c-Fos in magnocellular PVN and SON AVP and/or OXT neurons (Roberts, Robinson et al. 1993). The threshold required for hemorrhagic stress to induce c-Fos expression in AVP magnocellular neurons in the SON is lower than PVN AVP magnocellular neurons, whereas OXT magnocellular neurons require greater blood loss to induce c-Fos expression than AVP magnocellular neurons in both the PVN and SON (Roberts et al. 1993). Determining the proportion of activated AVP neurons that belong to the magnocellular or parvocellular division will also provide a better understanding of the role activated AVP neurons may be playing in the perinatal brain.

We also found neural activation shortly after birth in other brain areas including the CPu, LHb, PVT, and SFO, suggesting that these areas may also play important roles in the adaptation of the newborn to the extrauterine environment. For example, the LHb receives inputs from limbic forebrain structures important in pain processing, such as the nucleus accumbens and the basal ganglia (Chudler and Dong 1995, Becerra and Borsook 2008), and recent studies show that neurons within the LHb respond directly to nociceptive stimuli, further supporting a prominent role of the LHb in pain processing (Wu, Huang et al. 2005). The PVT has been identified as an important relay for arousal- and stress-related information to specific forebrain areas (Bubser and Deutch 1999, Parsons, Li et al. 2006). The SFO is a sensory circumventricular organ that responds to circulating hormones and neurotransmitters and influences water and energy homeostasis (Li and Ferguson 1993, Llewellyn, Zheng et al. 2012, Medeiros, Dai et al. 2012). Interestingly, the CPu, LHb, and SFO send or receive inputs from AVP neurons in other regions (i.e. the PVN) (Hawthorn, Ang et al. 1980, Braga, Medeiros et al. 2011, Hernandez, Vazquez-Juarez et al. 2015). Therefore, neural activation in CPU, LHb and SFO may occur in response to AVP signaling from/to hypothalamic areas, or may occur autonomously in response to the challenges of birth.

Notably, a significant proportion of Fos⁺ neurons remains unidentified at 3h postpartum, especially in the PVN. The PVN is remarkably heterogeneous, making the identification of the relevant neural phenotypes somewhat of a fishing expedition. Nonetheless, there are a few logical possibilities. For example, TRH and somatostatin neurons are prominent neural subtypes in the PVN with important roles in energy homeostasis (Lechan and Fekete 2006). TRH stimulates the release of thyroid hormones that control heat generation, lipogenesis and appetite regulation, which are of obvious importance to the newborn (Oppenheimer, Schwartz et al. 1991, Silva 1995). On the other hand, somatostatin signaling in the rat PVN has been shown to increase fat utilization and glucose levels and to modulate metabolic parameters for appropriate utilization of energy (Atrens and Menendez 1993).

In summary, we find (based on c-Fos immunoreactivity) that a vaginal birth triggers an acute and transient increase in neural activation in specific brain areas including the PVN, SON, and SCN, and that many of the activated cells are AVP⁺. These results suggest that AVP neurons within discrete hypothalamic areas are particularly sensitive to the stimuli of birth, and raise the question of what aspect(s) of birth trigger activation of AVP neurons at birth.

3 CHAPTER THREE: NEURAL ACTIVATION AT BIRTH OCCURS AS A RESULT OF THE TRANSITION TO THE EXTRA UTERINE ENVIRONMENT

Yarely C. Hoffiz, Alexandra Castillo-Ruiz, Megan A. Hall, and Nancy G. Forger

3.1 Abstract

Birth triggers dramatic physiological changes in key peripheral organs to ensure offspring survival in the *ex utero*, but it is less clear how the brain responds to the event of birth. We recently found that a vaginal birth triggers neural activation in various brain areas, but most prominently in discrete areas within the hypothalamus. Specifically, the PVN, SON, and SCN exhibited up to a 500% increase in neural activation 3h after birth compared to one day before birth or one day after birth, suggesting that these regions are particularly sensitive to the stimuli of birth. We also found that AVP neurons are strongly activated at 3h postpartum in the PVN, SON, and SCN, compared to OXT or CRH neurons. To begin determining what aspects of birth trigger the activation of vasopressin neurons within the hypothalamus, we manipulated birth delivery mode (vaginal vs. C-section delivery). C-section deliveries differ from a vaginal birth in many aspects including fetal exposure to the hormones associated with labor, and passage through the birth canal. We generated timed-pregnancies in C57BL/6 mice and collected the brains of male and female offspring *in utero* at embryonic day 18.5, and *ex utero* at 3h, or 1 day after vaginal or C-section delivery. The tissue was processed for IF double labeling of the immediate early gene product, c-Fos (as a marker of neural activation), and AVP or OXT. To determine whether neural activation is triggered by external factors or by an autonomous developmental program, we also collected brains 3h after C-section delivery at E18.5 and evaluated the effect of advancing birth by one day on neural activation. We found that the density of c-Fos-ir cells and the percentage of activated AVP and OXT neurons was always higher at 3h postpartum in the PVN, SON, and SCN than *in utero*

regardless of birth mode or timing of birth, suggesting that neural activation at birth occurs as a result of the exposure to the extra-uterine environment.

3.2 Introduction

Birth is an extraordinary event involving numerous vital modifications to the physical and physiological state of the newborn to ensure its survival outside the womb. While perinatal peripheral adaptations in the fetus/newborn have been described in detail (Liggins 1976, Liggins 1994, Murphy, Smith et al. 2006), it is less clear what changes the brain undergoes around the time of birth. Recent studies provide evidence that birth serves as an orchestrator of developmental processes in the brain. For example, birth plays a pivotal role in the initiation of barrel formation in the somatosensory cortex and regulates eye-specific segregation of projections from the retina to the midbrain via a serotonin-dependent mechanism (Toda, Homma et al. 2013). Additionally, we recently showed that deviations from a normal vaginal delivery, including variations in birth delivery mode or the timing of birth, affect the patterning of developmental cell death, and have potential long-lasting effects on the vasopressin system (Castillo-Ruiz, Mosley et al. 2018, Castillo-Ruiz, Hite et al. 2020). Together, these results suggest that a vaginal birth (and/or the signals that accompany it) is important for normal development and physiological function.

Given that birth marks the initiation of many peripheral functions in the newborn, and that the brain houses the respective control centers, we previously studied the perinatal pattern of neural activation via IHC detection of c-Fos. We found that birth triggers neural activation in specific brain areas. Notably, within the hypothalamus, discrete nuclei (PVN, SCN, and SON) showed marked changes in c-Fos-ir; particularly, AVP neurons were strongly activated at 3h postpartum in the PVN, SCN, and SON, compared to OXT or CRH neurons one day before or after birth (OXT neurons were also strongly activated in the SON at 3h postpartum). These data suggest that some

aspect of birth triggers neural activation in the perinatal brain, and that vasopressin and/or oxytocin neurons may play important roles at birth.

Birth is a complex stimulus, however, what aspect(s) of birth are important for triggering neural activation in the perinatal brain is not known. In the present study, we first addressed this question by manipulating birth delivery mode to begin depicting what aspect(s) of birth trigger neural activation. C-sections differ from a vaginal birth in many aspects, including fetal exposure to hormonal signals associated with labor, mechanical stimulation associated with uterine contractions and passage through the birth canal. Here we show increased neural activation at 3h postpartum compared to *in utero* levels regardless of manipulations in birth delivery mode. Additionally, we show increased neural activation at 3h postpartum when animals were delivered one day prior to the expected day of birth. Taken together, our data suggest that neural activation at birth is triggered by exposure to the extrauterine environment upon delivery.

3.3 Methods

3.3.1 *Animals*

Adult C57BL/6 mice were purchased from The Jackson Laboratory or obtained from our colony. Mice were kept in a 12h light and 12h dark cycle, and given access to water and food ad libitum. All procedures were approved by Georgia State University's Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

3.3.2 *Timed pregnancies and delivery mode*

We established timed-pregnancies by housing breeding pairs together within 2h of lights off. Males were removed from the cages the next morning 1-2h after lights on and that day was marked as embryonic day (E) 0. Overall health and body weight of expectant females was

monitored during pregnancy. Starting at E18.5, expectant females were checked hourly for any signs of labor until vaginal delivery. Brains of male and female pups were collected at 3h postpartum.

We also collected brains of pups delivered via C-section at E18.5 and E19. E19 C-sections were yoked to vaginal births to match the total gestation length and circadian time of birth given that c-Fos may be expressed in a circadian rhythm (Earnest and Olschowka 1993, Novak and Nunez 1998, van der Veen, van der Pol-Meijer et al. 2008). Dams were euthanized using 2% CO₂ followed by rapid decapitation. An aseptic abdominal incision was made to expose the uterine horns, the fetuses were removed one by one from their gestational sacs, and the brains were immediately collected. All C-section-delivered pups were kept on a heating pad kept at ± 32 °C until collection at 3h post-delivery on E18.5 or E19.

3.3.3 Tissue processing

Collected brains were fixed in 4% paraformaldehyde (PFA) for 24h and then transferred into 30% sucrose solution. Brains were coronally frozen-sectioned into four 40 μ m series using a rotary microtome, and sections were processed for IHC detection of c-Fos, or IF double labeling of c-Fos and AVP or c-Fos and OXT as previously described (see Chapter 2). Slides were also analyzed as previously described (see Chapter 2).

3.3.3.1 c-Fos immunohistochemistry

Briefly, free-floating sections were rinsed in PBS, submerged in concentrated blocking solution and incubated overnight in primary antibody solution (rabbit anti-c-Fos, Santa Cruz) at room temperature. The next day, sections were washed and incubated in secondary antibody solution (goat anti-rabbit, Vector Laboratories, Burlingame, CA). After washing in PBS, sections were incubated in ABC solution (Vectastain Elite ABC Kit; Vector Laboratories) and incubated

in DAB-nickel solution as the peroxidase chromogen. Finally, sections were mounted onto microscope slides and dehydrated before counterstaining with neutral red.

3.3.3.2 c-Fos and AVP or OXT immunofluorescent double labeling

Alternate free-floating sections were rinsed in PBS, and submerged in sodium citrate. Following rinsing, sections were placed in glycine, rinsed, incubated in a concentrated blocking solution, and incubated overnight in primary antibody solution against c-Fos (rabbit anti-c-Fos, Santa Cruz) at 4°C. The next day, sections were washed and incubated in secondary antibody solution (goat anti-rabbit Alexa 594, Vector Laboratories, Burlingame, CA). Sections were washed and incubated overnight in primary antibody solution against AVP or OXT (rabbit anti-OXT, Peninsula Labs, T-4084; or rabbit anti-AVP, EMD Millipore, PC234L) at 4°C. The next day, sections were washed and incubated in secondary antibody solution (goat anti-rabbit Alexa 488, Vector Laboratories, Burlingame, CA) at 4°C. To reduce background auto-fluorescence, sections were incubated in 100mM cupric sulfate in 50mM ammonium acetate. Finally, sections were rinsed and mounted onto microscope slides.

3.3.4 Image capture and analysis

3.3.4.1 Light microscopy

Brain regions of interest (PVN, SCN, and SON) were outlined in both hemispheres with Stereo Investigator software (MBF Bioscience Inc.) using anatomical landmarks (Paxinos 2007). The area of each brain region was recorded and the number of c-Fos-positive cells within each trace was recorded. The volume of each brain region was determined by summing the areas of all sections and multiplying by the section thickness. Cell density was calculated by dividing the total number of c-Fos-positive cells per animal by the volume of the brain region (c-Fos+ cells/mm³).

We used cell density to compare neural activation in the perinatal brain, as it allows us to perform direct comparisons even when the volume of the regions under study are different.

3.3.4.2 Fluorescent microscopy

Brain sections were magnified 100X and imaged using structured illumination microscopy (Zeiss ApoTome.2 Microscope) via the Stereo Investigator software (MBF Bioscience Inc). Focal series of Fos+AVP or Fos+OXT double labeled cells in wildtype brain sections were captured through the PVN, SON, and SCN using anatomical landmarks. Image size, z-steps, average optical sections, and excitation/emission spectra used to image Fos, AVP and OXT were as previously described (see Chapter 2).

First, we determined the total number of Fos+, AVP+ or OXT+ neurons on each z-stack using Image J and the Cell Counter plugin to mark each subtype differently within the areas of interest. Second, we determined the total number of Fos+ cells that are AVP+ or OXT+ by marking co-labeled neurons. Z-stacks were scrolled through and channels were turned on and off as needed to verify that Fos-positive nuclei were within neurons with the peptide of interest. Finally, the percentage of AVP or OXT cells that are Fos+ was determined by dividing total number of Fos+AVP or Fos+OXT co-labeled cells over total number of AVP or OXT cells multiplied by 100. Similarly, the percentage of Fos cells that are AVP+ or OXT was calculated by dividing total number of Fos+AVP or Fos+OXT co-labeled cells over total number of Fos+ cells multiplied by 100. All analyses were performed by researchers blinded to experimental conditions.

3.3.5 Statistical analyses

We performed student t-tests to evaluate effects of birth and birth mode on total neural activation and activation of specific neural subtypes at 3h postpartum in all brain areas of interest. Two-way ANOVAs were used to evaluate effects of age and neural phenotype on c-Fos labeling.

When applicable, ANOVA was followed by Fisher's least significant difference post hoc tests. Data transformations and nonparametric tests (Friedman test followed by Dunn's tests) were performed as necessary.

3.4 Results

3.4.1 Perinatal neural activation in the PVN, SON and SCN is not affected by birth mode

We took advantage of the inherent differences between a vaginal and a C-section birth (which include hormones associated with labor, mechanical stimuli associated with uterine contractions, and passage through the birth canal) to tease apart the aspect(s) of birth that trigger neural activation in the perinatal rodent brain. We, again, found increased neural activation at 3h postpartum in the PVN ($P = 0.005$) and SON ($P = 0.03$) (**Figure 3.1**). There was also an increase c-Fos labeling in the SCN, but it was not significant in this experiment due to large variability at 3h. We also found that the density of neural activation at 3 h post-delivery was similar between pups delivered vaginally and pups delivered via C-section in all brain areas examined. If anything, activation was slightly (non-significantly) higher in C-section delivered pups. This indicates that a vaginal delivery is not required for the increase in neural activation.

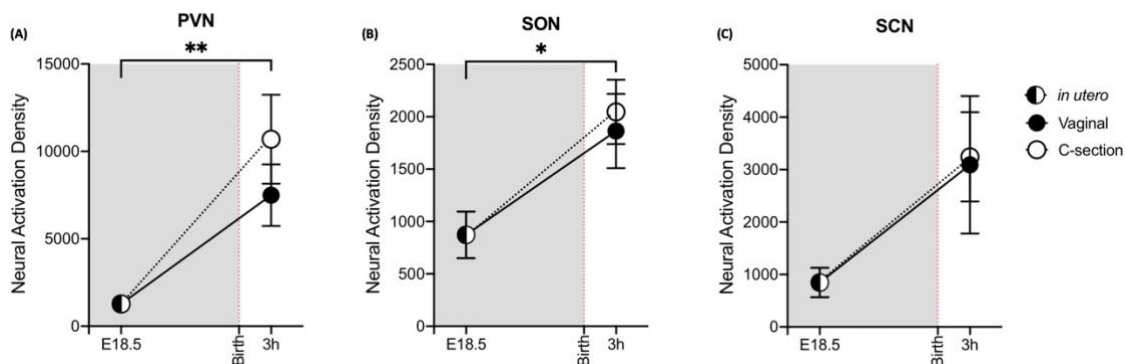


Figure 3.1 Birth mode does not affect neural activation (number of *c-Fos*⁺ cells/mm³) in the PVN, SCN or SON.

The density of *c-Fos*-ir cells was higher at 3h postpartum in the PVN (A) and SON (B) (asterisks over brackets, ***P* = 0.005, and **P* = 0.03, respectively), but not the SCN (C). We found no differences between pups delivered vaginally (black circles) and pups delivered via C-section (open circles) in all brain areas examined in any of the areas. Gray shading indicates *in utero* timepoints. Red dotted line indicates the timing of birth. Data are mean ± SEM. *n* = 11-12 animals per group.

To rule out the possibility that the transient increase in *c-Fos* labeling at 3h postpartum is not related to birth at all, but instead due to a developmentally-programmed induction of the *c-fos* protein product, we examined the neural activation pattern at 3h postpartum in animals that were delivered one day prior to the expected day of birth (E18.5). We, again, found an effect on neural activation at 3h postpartum in the PVN, SCN and SON: neural activation was significantly higher at 3h postpartum compared to *in-utero* levels in pups delivered one day before the expected day of birth (*P* < 0.0001 in all areas) (Figure 3.2).

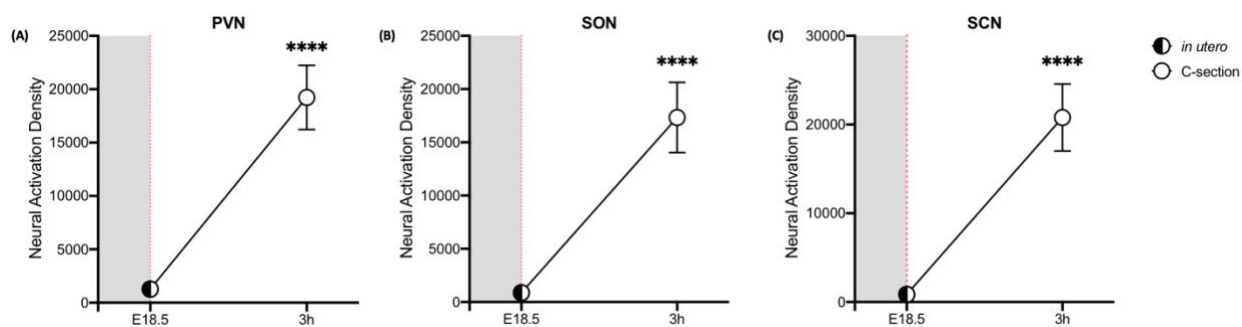


Figure 3.2 Neural activation (number of *c-Fos*⁺ cells per mm³) is higher at 3h post-delivery compared to *in utero* levels in animals delivered one day prior to the expected day of birth.

*The density of c-Fos-ir cells was higher at 3h post-delivery in the PVN (A), SON (B), SCN (C) (**** $P < 0.0001$) when animals were delivered on E18.5. Gray shading indicates in utero timepoints. Red dotted line indicates the timing of birth. Data are mean \pm SEM. $n = 12$ animals per group.*

3.4.2 Perinatal activation of AVP or OXT neurons in the PVN, SON and SCN is not affected by birth mode

While we did not find differences in the density of c-Fos-ir cells between vaginally- and C-section-delivered pups, it remained possible that there were differences in neural activation of specific neural subtypes. We therefore, analyzed the activation pattern of the two neural subtypes that were significantly activated within the hypothalamus in our previous study: AVP and OXT (see Chapter 2). In the PVN, there was a significant main effect of age [$F(1, 42) = 28.28$; $P < 0.0001$], and a significant main effect of neural phenotype [$F(1, 42) = 24.31$; $P < 0.0001$] (**Figure 3.3**). In pups born on E19, we again found greater activation of AVP ($P < 0.0001$) and OXT ($P = 0.023$) at 3h post-delivery compared to E18.5. Additionally, AVP neurons showed increased c-Fos labeling compared to OXT+ neurons at E18.5 ($P < 0.04$) and 3h ($P < 0.0001$). We, however, did not find significant differences in activation of AVP or OXT neurons in the SON and SCN at 3h postpartum. Moreover, manipulating birth mode via C-section did not significantly alter the activation pattern of AVP and OXT neurons in the PVN, SON and SCN, although C-section-delivered pups had a tendency to show higher neural activation, particularly of OXT neurons in the PVN and SON.

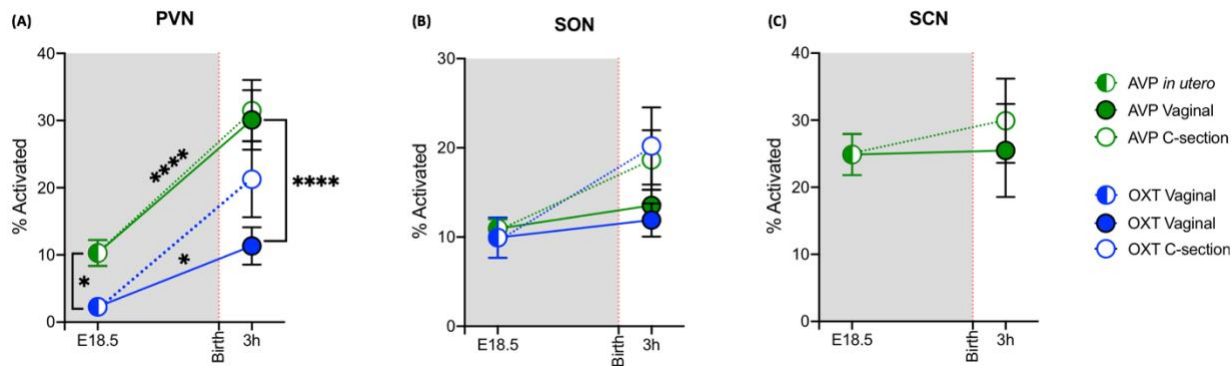


Figure 3.3 Birth mode does not affect the percent of vasopressin or oxytocin neurons in the PVN, SON or SCN that are *c-Fos*+

(A) Two-way ANOVA showed a significant main effect of age [$F(1, 42) = 28.28$; $P < 0.0001$], and a significant main effect of neural phenotype [$F(1, 42) = 24.31$; $P < 0.0001$]. In pups born on E19, activation of AVP and OXT was higher at 3h post-delivery ($P < 0.0001$ and $P = 0.023$, respectively). AVP neurons showed increased *c-Fos* labeling compared to OXT neurons at E18.5 (asterisk next to vertical bracket, $*P < 0.04$) and 3h (asterisks next to vertical bracket, $****P < 0.0001$). We did not find significant differences in activation of AVP or OXT neurons in the SON (B) and SCN (C) at any timepoint. C-section did not significantly alter the activation pattern of AVP and OXT neurons in the PVN (A), SON (B), or SCN (C). Gray shading indicates in utero timepoints. Red dotted line indicates the timing of birth. Data are mean \pm SEM. $n = 11-12$ animals per group.

In the PVN of pups delivered early, at E18.5, we found a significant main effect of age [$F(1, 43) = 84.25$; $P < 0.0001$], a significant main effect of neural phenotype [$F(1, 43) = 27.49$; $P < 0.0001$], and an age-by-neural phenotype interaction [$F(1, 43) = 9.123$; $P = 0.0042$]. Specifically, we found increased activation of both AVP and OXT neurons at 3h postpartum compared to E18.5 ($P < 0.0001$), but the percentage of AVP neurons showing *c-Fos* labeling at 3h was greater than OXT neurons ($P < 0.0001$). In the SON, we found a significant main effect of age [$F(1, 44) =$

33.63; $P < 0.0001$], with both AVP and OXT neurons showing increased c-Fos labeling at 3h post-delivery ($P = 0.0008$ and $P < 0.0001$, respectively). Similarly, AVP neurons in the SCN showed increased c-Fos labeling at 3h post-delivery ($P = 0.0003$) (**Figure 3.4**).

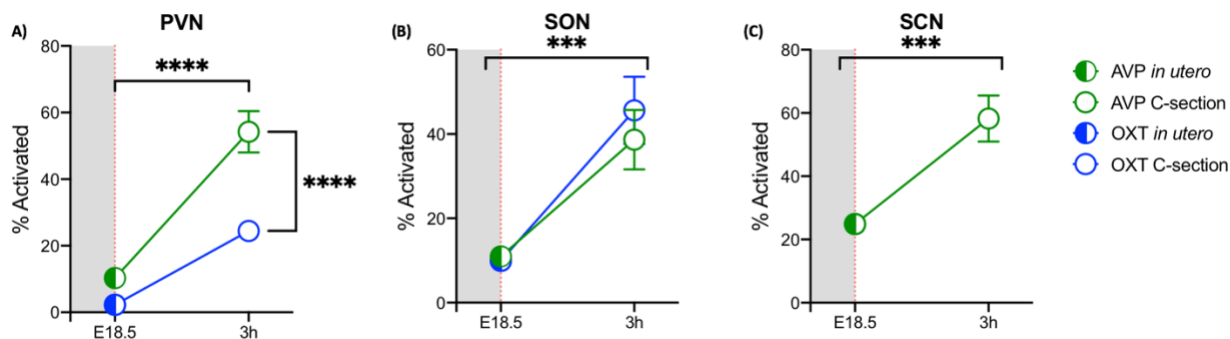


Figure 3.4 The percent AVP and OXT neurons showing c-Fos immunoreactivity increases 3h post-delivery in animals delivered one day prior to the expected day of birth in the PVN, SON and SCN.

(A) Two-way ANOVA showed a main effect of age [$F(1, 43) = 84.25$; $P < 0.0001$], a significant main effect of neural phenotype [$F(1, 43) = 27.49$; $P < 0.0001$], and an age-by-neural phenotype interaction [$F(1, 43) = 9.123$; $P = 0.0042$] in the PVN of pups delivered on E18.5. AVP and OXT neurons showed increased c-Fos labeling at 3h postpartum (asterisks over bracket, **** $P < 0.0001$), but the effect was greater in AVP neurons (asterisks next to vertical bracket, **** $P < 0.0001$). (B) In the SON, both AVP and OXT neurons showed increased c-Fos labeling at 3h post-delivery [$P = 0.0008$ and $P < 0.0001$, respectively; there was a significant main effect of age [$F(1, 44) = 33.63$; $P < 0.0001$]]. (C) AVP neurons in the SCN showed increased c-Fos labeling at 3h post-delivery ($P = 0.0003$). Gray shading indicates in utero timepoints. Red dotted line indicates the timing of birth. Data are mean \pm SEM. $n = 12$ animals per group.

We also determined the percentage of c-Fos-ir cells positive for each peptide analyzed in the PVN, SCN, and SON in pups delivered early, at E18.5 (**Figure 3.5**). For all brain areas of

interest, we used two-way ANOVA to evaluate whether c-Fos immunoreactivity varied significantly between neural phenotype groups over the two timepoints. In the PVN, we found a significant main effect of neural phenotype [$F(2, 66) = 31.7$; $P < 0.0001$], and an age-by-neural phenotype interaction [$F(2, 66) = 16.32$; $P < 0.0001$], reflecting increased c-Fos-ir cells that are OXT+ at 3h (15.3% at E18.5 vs 30.8% at 3h, $P = 0.001$), and a reduction in the percentage of unidentified cells at the same timepoint compared to E18.5 (57.7% at E18.5 vs 37.5% at 3h, $P < 0.001$); the percentage of c-Fos-ir cells that are AVP+ remained fairly constant across timepoints (27% at E18.5 vs 31.7% at 3h). In the SON, there was a significant main effect of neural phenotype [$F(2, 66) = 37.34$; $P < 0.0001$], and an age-by-neural phenotype interaction [$F(2, 66) = 5.23$; $P = 0.0078$], reflecting a greater percentage of c-Fos-ir cells that are AVP+ compared to OXT+ at E18.5 ($P = 0.0005$), and a greater percentage of c-Fos-ir cells that are AVP+ or OXT+ compared to unidentified cells at both timepoints ($P \leq 0.0002$). At E18.5 all c-Fos-ir cells at E18.5 were either AVP+ (66%) or OXT+ (34%). At 3h, the percentage of c-Fos-ir cells that are AVP+ decreased significantly compared to E18.5 (66% at E18.5 vs 43.7% at 3h, $P = 0.0122$); in contrast, the percentage of c-Fos-ir cells that are OXT+ and unidentified increased slightly but not significantly at 3h compared to E18.5 (34% at E18.5 vs 49.7% for OXT, 0% at E18.5 to 6.6% at 3h for unidentified cells). In the SCN, however, we did not find significant effects of age and/or neural phenotype in c-Fos labeling; the proportion of c-Fos-ir cells that are AVP+ or unidentified did not change or were significantly different than each other across timepoints.

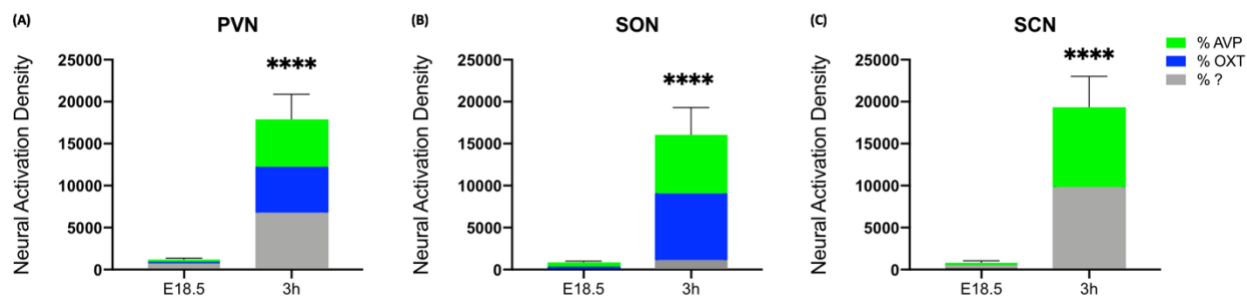


Figure 3.5 *The percent activation (c-Fos+) of AVP and OXT neurons increases 3h post-delivery in animals delivered one day prior to the expected day of birth.*

*Asterisks over bars represent the acute effect of birth on the density of c-Fos-ir cells identified previously (Figure 3.2): c-Fos immunoreactivity was higher at 3h post-delivery compared to immediately after delivery on E18.5 in the PVN (A), SON (B), and SCN (C) (****P < 0.0001). (A) PVN: There was a significant main effect of neural phenotype [$F(2, 66) = 31.7$; $P < 0.0001$], and an age-by-neural phenotype interaction [$F(2, 66) = 16.32$; $P < 0.0001$], reflecting increased c-Fos-ir cells that are OXT+ at 3h (15.3% at E18.5 vs 30.8% at 3h, $P = 0.001$), and a reduction in the percentage of unidentified cells at the same timepoint compared to E18.5 (57.7% at E18.5 vs 37.5% at 3h, $P < 0.001$); the percentage of c-Fos-ir cells that are AVP+ did not change significantly across timepoints (27% at E18.5 vs 31.7% at 3h). (B) SON: there was a significant main effect of neural phenotype [$F(2, 66) = 37.34$; $P < 0.0001$], and an age-by-neural phenotype interaction [$F(2, 66) = 5.23$; $P = 0.0078$], reflecting a greater percentage of c-Fos-ir cells that are AVP+ compared to OXT+ at E18.5 ($P = 0.0005$), and a greater percentage of c-Fos-ir cells that are AVP+ or OXT+ compared to unidentified cells at both timepoints ($P \leq 0.0002$). At E18.5 all c-Fos-ir cells at E18.5 were either AVP+ (66%) or OXT+ (34%). At 3h, the percentage of c-Fos-ir cells that are AVP+ decreased significantly compared to E18.5 (66% at E18.5 vs 43.7% at 3h, $P = 0.0122$); in contrast, the percentage of c-Fos-ir cells that are OXT+ and unidentified increased slightly but not significantly at 3h compared to E18.5 (34% at E18.5 vs 49.7% for OXT, 0% at E18.5 to 6.6% at 3h for unidentified cells) (C) SCN: we did not find significant effects of age and/or neural phenotype in c-Fos labeling; the proportion of c-Fos-ir cells that are AVP+ (39.57% at E18.5 vs 49.5% at 3h) or unidentified (60.43% at E18.5 vs 50.5% at 3h) did not change*

or were significantly different than each other across timepoints. Data are mean \pm SEM. $n = 12$ animals per group.

3.5 Discussion

We previously generated a profile of the neural activation pattern in the perinatal mouse brain using c-Fos immunoreactivity. We showed that discrete hypothalamic areas become significantly activated at 3h postpartum, and that many of the c-Fos-ir cells are AVP neurons in the PVN, SON, and SCN, or OXT neurons in the SON. Here we sought to determine the aspect(s) of birth that trigger the perinatal neural activation. We dissociated diverse stimuli by contrasting neural activation after vaginal vs C-section and timing of birth. We found that neural activation was always higher following birth (vaginal or C-section) compared to c-Fos immunoreactivity *in utero*, and the activation pattern did not differ between vaginally- and C-section-delivered pups. Moreover, the effect of birth on neural activation was replicated when animals were delivered one day prior to the expected day of birth. Together, these results rule out the requirement for a vaginal delivery and also demonstrate that the increased neural activation is not due to an intrinsic developmental program. Instead, neural activation occurs as a result of the exposure to the extra uterine environment.

C-sections provide a powerful experimental model for dissociating specific aspects of birth, which could not be otherwise possible by studying vaginally-delivered animals. For example, C-sections differ from vaginal deliveries in that offspring do not experience uterine contractions, passage through the birth canal, and labor (depending on the timing of the C-section). While previous studies have taken advantage of these inherent differences between vaginal and C-section deliveries to determine the role of birth in specific perinatal processes (Ringstedt, Tang et al. 1995, Tang, Ringstedt et al. 2000, Toda, Homma et al. 2013, Castillo-Ruiz, Mosley et al. 2018,

Castillo-Ruiz, Mosley et al. 2018), very few have taken into account variations in the timing of birth that may result from manipulating birth mode. For example, a recent study reported increased neural activation in the piriform cortex, caudate putamen, part of the cerebellum, and facial nucleus following a vaginal but not a C-section delivery (Ikeda, Onimaru et al. 2019). However, the groups were not really comparable given that the brains of vaginally- and C-section-delivered pups were collected at different times after birth (C-section-delivered animals were collected immediately at delivery, whereas the time between birth and brain collection of vaginally-delivered animals was not controlled for). Therefore, the reported effect of birth mode on c-Fos expression may correspond to differences in the number of hours ex utero in the two groups (Ikeda, Onimaru et al. 2019). Other studies have compared pups born vaginally to pups delivered by C-section before to the expected day of birth (done, presumably to ensure that none of the C-section group would give birth vaginally) (Ringstedt, Tang et al. 1995, Ikeda, Onimaru et al. 2019). To avoid this confound, we yoked C-section deliveries to vaginal deliveries to precisely match the total gestation length and circadian time of delivery, and found that c-Fos immunoreactivity increased similarly in vaginally- and C-section-delivered mice 3h after birth compared to *in utero* levels.

Interestingly, vaginal delivery triggers a massive release of peripheral AVP in human neonates (Chard, Hudson et al. 1971, Polin, Husain et al. 1977, Hoppenstein 1980, Leung, McArthur et al. 1980, Rees, Forsling et al. 1980). The increase in peripheral AVP is much smaller in C-section-delivered subjects (Parboosingh, Lederis et al. 1982, Evers and Wellmann 2016), suggesting that some aspect of a vaginal birth is necessary to trigger the release of AVP at birth. Only one study has examined this in rodents, as far as we are aware, and found higher AVP (measured via copeptin, a surrogate marker for AVP) in rat neonates sampled within 2 min of birth compared to C-sectioned-delivered pups sampled immediately after delivery on the expected day

of birth, or pups sampled 5h after a vaginal birth (Summanen, Back et al. 2018). We therefore predicted increased activation of AVP neurons in the hypothalamus of vaginally-delivered pups compared to C-section-delivered pups. However, we found similar activation patterns across groups. Importantly, as mentioned previously (see Chapter 2), c-Fos is not a perfect surrogate of neural activity, as only depolarizations, increases in firing rate, or synaptic activity that result in substantial neural influx of Ca^{++} , lead to induction of *c-fos* (Morgan and Curran 1986, Morgan and Curran 1988, Vendrell, Pujol et al. 1992, Zhu and Herbert 1997). In this regards, neural activation (or lack thereof) does not necessarily correspond to AVP secretion into systemic circulation or central release.

Interestingly, peripheral neonatal AVP levels correlate closely with AVP levels in human infant cerebrospinal fluid (Bartrons, Figueras et al. 1993, Carson, Howerton et al. 2014), suggesting simultaneous release of AVP centrally and peripherally. Centrally-released AVP (or OXT) can diffuse throughout the extracellular space and act on distant targets or within the site of origin (Ludwig and Leng 2006) by acting on AVP (or OXT) receptors on their cell surfaces to modulate their own release without necessarily triggering action potentials (Gouzenes, Desarmenien et al. 1998, Ludwig, Sabatier et al. 2002). Therefore, it is possible for vaginally- and C-section-delivered pups to show differences in AVP release without exhibiting differences in c-Fos immunoreactivity. Alternatively, equivalent expression of c-Fos in vaginally- and C-section-delivered pups possibly reflects a ceiling effect; activation of AVP neurons as a result of the stress of birth/transition to the extra uterine environment could be masking other effects of birth on the activation of AVP neurons. Only direct measurements of AVP in plasma and/or the brain will provide a better understanding of AVP concentrations in the perinatal period (see Chapter 4).

We ruled out the possibility of a developmentally-programmed induction of the *c-fos* gene and protein product at 19 days post-conception, because pups delivered one day prior to the expected day of birth (E18) also showed increased neural activation within 3h, suggesting that neural activation at birth occurs as a result of the exposure to the extrauterine environment. We also reason that hypoxia is unlikely to be the stimulus leading to c-Fos induction shortly after birth. Intensification of uterine contractions is associated with hypoxia in the uterus (Alotaibi, Arrowsmith et al. 2015) and while we did not measure fetal oxygen levels, low oxygen levels have been extensively reported at birth in several species, including rodents (Schmidt et al. 1985; Pimentel, Poore, and Nathanielsz 1989). Two studies evaluating changes in *c-fos* mRNA expression in the context of birth hypoxia show that *c-fos* mRNA is induced by prolonged hypoxia rather than intermittent short periods as occurs during a normal vaginal delivery (Alotaibi, Arrowsmith, and Wray 2015). Ringstedt et al. 1995 found that exposing newborn rats to hypoxic conditions (9% oxygen in nitrogen) for 1h immediately after a C-section delivery does not increase *c-fos* mRNA expression further than normoxic conditions in the pons, midbrain and neocortex. In contrast, Tang et al. 2000 did find increased in *c-fos* mRNA expression after exposing C-section-delivered mice to hypoxic conditions (water bath) for 15-16min, but not for 5min or 21 min, in the nucleus tractus solitarius, hippocampus, neocortex, striatum, and ventromedial hypothalamus. While blocking catecholamines, hormones important for lung liquid reabsorption (Faxelius et al. 1983; Walters and Olver 1978) did not reduce *c-fos* mRNA expression in animals exposed to hypoxic conditions, it did partially reduce *c-fos* mRNA expression in animals exposed to normoxic conditions, suggesting that birth-induced, but not asphyxia-induced *c-fos* mRNA expression may be mediated by catecholamine action (Tang et al. 2000).

In summary, we dissociated several aspects of birth including labor and delivery, and timing of birth to determine the what triggers neural activation in the neonatal brain. We found that vaginal and C-section delivered animals showed similar patterns of neural activation regardless of the timing of birth. Our results suggest that neural activation at birth measured via c-Fos immunoreactivity is triggered by fetal exposure to the extrauterine environment.

4 CHAPTER FOUR: VASOPRESSIN RELEASE AT BIRTH MAY BE NEUROPROTECTIVE IN THE NEWBORN BRAIN

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4.1 Abstract

We recently found that birth plays an important role in the patterning of naturally occurring neuronal cell death in the newborn mouse brain. In addition, manipulations to birth delivery mode (via C-section) alter cell death shortly after birth in many brain areas. The paraventricular nucleus of the hypothalamus (PVN) showed the largest effect of birth mode on cell death, with an abrupt ~3-fold reduction in cell death in vaginally-delivered mice, but not in those delivered by C-section. This was associated with a higher number of vasopressin immunoreactive cells at weaning in the vaginally-delivered animals. These results suggest that some aspect of a vaginal delivery might be neuroprotective. In Chapter 2, we also found that birth triggers activation of vasopressin (AVP) neurons within the hypothalamus, including in the PVN. Interestingly, in humans, a vaginal birth is accompanied by a large surge of vasopressin (AVP) that is never recapitulated later in life, and the surge is blunted in babies delivered via C-section. Here, we measured copeptin (a surrogate marker of AVP) in the plasma of newborn mice delivered vaginally or by C-section to determine whether vaginal birth is also associated with peripheral release of AVP in the mouse. We found that copeptin is markedly elevated perinatally compared to levels in adults, with highest values observed prenatally at E17.5 and E18.5. In addition, copeptin levels 3 hours after birth are significantly lower in pups delivered via C-section versus those delivered vaginally. Given that AVP plays an essential role in osmotic balance, we also measured plasma osmolality, and found an acute decrease in osmolality in the hours after a vaginal birth that is absent in C-section

delivered animals. We also asked whether AVP plays a role in the control of neuronal cell death by pharmacologically manipulating AVP, and found that intracerebroventricular (ICV) administration of AVP immediately after a Cesarean delivery significantly reduced cell death 3h later in the PVN and AHA. Preliminary data suggest that exogenous AVP may be acting on OXT receptors to reduce cell death in the PVN. These results provide evidence supporting the hypothesis that birth plays an important role in the developing brain.

4.2 Introduction

Developmental neuronal cell death is an essential feature of brain development. Approximately 50% of the neurons initially produced are eliminated by apoptosis, resulting in a profound pruning and restructuring of neural circuits in the brain (Clarke, Posada et al. 1998). Although potentially counterintuitive, cell death is beneficial to the developing nervous system, and defects in the cell death pathway can be lethal to the embryo (Kuida, Zheng et al. 1996). Despite the importance of cell death in neural development, it is unclear what signals guide the patterning of cell death in the developing nervous system and what triggers the initiation or termination of the cell death period.

To begin answering these questions, we previously described the pattern of cell death in the mouse brain at different developmental time points (Ahern, Krug et al. 2013, Mosley, Shah et al. 2017). Using immunohistochemical (IHC) detection of activated caspase 3 (AC3) as a marker of dying cells, we showed that cell death increases in most brain areas immediately after birth, but decreases in others, such as the PVN (Ahern, Krug et al. 2013, Mosley, Shah et al. 2017). We also manipulated the mode of birth and found significant differences in cell death between pups delivered vaginally or by C-section (Castillo-Ruiz, Mosley et al. 2018). A C-section birth differs from a vaginal birth in major ways (including hormonal surges that accompany labor and delivery,

mechanical forces associated with uterine contractions and passage through the birth canal, microbiota profile exposure and, often, gestation length), and we took advantage of these inherent differences to determine what aspects of birth play a role in the patterning of cell death. Contrary to C-section-delivered pups, vaginally-delivered pups showed a transient but significant decrease in cell death in most brain areas (Castillo-Ruiz, Mosley et al. 2018). The PVN showed the largest effect of birth mode on cell death, with a transient ~3-fold reduction in cell death in vaginally-delivered mice, suggesting that a specific aspect of a vaginal delivery might be neuroprotective. Furthermore, effects of birth mode on cell death may have long-lasting effects, as C-section-delivered pups showed a 20% reduction in the number of vasopressin immunoreactive cells in the PVN at weaning (Castillo-Ruiz, Mosley et al. 2018) and in adulthood (Castillo-Ruiz, Hite and Forger, unpublished) when compared to vaginally delivered pups.

Using c-Fos immunohistochemistry, we also found strong neural activation 3 hours after a vaginal birth in the PVN, with many of the activated cells being AVP-producing neurons (see Chapter 2). AVP neurons are one of the most prominent populations in the PVN, but whether they play an important role at birth has not been tested. High levels of peripheral AVP (or its surrogate marker, copeptin) have been widely reported in the human literature on the day of birth. Copeptin is derived from the same precursor molecule as AVP and is released into systemic circulation in equimolar amounts; it is used as a surrogate marker of AVP because it is a larger and more stable peptide (Morgenthaler, Struck et al. 2006, Morgenthaler, Muller et al. 2007, Balanescu, Kopp et al. 2011). Vaginally-delivered human neonates show extremely high levels of copeptin/AVP that are never recapitulated in life, including pathological levels (Polin, Husain et al. 1977, Rees, Forsling et al. 1980, Acher and Chauvet 1995). C-section-delivered human neonates also show elevated copeptin/AVP levels at birth, but the increase is muted compared to vaginally-delivered

newborns, with some studies reporting a 100-fold difference between vaginally- and C-section-delivered babies (Parboosingh, Lederis et al. 1982, Evers and Wellmann 2016). Interestingly, peripheral levels of AVP strongly predict levels of vasopressin in the CSF of human newborns (Bartrons, Figueras et al. 1993, Carson, Howerton et al. 2014), suggesting that AVP is also released centrally at birth.

Although the human data have been interpreted as a surge of vasopressin at birth, prenatal levels have not been examined in humans. Classic studies using chronic catheterization of lamb fetuses allowed investigators to repeatedly sample plasma AVP levels during the last stage of pregnancy and postpartum in the same individual. One study found marked increases in fetal AVP during the last few days of gestation (Alexander, Bashore et al. 1974), compared to undetectable or very low levels at earlier stages. A second group also reported that AVP increased prior to birth in fetal lambs, but only after the onset of spontaneous uterine contractions (Stark, Daniel et al. 1979, Stark, Daniel et al. 1981). AVP present in lamb circulation is of fetal origin because AVP levels are much lower in the pregnant ewes, and there is no correlation between the levels in ewes and their lambs (Stark, Daniel et al. 1981).

The only study we are aware of in rodents recently showed that copeptin levels are elevated in vaginally-delivered rat pups immediately after birth (Summanen, Back et al. 2018), and that levels drop rapidly after birth (within 5h). Taken together, these findings suggest an evolutionarily conserved endocrine response to birth across species, although the exact timing of the response is not clear. One of the goals of the current study was, therefore, to examine perinatal AVP release in mice by measuring copeptin in late prenatal fetuses and newborns born vaginally or by C-section.

Some studies link the surge of peripheral AVP at birth with birth-related hypoxia (Stark, Daniel et al. 1981, Schlapbach, Frey et al. 2011, Summanen, Seikku et al. 2017). *In vitro* studies suggest that AVP could protect neurons during hypoxic conditions (such as that experienced during a vaginal birth) by, for example, reducing neural activity in the neonatal hippocampus via its V1a receptor (V1aR) (Spoljaric, Seja et al. 2017). However, the direct effect of AVP on cell death has not been tested *in vivo* or *in vitro* during the perinatal period.

To test the hypothesis that the surge of AVP at birth plays a neuroprotective role in the newborn brain, we centrally administered AVP immediately after birth to C-section delivered pups and analyzed the pattern of cell death at 3 hours later (when we previously saw the effect of birth mode on cell death (Castillo-Ruiz, Mosley et al. 2018)). We found that pharmacological administration of AVP at birth reduces cell death in the newborn PVN and AHA, but not the SON and LHb. Moreover, our preliminary data show that the neuroprotective role of AVP at birth could be mediated by OXT receptors.

4.3 Methods

4.3.1 Animals

Adult C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, Maine) or obtained from our colony. Mice were kept in a 12h light : 12h dark cycle, and given access to water and food ad libitum. All procedures were approved by Georgia State University's Institutional Animal Care and Use Committee and performed in accordance with the National Institutes of Health's *Guide for the Care and Use of Laboratory Animals*.

4.3.2 Timed pregnancies and delivery mode

We established timed-pregnancies by pairing males and females within 2h of lights off. Males were removed from the cages the next morning 1-2h after lights on and the day was marked as E0. Overall health and body weight of expectant females was monitored during pregnancy.

Pups were collected at different developmental ages (E17.5, E18.5, E19, and postnatally 1h, 3h or 1 day after a vaginal or Cesarean delivery), depending on the experimental procedure. For C-sections, dams were euthanized using 2% CO₂ followed by rapid decapitation. An aseptic abdominal incision was made to expose the uterine horns, and the fetuses were removed one by one from their gestational sacs and brains were either collected immediately (for E18.5 and E19 time points) or 3h after delivery. For the 3h group, pups were kept on a heating pad at ± 32 °C until collection.

4.3.3 Plasma osmolality measurements

Blood samples of male and female newborns were collected at E18.5 or E19, and postnatally at 1h, 3h or P1 following a vaginal delivery or at 3h following a C-section delivery. Pups were rapidly decapitated and trunk blood was immediately collected in heparin-coated capillary tubes (Fisher), transferred into clean microcentrifuge tubes, centrifuged at 1300g at 4°C for 5 min, and plasma then collected and stored at -80°C until analysis. Plasma samples were diluted 2-fold in nanopure water and osmolality was measured using an osmometer (The Advanced Micro Osmometer; Model 3300; Advanced Instruments; Norwood, MA).

4.3.4 Mouse copeptin enzyme-linked immunosorbent assay (ELISA)

Copeptin concentrations in neonatal mouse plasma were measured using a commercially-available sandwich ELISA kit (Biomatik, Ontario, Canada). Plasma samples were collected as per

manufacturer's instructions. Briefly, pups were rapidly decapitated and trunk blood was immediately collected in EDTA-coated capillary tubes (Fisher) containing protease inhibitors (1X Complete-mini, Roche, Darmstadt, Germany). Blood samples were then transferred into clean microcentrifuge tubes, spun at 1000g at 4°C for 15 min, plasma collected and stored at -80°C until analysis. Groups included male and female fetuses collected at E17.4, E18.5 or E19, and pups collected postnatally immediately after birth (0h), at 3h and P1 following a vaginal delivery, or at 0h and 3h following a C-section delivery (C-sections were yoked to vaginal deliveries to match the timing of birth and gestational length). To confirm that the copeptin in offspring blood was indeed from the pups and not the dam, we also collected plasma from eight pregnant dams (two at E17.5, two at E18.5 and four at 0h postnatal (two vaginal and two C-section), as well as three non-pregnant adults (two female) as a control. Adults were euthanized using 2% CO₂ followed by rapid decapitation and the plasma was collected as described above. Because very low plasma volumes could be obtained from perinatal mouse pups, samples from 2-3 pups of the same sex from different litters were pooled to form single samples for analysis, and two male and two female samples were included for each timepoint analyzed. All plasma samples were diluted 4-fold in PBS and assayed in duplicate. First, 100µL of standard or sample was added to each well of the plate. After 2h incubation at 37°C, the liquid was replaced by 100µL of biotinylated mouse anti-copeptin and the plate was incubated for 1h at 37°C. After thorough washes, 100µL of HRP-avidin complex were added to each well and incubated for 1h at 37°C. Finally, after thorough washes, 90µL of the 3,3',5,5'-tetramethylbenzidine chromogenic substrate was added to each well for 30min of at 37°C, and the reaction was stopped by adding 50µL of the stop solution. The plate was read using a multimode plate reader (iMark Microplate Reader; Bio-Rad, Hercules, CA).

4.3.5 Tissue processing

Collected brains were fixed in 5% acrolein for 24h and then transferred into 30% sucrose solution. Brains were coronally frozen-sectioned into two 40 μ m series using a rotary microtome. Sections were processed for immunohistochemical (IHC) detection of AC3 as reported previously (Castillo-Ruiz, Mosley et al. 2018).

4.3.6 Intracerebroventricular injections

Pups delivered by C-section were previously shown to have higher cell death 3h after birth than those delivered vaginally (Castillo-Ruiz et al., 2018). To see if we could reduce cell death in C-section delivered pups, we and injected 250 ng AVP (Sigma V9879, in 1 μ L artificial cerebrospinal fluid total, Tocris Bioscience), or the vehicle alone, ICV into each hemisphere of cryoanesthetized pups within 30min of C-section delivery. In a separate experiment, to determine the receptor(s) that AVP acts through to influence cell death, C-section-delivered pups were cryoanesthetized and received one of five ICV treatments within 30 minutes of birth: 1) vehicle alone, 2) 250 ng AVP alone, 3) a mixture of 250 ng AVP and 1 μ g of an AVP V1a receptor antagonist [β -Mercapto- β , β -cyclopentamethylenepropionyl₁, O-me-Tyr₂, Arg₈] (Sigma V2255), 4) a mixture of 250ng AVP and 2.5ng of an OXT receptor antagonist β -Mercapto- β , β -cyclopentamethylene-propionyl-Tyr(Me)-Ile-Thr-Asn-Cys-Pro-Orn-OH trifluoroacetate (Bachem 4031339), or 5) a cocktail of AVP, the V1aR antagonist and the OXT receptor agonist (all in 1 μ L artificial cerebrospinal fluid total). As described previously (Mosley, Weathington et al. 2017), a 30 gauge needle attached to a 5 μ l Hamilton syringe was lowered 2 mm below the skull, at approximately 1 mm rostral to lambda and 1 mm lateral to the sagittal suture and one of the five treatments was injected at a rate of 33 nL/sec using a Micro4 micro syringe pump into the brain of each pup (World Precision Instruments, Sarasota, FL).

4.3.7 Statistical analyses

One-way ANOVAs were used to evaluate age differences in levels of plasma copeptin, and effect of pharmacological treatments on developmental cell death. Two-way ANOVAs were used to evaluate effects of age and birth mode on plasma copeptin levels, and effects of age and plasma source (pups or Dams) in levels of plasma copeptin. When applicable, ANOVAs were followed by Fisher's least significant difference post hoc tests. Mann-Whitney test was used to evaluate effects of birth mode on plasma osmolality. Student t-tests were used to test effects of AVP treatment on cell death in each brain area of interest. Kruskal Wallis followed by Dunn's post hoc test was used to evaluate age differences in plasma osmolality and the effect of pharmacological treatments on cell death.

4.4 Results

4.4.1 Copeptin levels are elevated in the perinatal mouse plasma

To determine whether birth is associated with a peripheral release of AVP in the mouse, we measured levels of copeptin at five perinatal timepoints (E17.5, E18.5, 0 h after birth, 3 h after birth and P1). Since lower AVP plasma levels have been reported in C-section-delivered human neonates compared to those delivered vaginally (Parboosingh, Lederis et al. 1982, Evers and Wellmann 2016), we also evaluated the effect of birth mode on AVP release in the neonatal mouse. We first assessed the acute effects of birth on copeptin levels. We found greatly elevated levels of plasma copeptin perinatally compared to non-pregnant adults in both groups (**Figure 4.1**). Specifically, plasma copeptin was below the detection level of the assay (3.12 pg/mL) in all three non-pregnant adults and was 50- to 100-fold higher in perinatal animals. Peak mean levels were found on E18.5, about half a day prior to expected delivery, although there was no significant difference in copeptin level between E17.5 and E18.5. Copeptin levels dropped rapidly at 0h after

a vaginal or C-section birth compared to E18.5 ($P < 0.0001$ and $P < 0.006$, respectively), and decreased further between 0h and 3h in both groups, but only significantly in C-sectioned pups ($P = 0.0002$). While copeptin levels dropped postnatally, they were still significantly higher at 0h in the vaginal and C-section groups ($P \leq 0.0002$), and at 3h in the vaginal group ($P < 0.009$) compared to non-pregnant adults (all of which had undetectable levels, $n = 3$). There was also a trend for higher levels at P1 than in non-pregnant adults in the vaginal group ($P = 0.05$).

In a second analysis, we used two-way ANOVA to determine whether copeptin levels varied significantly between the vaginal and C-section group at 0h and 3h. There was a significant main effect of age [$F(1, 19) = 30.83$; $P = 0.0002$], and an age-by-birth mode interaction [$F(1, 19) = 7.372$; $P = 0.023$], reflecting the fact that copeptin levels that were initially similar between groups at 0h, but decreased further in the C-section group at 3h compared to the vaginal group at the same timepoint ($P < 0.05$) (**Figure 4.1**).

Higher copeptin levels have been repeatedly reported in human subjects in blood collected from the umbilical artery compared to the umbilical vein (Wellmann, Benzing et al. 2010), suggesting neonatal and not maternal origin. To test that here, we collected blood samples from the eight dams whose offspring were assayed above. We found a significant main effect of plasma source (pups or Dams) [$F(1, 16) = 133.3$, $P < 0.0001$], and an age-by-plasma source interaction [$F(3, 16) = 4.807$, $P < 0.015$]. All pre-parturient pregnant dams (E17.5 and E18.5, $n = 4$) as well as dams that delivered by C-section at full term on E19 ($n = 2$) had undetectable levels of copeptin. Copeptin was detectable, however, in the two dams that delivered pups vaginally, although even these dams had levels that were significantly lower than that of their offspring at the same timepoint (0h, $P < 0.008$) (**Figure 4.1**).

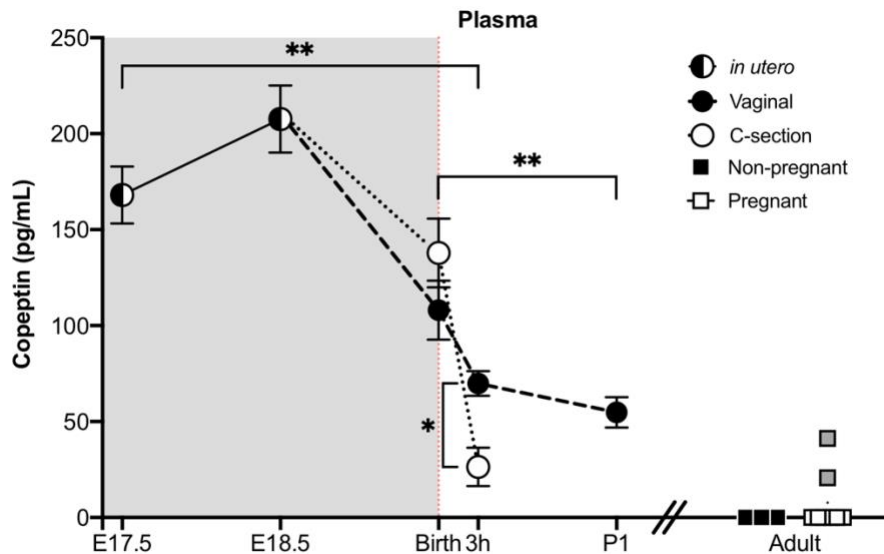


Figure 4.1 Copeptin levels in plasma are high in the perinatal period, and C-section-delivered mice have lower copeptin levels than vaginally-delivered mice at 3h post-delivery. Plasma copeptin levels were highest prenatally at E17.5 and E18.5, and dropped abruptly after a vaginal (black circles) or C-section (open circles) delivery (asterisks over lower horizontal bracket, $**P < 0.006$). Two-way ANOVA showed a significant main effect of age [$F(1, 19) = 30.83$; $P = 0.0002$], and an age-by-birth mode interaction [$F(1, 19) = 7.372$; $P = 0.023$], reflecting the fact that copeptin levels were initially similar between the vaginal and C-section group at 0h, but decreased further in the C-section group at 3h compared to the vaginal group at the same timepoint (asterisk next to vertical bracket, $*P < 0.05$). Despite the postnatal drop, copeptin levels were still significantly higher at 0h and 3h (except in the C-section group at 3h) than non-pregnant adults (asterisks over top bracket, $**P < 0.009$); there was also a trend for higher levels at P1 than in non-pregnant adults ($P = 0.05$). All non-pregnant adults (black squares at the right of the figure, $n = 3$) and all pre-parturient pregnant dams (open squares, E17.5 and E18.5, $n = 6$) had undetectable levels of copeptin. The two dams that delivered pups vaginally had

detectable levels (gray squares) that were significantly lower than that of their offspring at the same timepoint (0h, $p < 0.008$). Data are Mean \pm SEM. Perinatal copeptin, $n = 2-5$ per group.

4.4.2 Decreased plasma osmolality after a vaginal delivery

AVP is also known as antidiuretic hormone because it plays a key role in maintaining the concentration of dissolved particles and water volume in the blood. Therefore, the release of peripheral AVP at birth could increase water reabsorption from the kidneys and cause osmolality changes (Boone and Deen 2008). To test whether birth is associated with osmolality changes, we measured plasma osmolality prenatally at E18.5 and E19, and 1h, 3h and P1 after a vaginal delivery. We found that birth is associated with an acute and transient decrease in plasma osmolality after a vaginal delivery (E18.5 vs. 1h, $P < 0.002$; E18.5/E19 vs 3h, $P < 0.0001$; 3h vs P1, 0.0003) (**Figure 4.2**). However, the decrease in plasma osmolality is not observed in C-section-delivered pups, which had significantly higher plasma osmolality at 3 h postpartum ($P = 0.0015$).

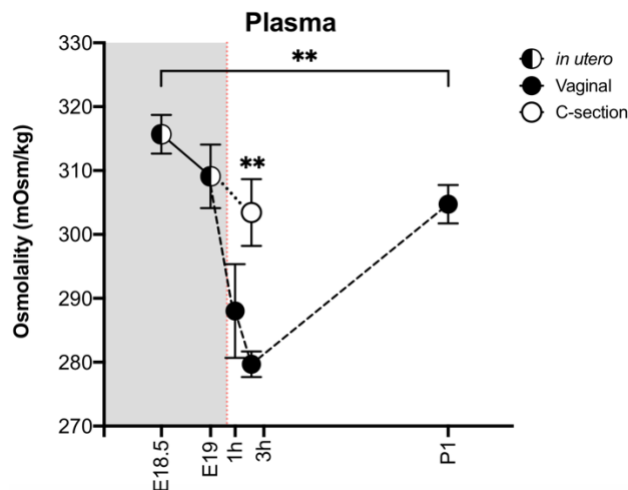


Figure 4.2 Plasma osmolality is acutely decreased after a vaginal delivery.

Relative to prenatal levels or levels at P1, plasma osmolality was decreased at 1h and 3h after vaginal birth (asterisks over bracket, $**P < 0.002$). A similar decrease was not observed in C-

section-delivered pups, which had significantly higher plasma osmolality at 3 h postpartum than did vaginally-delivered pups (** $P = 0.0015$). Data are Mean \pm SEM. $n = 6$ –11 per group.

4.4.3 Vasopressin treatment at birth reduces cell death in the PVN and AHA of Cesarean-delivered pups

One study showed that vasopressin promotes cell survival *in vitro* (Chen and Aguilera 2010). However, an *in vivo* protective role of vasopressin in the newborn brain has never, to our knowledge, been examined. To test this, we injected AVP or vehicle ICV to C-section-delivered mice immediately after birth, and measured effects on cell death at 3h postpartum [when we previously observed the effect of birth mode on cell death (Castillo-Ruiz, Mosley et al. 2018), and saw differences in copeptin levels between vaginally- and C-section-delivered pups, above]. We examined three brain areas (the PVN, lateral habenula (LHb) and the AHA), where we previously saw decreased cell death 3h after a vaginal birth and an effect of birth mode on cell death (Castillo-Ruiz, Mosley, Jacobs, et al. 2018); we also examined the SON, given that the SON houses AVP neurons. We found that ICV injections of AVP reduced cell death in the PVN and the AHA, but not the SON or the LHb (Figure 4.3).

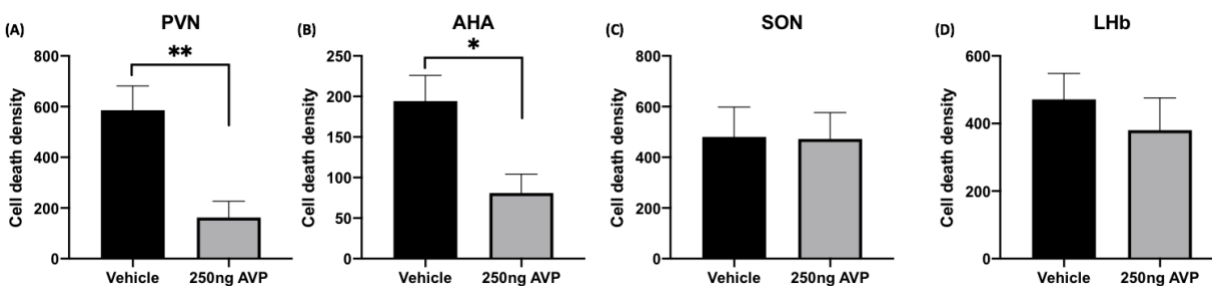


Figure 4.3 AVP reduces cell death in the PVN and AHA of C-section-delivered pups.

*ICV injections of 250ng of AVP at birth reduced cell death in the PVN [(A) **P = 0.008] and AHA [(B) *P = 0.02], but not in the SON (C) or LHb (D) of C-section-delivered pups. Data are Mean ± SEM. Vehicle group, n = 14, AVP group, n = 7.*

4.4.4 The role of V1aRs and OTRs on the neuroprotective effect of vasopressin at birth

To further investigate the neuroprotective mechanism of AVP in the newborn brain, we used antagonists to AVP receptors in a pilot study. AVP acts in the brain via V1aR or V1b receptors, but many of its roles in social behavior and analgesia are mediated via V1aRs (Veinante and Freund-Mercier 1995, Dumais and Veenema 2016, Song and Albers 2018). Moreover, AVP can bind to OXT receptors due to its remarkable similarity with OXT (Acher and Chauvet 1995, Gimpl and Fahrenholz 2001). Therefore, we antagonized AVP V1aRs [using the Manning Compound – (MC)] and OXT receptors to determine whether AVP provides neuroprotection via V1aRs, OTRs, or both. We injected vehicle or AVP alone, or a mixture of AVP with V1aR or OTR antagonists or a cocktail of both V1aR and OTR antagonists into C-section-delivered newborn pups immediately after birth. Our preliminary data again suggest that AVP treatment reduces cell death 3h after C-section delivery (Figure 4.4). In addition, AVP may act via OTRs to provide neuroprotection at birth, given that pups treated with AVP+OTR antagonist did not show reduced cell death (**Figure 4.4**). However, pups treated with the V1aR antagonist, MC, showed no cell death, suggesting that MC may be acting as an agonist rather than an antagonist in the newborn brain; reduced cell death in animals treated with AVP+cocktail (p = 0.05) further supports the idea that MC may be acting as an AVP agonist than an antagonist (**Figure 4.4**). However, since this analysis included only two animals per group and a single dose of each antagonist, clearly, more animals and doses are needed before firm conclusions can be drawn.

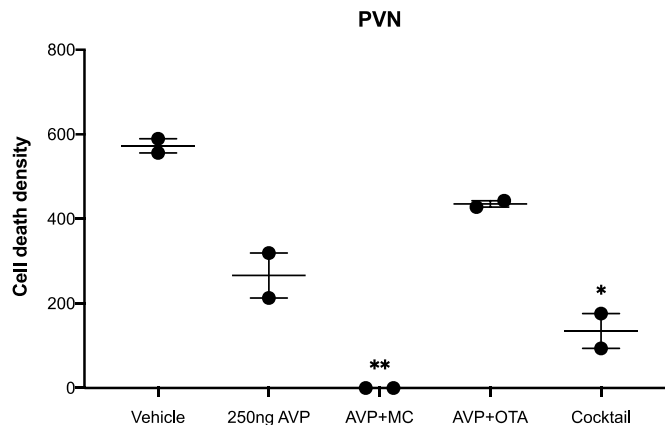


Figure 4.4 AVP may act via OTRs to reduce cell death in the newborn brain.

Although not statistically significant, C-section-delivered pups that received an ICV of 250ng of AVP at birth showed reduced cell death in the PVN compared to the vehicle treated group. Cell death density was completely abolished after ICV injections of AVP with the V1aR antagonist, Manning Compound (AVP+MC; $**P = 0.008$ compared to vehicle group). Cell death density in the pups injected with AVP and an OXT receptor antagonist (AVP+OTA) was not different than the Vehicle group. The group that was treated with a combination of AVP with both MC and OTA (Cocktail) had a cell death density that was intermediate to the AVP+MC and AVP+OTA groups, and significantly lower than the vehicle group ($*P < 0.05$). Data are Mean \pm SEM. $n = 2$ in all groups.

4.5 Discussion

Birth is a dramatic event involving remarkable changes in the newborn's environment, including changes in hormonal signals, the function of peripheral organs, and neural activity (Lagercrantz and Slotkin 1986, Liggins 1994, Tyzio, Cossart et al. 2006, Lagercrantz 2016, Spoljaric, Seja et al. 2017). Recent studies have identified important roles of birth in triggering developmental events. For instance, birth plays a pivotal role in the initiation of barrel formation in the somatosensory cortex and regulates eye-specific segregation of projections from the retina

to the midbrain via a serotonin-dependent mechanism (Toda, Homma et al. 2013). Moreover, we recently reported a transient decrease in cell death 3h after a vaginal but not a C-section delivery, which was associated with a reduction of AVP cell number in cesarean delivered pups at weaning and in adulthood (Castillo-Ruiz, Mosley et al. 2018). However, what aspect(s) of a vaginal birth confer neuroprotection to the newborn brain is not known. Interestingly, there is a remarkable difference in the levels of peripheral vasopressin at birth between vaginally and C-section-delivered human newborns, with vaginally-delivered newborns showing higher levels (Parboosingh, Lederis et al. 1982, Evers and Wellmann 2016, Summanen, Back et al. 2018). AVP can protect neurons from nutrient deprivation (Chen and Aguilera 2010) and suppress neural network activity to potentially protect neurons from excitotoxicity *in vitro* (Spoljaric, Seja et al. 2017). Therefore, we hypothesized that there is a surge of AVP in mouse pups at birth that is larger in vaginally-delivered than in Cesarean-delivered pups, and that is neuroprotective.

To test this, we first determined whether copeptin (a surrogate marker of AVP) is indeed elevated in the neonatal mouse, which, to our knowledge, has never been tested. We found that perinatal levels of copeptin are greatly elevated compared to adults. Contrary to what is implied by the human literature, however, we saw the highest levels prenatally, at E17.5 and E18.5. These results are in agreement with previous studies reporting elevated levels of circulating AVP in the last few days of gestation in the fetal lamb (Alexander, Bashore et al. 1974, Sinding, Robinson et al. 1980). Our current data do not allow us to determine when in gestation levels begin to increase. It would be interesting to examine earlier time-points, although the volume of sample obtainable will be a problem, as even at E17.5, very small volumes of plasma were obtained (on average 13 μ l per fetus) and plasma from multiple animals had to be pooled to obtain a single sample.

Maternal plasma samples were also collected at the time of Cesarean or vaginal delivery, and copeptin levels of all but two dams that delivered pups vaginally were undetectable; levels in those two dams were still significantly lower than their pups at the same timepoint. High levels of copeptin in the offspring and significantly lower copeptin levels in the respective dams at the same timepoint provide evidence that the observed copeptin in the fetus/neonate are not of maternal origin, which also what has been concluded for humans (Wellmann, Benzing et al. 2010) and sheep (Stark, Daniel et al. 1981).

While we did not identify the factors that trigger the release of AVP perinatally, labor and the events that accompany it, such as hypoxia, seem to be good candidates given that inducing labor prematurely increases the levels of AVP in fetal lambs (Stark, Daniel et al. 1981). Experimentally-induced intermittent hypoxic episodes also cause an increase in copeptin levels in the P6 rat (Summanen, Back et al. 2018), and copeptin is higher at birth in human newborns suffering from birth asphyxia (reduced O₂ and increased CO₂ levels) (Summanen, Seikku et al. 2017) or exposed to uterine contractions induced by OXT treatment of the mother prior to C-section delivery (Wellmann, Koslowski et al. 2016, Summanen, Seikku et al. 2017, Summanen, Back et al. 2018).

However, while early stages of labor might be present at E18.5, labor itself is unlikely to explain the elevated copeptin levels we observed at E17.5. A possible explanation is that AVP is released at E17.5 in connection to HPA axis activation and/or in response to perinatal inflammation. Accumulating evidence suggest that birth is an inflammatory event, as expression of pro-inflammatory cytokines increase significantly near the end of gestation and immune cells infiltrate the myometrium and cervix (Thomson, Telfer et al. 1999, Osman, Young et al. 2003, Golightly, Jabbour et al. 2011, Kobayashi 2012, Shynlova, Nedd-Roderique et al. 2013). For

instance, expression of prostaglandin (PG) F₂alpha and the cyclooxygenase (COX) synthase enzyme, COX-1, are upregulated in the pregnant uterus prior to birth (E16.5-E18.5 in mouse), and play essential roles in labor induction (Sugimoto, Yamasaki et al. 1997, Gross, Imamura et al. 1998). Fetal membranes and amniotic fluid also show an increase in pro-inflammatory cytokines, including interleukin (IL)-8, IL-1 β , and IL-6 (Young, Thomson et al. 2002). Many studies show that major mediators of inflammation, including PGs and ILs, activate AVP neurons (Gatti and Bartfai 1993, Mastorakos, Weber et al. 1994, Landgraf, Neumann et al. 1995), and PGs enhance AVP secretion in sheep (Brooks and Gibson 1992). A more recent study showed via electrophysiological recordings that ICV injections of IL-6 increase the activity of SON AVP neurons similar to LPS challenge, and that ICV injections of IL-6 antibodies prevented the LPS-induced activation of AVP neurons (Palin, Moreau et al. 2009). Therefore, it is possible that prenatal immune activation signals the fetus and influences AVP release. It might be possible to test this hypothesis in future studies by examining AVP release and/or Fos activation in AVP neurons in pups with a dampened immune response.

Copeptin levels dropped immediately after birth compared to E18.5, and even further at 3h post-delivery in both vaginally and C-section-delivered pups. However, neonatal copeptin levels remained significantly higher after a vaginal delivery compared to adults. While there are no data reporting the levels of AVP/copeptin prenatally for humans, AVP levels in babies drop rapidly within a few hours of birth, and reach adult levels within one day of birth (Leung, McArthur et al. 1980, Rees, Forsling et al. 1980). Similarly, we found that copeptin levels at P1 in mice were not

significantly higher than non-pregnant adults, further supporting an evolutionarily conserved, acute endocrine response to birth.

Copeptin levels were similar in pups delivered vaginally and via C-section at 0h, but vaginally-delivered pups had higher copeptin levels than C-section-delivered pups at 3h post-delivery. Although these data are in general agreement with the human literature reporting significantly lower AVP/copeptin levels in C-section-delivered neonates (Parboosingh, Lederis et al. 1982, Wellmann, Koslowski et al. 2016), the difference we found was more subtle than the large differences reported in the human literature. This may be a species difference, or there may be something about the C-section procedure in humans (such as use of anesthesia) that attenuates AVP production by the newborn. While pups delivered vaginally or by C-section will both experience hypoxia, it is usually greater after a vaginal delivery (Berger, Vaillancourt et al. 2000), which would explain differences in copeptin levels, and further suggest that hypoxia may serve as a stimulus for perinatal AVP release.

AVP is also known as antidiuretic hormone due to its role in maintaining water homeostasis. Given the reported surge in AVP levels in the fetus/neonate in several species (Alexander, Bashore et al. 1974, Sinding, Robinson et al. 1980, Wellmann, Benzing et al. 2010, Summanen, Back et al. 2018), we examined plasma osmolality perinatally, and found an acute and transient decrease at 3h postpartum. We also measured osmolality in C-section-delivered pups at 3h, and found significantly higher osmolality than vaginally-delivered pups. The observed difference in plasma osmolality between vaginally- and C-section-delivered mice at 3h post-delivery correlates with the observed difference in copeptin levels at the same time, suggesting that increased AVP leads to decreased plasma osmolality. However, further experiments comparing electrolyte profiles, blood volume, and blood pressure between vaginally- and C-

section-delivered mice will be needed to determine what factors contribute to the plasma osmolality results. Human studies report lower blood pressure in C-section-delivered neonates compared to vaginally-delivered neonates, suggesting that the surge of AVP at birth may protect the neonate against hypovolemia (Holland and Young 1956). In addition, vaginally-delivered neonates are less likely to suffer from hypernatraemic dehydration than C-section-delivered neonates (Konetzny, Bucher et al. 2009), further supporting the possibility that higher AVP release following a vaginal delivery may be important for neonatal fluid balance.

Prenatally, however, copeptin levels do not correlate with the expected response in plasma osmolality. According to AVP's antidiuretic effect, high copeptin levels should result in decreased osmolality measurements, but in contrast, we observe normal osmolality values at E18.5 and E19 (average of ~316 mOsm/kg) (Silverstein, Sokoloff et al. 1961, Morishita, Tsutsui et al. 2005), compared to 3h postpartum. A possible explanation of this unexpected result is that fetal water and/or electrolyte balance is closely maintained by maternal/placental factors rather than fetal AVP production. Salt retention in the dam is known to increase in the last stage of pregnancy in rats (Atherton, Dark et al. 1982). Remarkably, more than half of the retained sodium is sequestered by the fetus (Lichten 1961, Churchi-1, Bengel et al. 1980), and even when the dam is fed a low sodium diet, maternal plasma sodium levels drop further in order to maintain fetal plasma sodium levels (Kirksey, Pike et al. 1962). Therefore, osmolality levels in the fetus may be maintained at a certain threshold by maternal/placental supplies regardless of increased fetal AVP levels.

AVP can also act on central targets where it is known to mediate social behaviors, including aggression and pair bond formation (Heinrichs and Domes 2008, Veenema and Neumann 2008). While measuring centrally-released AVP presents a challenge with currently available techniques, especially in neonatal rodents, two separate studies showing that peripheral levels of vasopressin

strongly predict levels in CSF of newborns suggest that peripheral levels of AVP may serve as a proxy for centrally-released AVP in the perinatal brain (Bartrons, Figueras et al. 1993, Carson, Howerton et al. 2014). Therefore, it is likely that a vaginal birth also triggers central discharge of AVP at birth via synaptic and/or volume transmission, where it may play an important role in brain development.

We recently showed that manipulations of birth mode have effects on developmental neuronal cell death (Castillo-Ruiz, Mosley et al. 2018). Specifically, birth triggers an acute and transient decrease in cell death in vaginally-delivered pups, but not C-section-delivered pups, suggesting that a specific aspect of a vaginal birth is neuroprotective (Castillo-Ruiz, Mosley et al. 2018). Moreover, AVP protects neurons from nutrient deprivation and glutamate-induced apoptosis *in vitro* (Chen and Aguilera 2010). However, whether AVP is neuroprotective *in vivo* has not been tested. We found that ICV AVP treatment decreased cell death in the PVN and AHA of C-section-delivered pups, but not the SON and LHb, suggesting that brain-region specific mechanisms regulate neuronal cell death. In fact a recent study showed that electrical activity was necessary for dictating the pattern of cell death in specific cortical areas (Blanquie, Yang et al. 2017), and AVP may play a role in regulating electrical activity in the perinatal brain (Spoljaric, Seja et al. 2017).

Our pilot study examining potential receptors mediating the neuroprotective effects of vasopressin suggested that an oxytocin receptor antagonist blocked the neuroprotective effect of AVP in the PVN. Intriguingly, antagonizing V1aRs with AVP+MC completely eliminated cell death, suggesting that MC could be acting as an agonist rather than an antagonist. The MC is actually a weak V2R agonist (Kruszynski, Lammek et al. 1980, Manning, Misicka et al. 2012), but has never been reported to act as a V1aR agonist. However, most studies reporting antagonizing

effects of the MC in V1aRs have used adult rodents, and the compound may have different actions when applied to the developing brain. When antagonists to both vasopressin and oxytocin were administered along with AVP, we observed intermediate levels of cell death compared to the OTA and the MC groups, further pointing to the possibility that the MC acts as a V1aR agonist in the developing brain. However, given the promiscuity of the MC [MC is a fairly potent OTR antagonist (Koshimizu, Nakamura et al. 2012, Manning, Misicka et al. 2012)], the results of the treatment with MC on developmental cell death remain puzzling. Further experiments with larger groups, other/more selective V1aR antagonists, and potentially including OXT as a control treatment (due to the close proximity in sequence homology with AVP), will shed more light to the mechanism of action of AVP as a neuroprotective agent in the neonatal brain.

5 CHAPTER FIVE: GENERAL DISCUSSION / SYNTHESIS

5.1 Overview

At delivery, the newborn mammal is forced to initiate autonomous activity of vital peripheral processes, including air breathing, thermoregulation, mature circulation, and changes in energy expenditure and metabolism, in compensation for the loss of the placenta and life outside the womb (Gluckman, Sizonenko, and Bassett 1999; Morton and Brodsky 2016). Naturally, organs that carry out these functions, namely, the lungs, heart, gut, and liver must develop during the perinatal period in order to function adequately at birth, and birth signals play important roles in triggering their maturation (Fowden, Mijovic, and Silver 1993; Liggins 1976, 1994). It is less clear what changes occur in the brain in preparation for the challenges of birth or in response to it, and addressing this question was the overarching goal of this dissertation.

The current findings show that birth triggers activation in specific brain areas, including discrete hypothalamic areas in the mouse and rat (Chapter 2), and that activation occurs regardless of birth mode or birth timing (Chapter 3). Our data also show that a large portion of the activated neurons in the mouse hypothalamus are vasopressinergic (Chapter 2 & 3), and demonstrate for the first time in the mouse a perinatal surge of peripheral AVP (Chapter 4). To determine possible peripheral and central functions of AVP at birth, we examined its effect on perinatal levels of plasma osmolality and neuronal cell death. We found that plasma osmolality is acutely decreased following a vaginal birth, but not a Cesarean birth, and show that vasopressin decreases cell death in specific hypothalamic areas of Cesarean-delivered pups, possibly acting via oxytocin receptors (Chapter 4). Collectively, the studies outlined in this dissertation provide support for the hypothesis that birth triggers a neuroendocrine response that provides neuroprotection to the newborn via vasopressinergic signaling.

5.2 Birth activates hypothalamic neurosecretory cells, including vasopressin neurons

Contrary to our prediction that *c-Fos* expression might be widespread, we found that neural activation is localized to specific brain areas shortly after birth. Neural activation was significantly increased within 3h of birth compared to E18.5 or 1h after birth in the PVN, SON and SCN, and in all cases neural activation returned to baseline levels within 24h of birth (P1). Previous reports show induction of *c-fos* mRNA or *c-Fos* protein in connection with birth. However, some of these reports focus on the analysis of whole brain homogenates, and thus do not address the specific brain areas where *c-fos* mRNA expression changes (Kasik, Wan, and Ozato 1987; Levi et al. 1989). Those studies that do report the expression pattern of *c-fos* mRNA or *c-Fos* protein altered important signals of birth (the majority via manipulations of birth mode), making it challenging to rule out the possibility that the reported effects are due to experimental manipulations (Tang et al. 2000; Ringstedt et al. 1995; Ikeda et al. 2019). Our initial study differs from previous work in that we examine expression of the *c-Fos* protein (as opposed to *c-fos* mRNA) without manipulation of the normal progression of birth, and provide a more fine-grained analysis of the brain areas and neural phenotypes that become activated in the perinatal mouse brain. In this study, we also found a similar pattern of neural activation in the rat brain, suggesting an analogous neural response across species.

Remarkably, all the hypothalamic areas that showed a significant increase in neural activation at 3h postpartum are prominent in AVP, OXT, and/or CRH neurons; extra-hypothalamic areas that showed a similar increase in neural activation (CPu, LHb, PVT, and SFO), also receive input from at least one of these populations (Hernandez et al. 2015; Braga et al. 2011; Hawthorn, Ang, and Jenkins 1980). We therefore performed phenotyping of the activated neurons, and found that AVP neurons are significantly more activated than OXT or CRH neurons in the PVN at 3h

postpartum (when we see the highest levels of c-Fos expression); we also found increased activation of AVP neurons in the SCN at 3h. Importantly, AVP neurons in the PVN and SON, along with CRH neurons in the PVN, play essential roles in the endocrine cascade that facilitates the adaptation to the *ex utero* environment (Kota et al. 2013; Liggins 1994). The only previous study evaluating the activation pattern of any of these neuropeptides was done in sheep, and found that CRH neurons in the PVN are strongly activated during labor, and that c-Fos immunoreactivity declines rapidly after delivery (Hoffman et al. 1991). Similarly, we found that approximately half (48.5%) of all the c-Fos-ir neurons in the PVN at E18.5 are CRH positive, but the percentage decreased significantly by 3h of birth. Taken together, this suggests an important role of CRH neurons in labor, whereas AVP may play an important role at birth.

5.3 Neural activation at birth is triggered by the transition to the extrauterine environment

Considering the induction period of the c-Fos protein (>90 minutes) (Morgan and Curran 1991; Sheng and Greenberg 1990), our results suggest that a stimulus/stimuli related to delivery and the immediate postpartum period triggers perinatal neural activation. However, since birth is a complex, multi-step process, our first study alone was not sufficient to determine the exact aspect(s) of birth that trigger the perinatal neural activation. Therefore, in our second study (Chapter 3) we manipulated birth delivery mode to begin to dissociate the stimuli accompanying a vaginal delivery. We showed that neural activation increases at 3h postpartum compared to *in utero* levels in both pups born vaginally and by C-section. Since C-section-delivered pups do not experience labor or travel through the vaginal canal, these stimuli are not required for the neural activation. We also ruled out the possibility that neural activation is triggered by an autonomous developmental program at 19 days post-conception rather than by external factors, as we also found increased neural activation at 3h post-delivery in animals delivered one day prior to the

expected day of birth. Taken together, these results suggest that neural activation at birth is triggered by exposure to the extrauterine environment.

A surge of peripheral AVP has been widely reported in human neonates after a vaginal delivery (Chard et al. 1971; Hoppenstein 1980; Leung et al. 1980; Polin et al. 1977; Rees, Forsling, and Brook 1980) but is blunted in C-section-delivered babies (Evers and Wellmann 2016; Parboosingh et al. 1982). A recent study also found a similar effect in rats (Summanen et al. 2018), suggesting that some aspect of a vaginal birth is necessary to trigger the release of AVP at birth. This might suggest increased activation of AVP neurons in the hypothalamus of vaginally-delivered pups compared to C-section-delivered pups. Contrary to this prediction, we did not find significant differences in the activation pattern of AVP (or OXT) cells within the hypothalamus between birth mode groups (Chapter 3). However, neural activation (or lack thereof) does not necessarily correspond to AVP release (Morgan and Curran 1986, 1988; Vendrell et al. 1992; Zhu and Herbert 1997). It is possible that AVP released centrally modulates further AVP release without triggering action potentials (Gouzenes et al. 1998; Ludwig et al. 2002; Ludwig and Leng 2006), which would allow vaginally- and C-section-delivered pups to show differences in AVP release without necessarily exhibiting differences in c-Fos immunoreactivity. Alternatively, similar c-Fos expression in vaginally- and C-section-delivered pups may reflect a ceiling effect; activation of AVP neurons as a result of the stress of birth/transition to the extra uterine environment could be so large that it masks effects of other factors (that differ between Cesarean and vaginal delivery) on the activation of AVP neurons.

5.4 Vasopressin is elevated in the perinatal mouse plasma

An important question is whether the activation of AVP neurons at birth results in peripheral or central AVP release. Unfortunately, measuring central release of AVP via

conventional micro invasive perfusion techniques is extremely challenging due to the small size of the fetal/neonatal brain and the short time window of our study (3h). In contrast, AVP released into systemic circulation can be indirectly measured using a sandwich ELISA for its more stable surrogate marker, copeptin.

Our data show copeptin levels are greatly elevated perinatally compared to those in adults. Copeptin was undetectable in adults, with the exception of two dams that had vaginal deliveries, and even in those two cases, levels were significantly lower than in their pups at the same timepoint (0h). Thus, we conclude that copeptin in the fetus/neonate is not of maternal origin, which is also the conclusion that has been reached for human newborns (Wellmann et al. 2010) and perinatal sheep (Stark et al. 1981). Contrary to what is implied by the human literature, however, we saw the highest copeptin levels prenatally at E17.5 and E18.5. These results are in agreement with previous studies reporting elevated levels of circulating AVP in the last few days of gestation in the fetal lamb (Sinding et al. 1980; Alexander et al. 1974). In addition, a study that induced labor prematurely in the fetal lamb showed a temporally coincident increase in AVP release (Stark et al. 1981), suggesting that perinatal AVP is triggered by labor. Similarly, experimentally-induced intermittent hypoxic episodes cause an increase in copeptin levels in the P6 rat (Summanen et al. 2018). Moreover, copeptin is higher at birth in human newborns suffering from birth asphyxia (reduced O₂ and increased CO₂ levels) (Summanen et al. 2017) or exposed to uterine contractions induced by OXT treatment of the mother prior to C-section delivery (Summanen et al. 2018; Wellmann et al. 2016; Summanen et al. 2017) compared to controls. Together, these results suggest that labor and the events that accompany it, trigger the release of AVP in the offspring, perinatally. The reason, however, why we do not see increased c-Fos expression at E18.5 could be

because AVP neurons are activated earlier in development. Our results showing high plasma copeptin levels at E17.5 support this prediction.

While early stages of labor might be present at E18.5, labor itself is unlikely to explain our finding of elevated copeptin levels at E17.5. AVP at late prenatal stages may instead be related to HPA axis activation and/or in response to perinatal inflammation. Pro-inflammatory cytokines rise near the end of gestation and immune cells infiltrate the myometrium, cervix, and fetal membranes (Osman et al. 2003; Thomson et al. 1999; Golightly, Jabbour, and Norman 2011; Kobayashi 2012; Shynlova et al. 2013; Young et al. 2002). Studies show that major mediators of inflammation, including prostaglandins and interleukins (ILs), activate AVP neurons (Gatti and Bartfai 1993; Landgraf et al. 1995; Mastorakos et al. 1994), and prostaglandins enhance AVP secretion in sheep (Brooks and Gibson 1992). A more recent study showed via electrophysiological recordings that ICV injections of IL-6 increase the activity of SON AVP neurons similar to LPS challenge, and that ICV injections of IL-6 antibodies prevented the LPS-induced activation of AVP neurons (Palin et al. 2009). Therefore, it is possible that prenatal immune activation signals the fetus and influences AVP release; this could be tested in future studies by examining AVP release and/or Fos activation in AVP neurons in pups with a dampened immune response.

We found that copeptin levels dropped immediately after birth compared to E18.5, and even further at 3h post-delivery in both vaginally and C-section-delivered pups. While copeptin levels were similar in pups delivered vaginally and via C-section at 0h, vaginally-delivered pups showed higher copeptin levels at 3h post-delivery. Pups delivered vaginally or by C-section both experience hypoxia, but it is usually greater after a vaginal delivery (Berger, Vaillancourt, and Boksa 2000), which could explain differences in copeptin levels, and further suggest that hypoxia may serve as a stimulus for perinatal AVP release.

In vitro work suggests a potential mechanism for the rapid drop in AVP release after birth. GABA is known to be excitatory during early life. However, cell-attached recordings on slice preparations of the perinatal rat hippocampus show a transient excitatory-to-inhibitory switch in GABA polarity that occurs during birth (Tyzio et al., 2006). Consequently, neurons that would normally be depolarized by GABA become transiently inhibited during birth. When, exactly (how long after birth), the GABA switch occurs is not clear, but by 6 hours after birth (compared to 5-15min after birth), GABA is again excitatory. A similar effect was also observed in the neocortex, suggesting that the transient switch in GABA polarity at birth may also occur in other brain areas.

Excitatory GABA signaling in the developing brain leads to a rise in intracellular Ca^{++} concentrations and induces c-Fos in hippocampal or cortical slice preparations or cultured hippocampal neurons (Berninger et al. 1995; Lin et al. 1994; Segal 1993; Yuste and Katz 1991). Therefore, transient inhibitory actions of GABA signaling during delivery may cause transient inhibition of AVP neurons and therefore decreased levels of AVP/copeptin at birth compared to prenatal levels. The switch in GABA polarity back to being excitatory immediately after birth, provides a potential explanation for increased c-Fos expression in AVP neurons at 3h postnatal. Copeptin values at 3h, and even at P1, remained significantly higher than in adults, suggesting some sustained release. Additional electrophysiological experiments (measuring both GABA polarity and AVP-releasing activity) are necessary to test these predictions. In addition, since the GABA polarity switch is thought to be mediated by the surge in maternal oxytocin during labor (Tyzio et al. 2006), further experiments should determine whether the switch occurs in pups delivered by C-section to dams that do not experience labor. Our finding of similar levels of c-Fos immunoreactivity between vaginally- and C-section-delivered pups suggest that both groups experience the switch in GABA polarity at birth.

1.5 Vasopressin may affect plasma osmolality and be neuroprotective at birth

The c-Fos labeling in AVP neurons and high levels of AVP/copeptin in perinatal plasma prompted us to examine peripheral and central processes that might be impacted by AVP. First, we examined perinatal changes in plasma osmolality. Normal plasma osmolality in mice is reported to be ~310-315 mOsm/kg (Morishita et al. 2005; Gooch et al. 2006; Yang et al. 2001), which is very similar to what we measured in at E18.5 and E19 (315.7 ± 3.033 mOsm/kg and 309.1 ± 4.97 mOsm/kg for E18.5 and E19, respectively). In vaginally-delivered mice, we found an acute and transient decrease within 1-3h after birth (~280-290 mOsm/kg) that, as far as we know, has not been previously reported. The decline was not as pronounced (and, if fact, was not statistically significant) in C-section-delivered pups at 3h (303.4 ± 5.227 mOsm/kg). The observed difference in plasma osmolality between vaginally- and C-section-delivered mice at 3h post-delivery correlates with the observed difference in copeptin levels at the same time.

An important question that arises from these data is whether changes in plasma osmolality after a vaginal delivery are a cause or a consequence of the AVP release. Despite the elevated AVP/copeptin prenatally, plasma osmolality was within the normal range *in utero*. Given that the fetus receives blood supply from mother, it is likely that maternal factors have direct influence in fetal osmotic balance. The sudden drop at birth may reflect the loss of placental supplies which, combined with high fetal/neonatal production of AVP leads to the substantial decrease in plasma osmolality at birth. The much less pronounced drop seen in the C-section group at 3h may be due to the lower AVP/copeptin levels we measured at that time. Alternatively, the drop in osmolality is due to other factor(s) related to vaginal delivery, which may, in turn, induce c-Fos expression in AVP cells shortly after birth.

It is likely that AVP is also released centrally in perinatal pups, as peripheral levels of vasopressin strongly predict levels in CSF of human newborns (Bartrons et al. 1993; Carson et al. 2014). Previously, we showed that birth is associated with an acute and transient decrease in cell death in vaginally-delivered pups, but not C-section-delivered pups at 3h postpartum (the same timepoint at which we find a significant effect of birth mode on plasma copeptin and osmolality) (Castillo-Ruiz et al. 2018). We therefore examined the effect of AVP signaling on neuronal cell death in C-section-delivered pups. AVP treatment decreased cell death in the PVN and AHA, but not the SON and LHb, suggesting that brain-region specific mechanisms determine the pattern of neuronal cell death. In fact, a recent study showed that electrical activity was necessary for dictating the pattern of cell death in specific cortical areas (Blanquie et al. 2017), and AVP may play a role in regulating electrical activity in the perinatal brain (Spoljaric et al. 2017). A follow-up pilot study suggested that AVP may act via OXT receptors to suppress cell death, but results are preliminary. Further experiments with larger groups, other/more selective V1aR antagonists, and potentially including OXT as a control treatment (due to the close sequence homology with AVP), will shed more light on the mechanism of action of AVP as a neuroprotective agent in the neonatal brain.

Another topic for future study is the possibility that central AVP release at birth mediates newborn analgesia. Analgesic effects of central AVP have been widely reported in adult animals. For example, ICV injections of AVP, but not intrathecal or intravenous injections of AVP, increase pain threshold in rats (Yang et al. 2006). Similarly, ICV injections of AVP but not AVP antiserum into the AVP-deficient Brattleboro rat, results in increased pain threshold (Bodnar et al. 1982). Several studies report increased levels of the opioid beta-endorphin in human babies following vaginal delivery but not a C-section delivery (Bacigalupo et al. 1987; Facchinetti et al. 1986;

Raisanen et al. 1986), and a more recent study showed that vaginally-delivered human newborns display decreased physiological and behavioral responses to high- and low-intensity pain stimulation compared to C-sectioned babies (Bergqvist et al. 2009). Given the reported difference in AVP/copeptin levels between vaginally- and C-section-delivered offspring on the day of birth in several species, AVP may be responsible for analgesia at birth.

5.5 Working hypothesis, limitations, and future research

The studies within this dissertation have addressed many gaps in the literature and allow us to portray a more detailed picture of the role of birth and birth-related signals in guiding neural development in the fetal/neonatal mouse. Our working hypothesis is that specific signals of labor/the final stage of gestation, likely HPA axis and/or immune system activation (Gross et al. 1998; Sugimoto et al. 1997; Young et al. 2002; Gatti and Bartfai 1993; Landgraf et al. 1995; Mastorakos et al. 1994; Brooks and Gibson 1992), trigger a perinatal surge of AVP that is important for neurodevelopment, and the transition of the fetus to life *ex utero* (Chapter 4). As labor progresses and the time of birth approaches, additional birth signals [which are thought to be specific to parturition given that the switch in GABA is not observed in cultured neurons (Tyzio et al. 2006)], mediate a transient switch in GABA polarity (from depolarizing to inhibitory), that may result in reduction of AVP release at birth (Chapter 4). Upon delivery, GABA signaling becomes excitatory again, thus resuming AVP release (although reduced) and inducing c-Fos immunoreactivity in specific brain areas and cell types, including AVP neurons (Chapter 2 & 3). High levels of AVP in the immediate postnatal period may play important roles in physiology and development of the neonate, including protection against dehydration and neuronal cell death (Chapter 4), and manipulations in birth mode have direct effects on these processes (Castillo-Ruiz et al. 2018), possibly due to alterations in AVP signaling (Chapter 4).

Future research should aim to elucidate the mechanism by which AVP is released and exerts neuroprotection in the newborn brain. Since our pilot data suggest that AVP may reduce cell death by signaling through OXT receptors, and OXT has been implicated in several important processes within the perinatal period (i.e. analgesia, GABA switch), future experiments should also examine the role of OXT in neuroprotection. However, given that perinatal OXT levels in the newborn remain relatively unchanged (in stark contrast to AVP), and that maternal OXT may not reach the fetal brain (Brown and Grattan 2007; Carbillon 2007), the involvement of OXT in neuroprotection at birth is uncertain.

A significant limitation of our studies is that we did not measure neural activity directly via electrophysiological experiments. However, current electrophysiological and whole-brain functional imaging techniques have significant limitations for birth-related studies (i.e. duration of tissue processing, limited spatial resolution). Therefore, using c-Fos as a marker of neural activity was the best practical experimental tool to assess how the brain responds to birth using a whole brain approach. Still, such studies are necessary, especially to determine the mechanism by which alterations in birth mode lead to differences in AVP release shortly after birth, as we hypothesize this is what leads to the effect of birth mode on developmental neuronal cell death (Castillo-Ruiz et al. 2018). A starting point could be determining whether C-section delivered pups experience the switch in GABA polarity, and if so, whether it affects hypothalamic AVP neurons. Future experiments should also aim to determine the concentration of central copeptin/AVP in the perinatal mouse or rat brain, and define whether central levels of AVP correlate with peripheral levels as in humans (Carson et al. 2014).

5.6 Final remarks

As mentioned previously, birth is a dramatic experience. Regardless of a vaginal or C-section delivery, birth involves extraordinary changes in the newborn external and internal environments, and the events immediately preceding (namely, labor and delivery) inform the fetus of the massive changes to come. Before the introduction of C-sections as a birth delivery mode, every mammal on earth arrived via a vaginal birth. Therefore, it would be surprising if there were not conserved mechanisms across species to prepare the brain for the upcoming challenges. It is likely that the ubiquitous nature of birth led us to disregard its importance as a developmental signal, or more importantly, as an orchestrator of neurodevelopmental processes. Future research should therefore, continue to elucidate the importance of birth and/or birth signals in guiding developmental processes, and determine how manipulations to the normal progression of birth (i.e. birth mode, timing of birth, analgesics) affect them, which will likely have implications for current obstetric practices.

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