Effects and Interactions of Early-Life and Adult Stress on Neuroendocrine Control of Reproduction

by

Amanda G. Gibson

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Doctoral Committee:

Professor Suzanne M. Moenter, Co-Chair
Professor Audrey Seasholtz, Co-Chair
Assistant Professor Christian Burgess
Assistant Professor Kevin Jones
Assistant Professor Joanna Spencer-Segal

Amanda G. Gibson

gibsonag@umich.edu

ORCID iD: 0000-0003-0817-2141

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Table of Contents

Acknowledgements ii

List of Tables xii

List of Figures xiv

List of Abbreviations xv

Abstract xvii

Chapter 1 Introduction 1

Reproductive axis overview 1

Stress axis overview 2

Early-life stress and reproduction 2

Rodent models of early-life stress 3

Reproductive consequences of early-life stress in rodents 4

Stress hyporesponsive period 5

Effects of early-life stress on the neuroendocrine stress axis 6

Effects of adult stress on the reproductive axis 8

Humans 8

Non-human primates 9

Sheep 11

Rodents 14

Hypothalamic synaptic physiology and the switch to positive feedback 16

Interactions between early-life and adult stress on reproductive outcomes 17

Stimulatory effects of stress on the reproductive axis 18

Stress-related changes in synaptic physiology 19

Dissertation preview 21

Chapter 2 Limited Bedding and Nesting Does Not Alter Adult Corticosterone Response to Acute Psychosocial Stress in Male or Female Mice or the Stress-Induced Disruption of the Preovulatory Luteinizing Hormone Surge 25

Abstract 25

Significance Statement 26

Introduction 26

Materials and Methods 29

Animals 29

Experimental design 29

Limited bedding and nesting (LBN) paradigm 31

Dam behavior monitoring 32

Body mass 32

Offspring maturation 33

Adult female estrous cycles 33

Acute, layered, psychosocial stress (ALPS) paradigm 33

Corticosterone administration 35

Corticosterone enzyme immunoassay 35

Ultra-sensitive LH assay 36

Electrophysiology 36

Statistical analysis 38

Code accessibility 41

Results 41

LBN dams exited the nest more frequently 41

LBN affected pup mass 44

LBN did not alter reproductive maturation or estrous cycles 47
Early-life stress did not alter the corticosterone response to adult stress........... 48
ALPS decreased afternoon LH in proestrous mice, LBN had no additional effect 55
Neither LBN nor ALPS reduced the frequency of GABA PSCs in GnRH neurons 56
Discussion 60
Chapter 3 Conclusion 87
LBN did not disrupt reproductive maturation 87
STD and LBN mice had similar corticosterone responses to ALPS 89
Timing of limited bedding and nesting paradigm 89
Diurnal and sex steroid effects on corticosterone release 90
Does ALPS suppress LH pulses? 92
Mechanisms of ALPS-induced disruption of the LH surge 92
Hypothalamus 92
Pituitary 94
Actions of corticosterone 94
Activation of CRH neurons 97
Other components of the stress response 100
Summary 101
Bibliography 102

List of Tables

Table 1.1. Summary of reproductive effects of stress studies in non-human primates. 22

Table 1.2. Summary of reproductive effects of stress studies in sheep. 23

Table 1.3. Summary of reproductive effects of stress studies in rats. 24

Table 1.4. Summary of reproductive effects of stress studies in mice. 24

Table 2.1. Type of statistical test used to analyze each outcome................................ 67

Table 2.2. Statistical table for pairwise comparisons for post-hoc tests........................ 71

Table 2.1.1. Number of dams in each group for studies in Figure 2.1. 75

Table 2.1.2. Statistics for tests of dam mass and dam behavior over time. 75

Table 2.2.1. Linear mixed models of offspring mass on PND11 and anogenital distance. 75

Table 2.2.2. Linear mixed model of the offspring mass from PND11-72 fit with the equation mass ~ early-life treatment * PND@ + (1 | dam) + (1 | mouse). 76

Table 2.2.3. Linear mixed models for maturation with the equation maturation feature ~ early-life treatment + (1 | dam). 76

Table 2.3.1. Statistics for estrous cycles from postnatal days 70-90. 76

Table 2.4.1. Number of litters and mice with serum corticosterone measurements before and after adult treatment. 77

Table 2.4.2. Statistics for serum corticosterone in male and female offspring. 78

Table 2.4.3. Number of litters and male mice with mass measurements on the day of adult treatment. 79

Table 2.4.4. Statistics from linear mixed models of male masses on day of adult treatment. 80

Table 2.4.5. Number of litters and female mice with mass measurements on the day of adult treatment. 81

Table 2.4.6. Statistics from linear mixed models of female masses on day of adult treatment. 82

Table 2.4.7. Statistics from linear mixed models of male masses on day of vehicle (0mg/kg) or corticosterone (2mg/kg) treatment. 84

Table 2.4.8. Statistics for serum corticosterone in males with vehicle or corticosterone administration. 84

Table 2.5.1. Statistics from linear mixed models of average LH in diestrous mice on day of adult treatment. 84

Table 2.6.1. Statistics from linear mixed models of electrophysiology properties on day of adult treatment. 85

Table 2.7.1. Statistics for number of postsynaptic current (PSC) events per 240s in GnRH neurons on the day of adult treatment. 85

Table 2.7.2. Pairwise comparisons of distributions of interevent interval and amplitude for PSCs recorded in GnRH neurons. 86

List of Figures

Figure 2.1. The LBN paradigm altered dam behavior. 43

Figure 2.2. LBN decreased PND11 body mass and lowered body mass in adult males but did not affect reproductive maturation. 46

Figure 2.3. LBN did not alter estrous cyclicity from PND70-90. 48

Figure 2.4. LBN does not change the corticosterone response to ALPS. 51

Figure 2.4.1. The ALPS paradigm caused small changes in tissue mass in males. 52

Figure 2.4.2. LBN and ALPS cause limited changes in tissue masses in females. 53

Figure 2.4.3. Acute elevation of serum corticosterone decreases testicular mass in males. 54

Figure 2.5. The LH surge is disrupted by adult stress. 56

Figure 2.6. Recording quality and passive properties of GnRH neurons were similar among groups. 58

Figure 2.7. Stress treatments did not alter overall GABA PSC frequency, but ALPS may lengthen the interevent interval in GnRH neurons. 59

Figure 2.3.1. Corticosterone administration on proestrus does not disrupt the LH surge or ovulation. 96

List of Abbreviations

ACE	adverse childhood experience
ACSF	artificial cerebrospinal fluid
ACTH	adrenocorticotropic hormone
ALPS	acute, layered, psychosocial stress
ARC	arcuate nucleus of the hypothalamus
AVPV	anteroventral periventricular nucleus
CRH	corticotropin-releasing hormone
CRHR	corticotropin-releasing hormone receptor
DREADDs	designer receptor exclusively activated by designer drugs
ERα	estrogen receptor alpha
ERβ	estrogen receptor beta
FSH	follicle-stimulating hormone
GABA	gamma-aminobutyric acid
$GABA_A$	A-type gamma-aminobutyric acid receptor
$GABA_B$	B-type gamma-aminobutyric acid receptor
GFP	green fluorescent protein
GnRH	gonadotropin-releasing hormone
HPA	hypothalamic-pituitary-adrenal
HPG	hypothalamic-pituitary-gonadal
ICV	intracerebroventricular

IV	intravenous
KNDy	arcuate neurons co-expressing kisspeptin, neurokinin B, and dynorphin
LBN	limited bedding and nesting
LBN-CON	limited bedding and nesting; subsequent control treatment
LBN-ALPS	limited bedding and nesting; subsequent acute, layered, psychosocial stress
LH	luteinizing hormone
LPS	lipopolysaccharide
mPSC	miniature postsynaptic current; recorded in presence of tetrodotoxin
OVX	ovariectomized
OVX+E	ovariectomized with replaced estradiol
POA	preoptic area
PSC	postsynaptic current
PVN	paraventricular nucleus of the hypothalamus
STD	standard-reared
STD-CON	standard-reared; subsequent control treatment
STD-ALPS	standard-reared; subsequent acute, layered, psychosocial stress

Abstract

The neuroendocrine systems regulating stress and reproduction are important for organisms to respond to their environments and ensure the continuation of the species. Gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus integrate many inputs and serve as the common central output to the downstream reproductive axis. The pulsatile release of GnRH leads to the synthesis and release of luteinizing hormone (LH) and follicle stimulating hormone from the anterior pituitary; these gonadotropins regulate the synthesis of sex steroids by the gonads. The frequency of GnRH/LH pulses changes throughout the ovarian reproductive cycle, and a high frequency of pulses in the preovulatory stage leads to a sustained rise in estradiol. This leads to a switch from negative to positive feedback effects of estradiol, inducing the LH surge and subsequent ovulation. The stress axis is organized similarly to the reproductive axis, beginning with corticotropin-releasing hormone (CRH) neurons in the hypothalamus. CRH stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary, and ACTH stimulates the synthesis of glucocorticoids by the adrenal glands.

Both early-life and adult stress in humans can affect the reproductive system, and early-life stress may also change the response to adult stress. Because causal and mechanistic studies cannot be conducted in humans to understand these relationships, we used models for early-life and adult stress in rodents. Though the length of the reproductive cycle is different in humans and rodents, the pattern of hormonal changes

is similar. We tested the hypotheses that limited bedding and nesting (LBN) from postnatal days 4-11 would delay sexual maturation in male and female mice, and would alter the response to an acute, layered, psychosocial stress (ALPS) in adulthood. ALPS disrupts the LH surge in most mice on proestrus. We also investigated a possible mechanism underlying ALPS disruption of the surge.

LBN dams exited the nest more often than standard dams. Contrary to the hypotheses, however, the age and mass at vaginal opening, first estrus, and preputial separation were not affected by LBN. In males, diestrous females, and proestrous females, basal corticosterone (the primary glucocorticoid in rodents) concentrations were similar between standard and LBN reared mice. Further, ALPS increased serum corticosterone similarly in standard and LBN reared offspring. The LH surge was disrupted by ALPS in most mice when applied on the morning of proestrus, but this effect was not changed by LBN. To test if ALPS disrupts the LH surge by blunting the observed increase in excitatory GABAergic input to gonadotropin-releasing hormone (GnRH) neurons on the afternoon of proestrus, whole-cell voltage-clamp recordings were conducted following ALPS treatment. The frequency of GABA PSCs in GnRH neurons was not altered by LBN, ALPS, or their interaction.

These studies suggested that LBN did not confer susceptibility or resilience to ALPS, and questions remain about how ALPS disrupts the LH surge. ALPS may act upstream of GnRH neurons, change the responsiveness of GnRH neurons to input, or decrease pituitary responsiveness to GnRH. We also tested and rejected the hypothesis that elevated serum corticosterone disrupts the LH surge and ovulation, as there were no differences between the incidence of the surge or ovulation between vehicle- and

corticosterone-treated proestrous mice. Future studies should consider other potential mechanisms for ALPS-induced disruptions such as central actions of CRH or endogenous opioid peptide signaling.

Chapter 1 Introduction

This chapter describes the knowledge of the field at the time that the dissertation project was developed.

Reproductive axis overview

Gonadotropin-releasing hormone (GnRH) neurons form the final common pathway for the hypothalamic component of the reproductive axis. In both sexes, GnRH is released in a pulsatile manner (1–7) that drives release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the pituitary; the frequency of pulses shapes the relative release of these gonadotropins, with higher frequencies favoring LH and lower favoring FSH (8–10). LH and FSH stimulate synthesis of sex steroids by the gonads; these sex steroids feed back to the brain to regulate GnRH pulse frequency. Collectively, this system is known as the hypothalamic-pituitary-gonadal (HPG) axis.

The frequency of GnRH/LH pulses fluctuates throughout the ovarian reproductive cycle. The pulse frequency increases during the follicular phase and then decreases dramatically during the luteal phase, which is also characterized by higher amplitude pulses (11–15). The increased frequency of LH pulses during the preovulatory stage (late follicular phase in humans, proestrous in rodents) leads to a sustained rise in estradiol levels (11,14,15), which induces a prolonged surge of GnRH and LH release (16–20) . Ovulation of mature follicles is triggered by the resulting LH surge (21,22). Positive feedback is likely conveyed to GnRH neurons via estradiol-sensitive neurons in

the anteroventral periventricular (AVPV) region that make kisspeptin, a potent stimulator of GnRH neurons (23–25). Stress during the follicular phase may be particularly detrimental for fertility, and it has been associated with a decreased probability of conception (26).

Stress axis overview

The organization of the neuroendocrine stress axis, the hypothalamic-pituitary-adrenal (HPA) axis, is similar to that of the reproductive axis. Corticotropin-releasing hormone (CRH) neurons in the paraventricular hypothalamus stimulate release of adrenocorticotropic hormone (ACTH) from pituitary corticotropes; ACTH promotes synthesis of glucocorticoids by the adrenal cortex (27). The primary glucocorticoid in humans is cortisol; in rodents, it is corticosterone. This dissertation focuses primarily on this neuroendocrine aspect of the stress response. While the neuroendocrine aspect is an important component of the stress response, helping to mobilize energy resources to react to threats, it must be noted that it does not encompass the entirety of the stress response. Unique circuits govern other components of the stress response, such as behavioral aspects and the sympathetic response; these components may have distinct interactions with the reproductive axis.

Early-life stress and reproduction

Most of the population experiences at least one adverse childhood experience (ACE), including experiences of abuse, neglect, and household dysfunction (28,29). ACEs can affect age at menarche, but the direction of the association (advance vs. delay) depends on the type and timing of stress (30–33). ACEs are also linked with infertility,

including risk for amenorrhea and increased time to pregnancy (34,35), indicating underlying physiological dysfunction. The causal relationships and mechanisms by which early-life stress could be leading to this dysfunction cannot be addressed in human research. Due to the limitations of studying early-life stress in humans, researchers have turned to animal models. Rodents offer additional methodological advantages, such as the availability of genetic tools and shorter lifespans to study development.

Rodent models of early-life stress

There are many ways to model early-life stress of various durations in rodents, including mimicking the adrenal component of the neuroendocrine stress response through corticosterone or dexamethasone administration, activating the immune system, or altering maternal-infant interactions. Maternal separation is a commonly used model, though there are limitations to this paradigm. This stress cannot be continuously applied throughout development, as pups and lactating dams cannot be indefinitely separated. It thus relies on daily experimenter manipulations to separate and then reunite the dams and pups, introducing a source of human variability. The duration of daily separation and whether the pups or the dam remain in the home cage could also impact how the paradigm influences subsequent interactions between the dam and pups. Further, effects of maternal separation in rat offspring, such as reducing exploratory behavior in the open field test and elevated plus maze, have not been successfully replicated in mice (36), suggesting that maternal separation may affect rats and mice differently.

Another model that alters dam-pup interactions is the limited bedding and nesting (LBN) paradigm. In contrast to maternal separation, the LBN paradigm is continuous through the treatment period and does not require repeated experimenter intervention. In this model, the dam and pups are moved into a new “low resource” cage that typically contains a raised wire platform over a single layer of bedding material and reduced nesting material; for review of this model, the reader is directed to Molet et al. (37) and Walker et al. (38). The timing of this treatment varies among research groups, but two of the most common windows are from postnatal day (PND) 2-9 or 4-11. The key finding of fragmented maternal behavior during this paradigm has been demonstrated in both rats (39–41) and mice (42).

Reproductive consequences of early-life stress in rodents

As suggested by the correlational studies in humans, models of early-life stress in rodents can lead to reproductive consequences. Disruptions to the reproductive axis have been demonstrated with prenatal dexamethasone exposure in rats and mice (43–46), perinatal lipopolysaccharide (LPS) exposure in rats and mice (47–53), and maternal separation in rats (54,55). In mice, results are mixed. LBN from PND4-11 was found to delay vaginal opening, an external indicator of sexual maturation (56). Another group using LBN from PND2-9 observed a delay in preputial separation, a marker of sexual maturation in males, but they did not observe a change in the age at vaginal opening (57).

The interpretation of the effects of LBN on reproductive maturation is challenged by the common finding that the paradigm can also lead to smaller offspring (38). This reduction

in body mass by LBN was observed in mice in both Manzano Nieves et al. (56) and Knop et al. (57). Body mass is affected by litter size (smaller litters lead to larger pups, and vice versa) and pups from larger litters have delayed vaginal opening (58–62). Litter sizes in Manzano Nieves et al. (56) are unknown, but were restricted to 6-7 pups in Knop et al. (57). This makes it difficult to determine if the delayed reproductive maturation with LBN is secondary to the reduction in body mass, which cannot be solely explained by a lack of litter size standardization, or if the early-life stress is directly altering maturation.

Stress hyporesponsive period

The neonatal period is an important developmental period and a time of dynamic changes. In altricial species, including humans and rodents, the neonate is dependent upon caregivers for fulfilling their basic needs, and thus must form successful attachments with these caregivers (63). It is often in the immediate interest of neonates to form these attachments even in the face of substandard care. This sensitive period for attachment in rodents is demonstrated by rat pups from PND1-9 forming a positive association to odors paired with a foot shock (63). This positive association with negative stimuli helps to ensure that pups attach to dams despite potentially aversive experiences such as being stepped on in the nest.

The stress hyporesponsive period occurs at a similar time in development as the sensitive period for attachment, and it is characterized by low basal corticosterone levels in neonates and decreased responsiveness to stressors (63–65). During the stress hyporesponsive period, neonatal rodents react more discriminately to different

stressors. Whereas stressors such as novel environments (66,67) do not reliably elevate corticosterone in neonates, others such as cold stress (68,69) and saline injections (70) can elicit elevated corticosterone concentrations during this period. This discrimination may be related to the intensity and/or the type of stressors. For example, cold stress likely represents an ethologically-relevant stressor to which pups must be able to respond. Maternal interactions such as nursing and licking also sculpt the HPA axis of pups (71), which could in part explain how paradigms such as maternal separation or limited bedding and nesting that alter normal maternal behavior lead to lasting developmental changes in offspring (63).

When assessing the impacts of different early-life stressors, it is important to consider the timing relative to this stress hyporesponsive period. As this is a critical window of HPA axis development, stressors applied during the stress hyporesponsive period may lead to distinct outcomes from the same stressors applied after this period. The stress hyporesponsive period is from approximately PND4-14 in rats (64,65) and PND1-12 in mice (64,65,67). LBN paradigms that run from PND2-9 in rats, thus, begin prior to the onset of the hyporesponsive period, whereas those from PND2-9 or 4-11 in mice are likely to occur primarily within this hyporesponsive period.

Effects of early-life stress on the neuroendocrine stress axis

There is evidence of perturbations in the hypothalamic-pituitary-adrenal axis in offspring at the end of the LBN paradigm. In offspring from both mice and rats, basal corticosterone concentrations were elevated at the end of the LBN paradigm (39,72–75). Normalized adrenal mass, another measure of adrenal axis activation, was

unchanged by LBN in PND9 mice (74), but was elevated in LBN rats at the end of the paradigm on PND9 (39,72). In female, but not male, Wistar rats, these elevations in basal corticosterone and normalized adrenal mass persisted to weaning at PND21 (76). Adult (4-7mo-old) males in the original study of LBN in mice had elevated basal corticosterone, though group size (3 mice per group) was small (75). Another laboratory observed a small increase in basal corticosterone of male adult LBN mice that approached the level set for significance; in contrast, female adult LBN mice had lower basal corticosterone concentration compared to STD females (77). However, by adulthood in both mice and rats, most studies have found that control and LBN offspring have similar basal corticosterone levels (39,57,74,78–80), suggesting that the effects of LBN on basal stress axis activity are predominantly transient.

LBN can change how rodents respond to subsequent stressors. In one study, standard-reared (STD) and LBN rat offspring on PND9 were exposed to either stress-free control conditions or cold stress for 30-40 minutes, resulting in four groups: STD-CON, STD-COLD, LBN-CON, and LBN-COLD (73). Before treatment, STD-CON and LBN-CON pups had similar corticosterone concentrations (73). STD-CON pups killed at 90min, 240 min, and 360 min did not have higher corticosterone concentrations than STD-CON pups killed before treatment (73). Concentrations in STD-COLD pups were higher than in STD-CON pups by 90min (73). In contrast, neither LBN-CON nor LBN-COLD pups had elevated corticosterone concentrations at 90min, and both had similarly elevated corticosterone at 240min, making it difficult to determine if the cold stress had an effect in LBN pups at 240min (73). The rise in LBN-CON corticosterone concentration at 240min could be due to LBN pups being more sensitive to the disruption of littermates

being removed from the nest, or secondary to changes in LBN dam behavior as pups are removed. Though the onset of the corticosterone response was delayed in LBN-COLD pups, these pups had higher corticosterone concentrations at 360min than both LBN-CON and STD-COLD pups, suggesting altered termination of the corticosterone response and/or clearance of corticosterone in LBN pups after cold stress (73). This initial study only tested the short-term neuroendocrine consequences of LBN exposure and did not address how LBN affects response to stress in adulthood.

The neuroendocrine consequences of LBN in adulthood are likely to vary by stressors and may be different in male and female offspring. Adult female mice that experienced LBN from PND2-9 had similar corticosterone levels as STD females immediately after a 10-min restraint stress, but LBN females had a blunted corticosterone response at 20 and 30min, before again approaching similar concentrations as STD females at 60min, when corticosterone has begun declining in both groups (79). Overall, males had lower corticosterone responses than females, and STD and LBN males did not differ from each other (79). In a different study using a 6min forced swim stress, adult male mice treated with LBN from PND2-12 had lower corticosterone concentrations than standard-reared offspring 1h after the swim stress (80). These studies suggest that LBN may dampen the responsiveness of the neuroendocrine stress response upon exposure to a brief stressor in adulthood, but that this could depend on sex and the type of stressor.

Effects of adult stress on the reproductive axis

Humans

Adult stress can independently affect reproductive outcomes. The likelihood of both natural conception and successful assisted reproductive technology pregnancies are related to women's perceived stress (26,81,82). Acute glucocorticoid exposure reduces mean LH release and pulse frequency in early-follicular-phase women (83), and LH release in response to acute GnRH administration is decreased in women taking the corticosteroid prednisolone (84).

As with studies of early-life stress, animal models are necessary to investigate the neurobiological mechanisms that tie together stress and fertility; such studies are not ethical in humans. Core aspects of fertility shared by spontaneously-ovulating mammals include central control by GnRH stimulating LH release from the pituitary, and a preovulatory shift from a negative feedback to positive feedback action of estradiol leading to a surge in LH that promotes ovulation (17,18,20,85–87). While the reproductive cycle length is different between species, the sequence of hormonal changes is similar. The summary of reproductive effects of stress studies in non-human primates (Table 1.1), sheep (Table 1.2), rats (Table 1.3), and mice (Table 1.4) are described below.

Non-human primates

An early indication that the neuroendocrine stress axis could impact the reproductive axis came from the finding that daily administration of hydrocortisone acetate to orchidectomized rhesus macaques for two months elevated serum cortisol concentrations and decreased serum LH and FSH concentrations (88). As hydrocortisone acetate increases serum cortisol in adrenalectomized macaques (89),

the elevated cortisol is likely due in part to metabolism of the drug. More acutely, CRH administration to ovariectomized (OVX) females also decreased mean LH by decreasing LH-pulse frequency (90,91). CRH administration increases serum cortisol (90–92), which could be partially responsible for the effects on the reproductive axis. However, even when OVX rhesus macaques are adrenalectomized, CRH still suppressed mean LH release (92). This indicates that CRH can affect the reproductive axis independent of elevating cortisol. CRH has central effects on the reproductive axis, as corresponding multiunit activity in the putative GnRH/LH pulse generator region of the hypothalamus (93) was also suppressed after CRH administration (94). The opioid antagonist naloxone reversed these effects of CRH (90,91,94), suggesting the involvement of endogenous opioid peptides in stress-induced suppression of the reproductive system. Activation of the immune system by the bacterial endotoxin lipopolysaccharide (LPS) increases cortisol in rhesus macaques, and decreases the frequency of LH pulses (95). As naloxone reverses this effect, opioid signaling likely also mediates the response to this immune stressor (95).

Psychosocial stressors also affect the reproductive system in nonhuman primates. In OVX marmosets with estradiol replacement (OVX+E), receipt of aggression from a female conspecific followed by restraint decreased mean LH (96,97), and with longer sampling times, decreased LH pulse amplitude (96). In gonad-intact rhesus macaques, restraint decreased mean LH in males and follicular-phase, but not luteal-phase, females (98,99); the researchers did not assess pulse parameters in these studies, though qualitatively, pulsatile release appears to be disrupted. The above effects of

restraint are also mediated by endogenous opioid signaling, as they are reversed by antagonizing opioid receptors (97–99).

Sheep

Immune stress

Studies in sheep provide great mechanistic detail about how stress interacts with the reproductive system, as sampling techniques allow for the simultaneous monitoring of GnRH and LH pulses. LPS decreased GnRH and mean LH (100). The amplitude of both GnRH and LH pulses was suppressed, but only LH-pulse frequency was reduced (100). This decoupling of the frequency of GnRH and LH pulses suggested that LPS altered how the pituitary gonadotropes responded to GnRH. After blocking endogenous GnRH release, LH release in response to hourly GnRH administration could be assessed. LPS decreased amplitude of LH pulses induced by GnRH injection (101), consistent with the hypothesis that LPS reduced pituitary responsiveness to GnRH. In addition to, and perhaps in part as a consequence of, the disruption to LH pulses, the GnRH/LH surge can also be delayed and/or blunted by LPS exposure prior to the onset of the surge (102,103).

Glucocorticoids

Increased glucocorticoids following LPS exposure could be a source of reproductive disruptions. Yet, in ewes, increased cortisol is not necessary for LPS to suppress LH release, as even when cortisol synthesis is blocked, LPS exposure still reduced LH-pulse frequency and mean LH levels (104). While increased cortisol concentrations

following LPS exposure may not be necessary to disrupt the reproductive axis, it may be sufficient, as demonstrated by studies that artificially elevate serum cortisol levels in ewes without additional stressors. In OVX ewes, serum cortisol concentrations that mimic those induced by LPS exposure decreased mean LH (104,105), variably suppressing either LH-pulse amplitude (105) or LH-pulse frequency (104). While neither GnRH-pulse amplitude nor frequency were suppressed in cortisol-treated OVX ewes (105), replacement of estradiol resulted in cortisol-induced suppression of both GnRH and LH-pulse frequency (106,107). As the results in OVX ewes without estradiol replacement demonstrated that features of LH pulses could be affected by cortisol without altering GnRH pulses (105), this suggested that cortisol alters the responsiveness of pituitary gonadotropes to GnRH. Indeed, when endogenous GnRH release was blocked, the amplitude of LH pulses in response to hourly GnRH administration was reduced following cortisol treatment (105). This effect is dependent on glucocorticoid receptors (108).

Studies of cortisol effects in ovary-intact, follicular-phase ewes offer insight into how stress may disrupt both LH pulses and the LH surge. Stress-like levels of cortisol at the start of the follicular phase prevented the increase in LH-pulse frequency and decrease in LH-pulse amplitude that typically occurs as ewes progress through the follicular phase (106,109). With these disrupted LH pulses, the ewes exposed to cortisol had either a delayed or blocked rise in estradiol, which then delayed or blocked the LH/FSH surge (106,109,110). Thus, one mechanism by which stress disrupts the LH surge may be by suppressing the estradiol signal necessary for a switch to positive feedback. It is also possible, however, that stress could also alter the responsiveness of the central

reproductive axis to the preovulatory rise in estradiol. Indeed, in OVX ewes in which artificial estrous cycles were generated using progesterone and estradiol replacement, ensuring a rise in estradiol in the artificial follicular phase, cortisol still delayed the LH surge (111).

Psychosocial stressors

As observed in nonhuman primates, psychosocial stressors disrupt LH pulses in ewes. In gonadectomized male and female sheep, restraint stress suppressed many aspects of pulsatile LH release; the exact effects on mean LH, pulse amplitude, and pulse frequency depended on steroid milieu (112,113). A sequence of layered, psychosocial stressors including isolation, blindfolding, and predator cues decreased mean LH release and GnRH and LH-pulse amplitude without changing GnRH and LH-pulse frequency (114,115). The decrease in GnRH and LH-pulse amplitude with this paradigm was not dependent on glucocorticoid receptors (114,115).

Psychosocial stress can also change the responsiveness of pituitary gonadotropes to exogenous GnRH, as measured by LH release. Gonad-intact and orchidectomized males exhibited decreased LH release in response to a single IV injection of GnRH after restraint compared to controls (113,116). In OVX ewes, whereas restraint alone did not alter pituitary responsiveness to this GnRH injection (113), the layered psychosocial stress decreased the amplitude of LH pulses in response to hourly GnRH administration in a glucocorticoid receptor-dependent manner (114). This contrasts with the central actions of the layered stress to decrease GnRH-pulse amplitude being independent of glucocorticoid receptors, as described above.

Interestingly, the LH surge appears to be more resistant to the effects of psychosocial stress in ewes. All ewes had typical LH surges after repeated isolation during the early, mid, and late follicular phase (117). Two exposures to the layered stress paradigm similarly did not alter the LH surge, nor did repeated, variable psychosocial stress (117). These findings highlight the specificity with which the reproductive system appears to respond to different stressors, offering an example where animals may be susceptible to the effect of a stressor on one outcome but resilient to the effects on a different outcome.

Rodents

The use of rodent models to study the effects of stress on reproduction offers different methodological advantages including access to genetic tools to probe pathways and shorter lifespans and reproductive cycles. LPS and restraint stress both decreased LH-pulse frequency in OVX rats with (118–120) and without (121) estradiol replacement. Whereas LPS acts via CRH receptor 2 (CRHR2) to suppress LH pulses (119) and depends on activity in the central amygdala (121), restraint acts via both CRHR1 and CRHR2 (119,122) and depends on the medial amygdala (121).

LPS suppresses multiunit activity in the putative GnRH/LH pulse generator region of the hypothalamus in OVX rats (123). Neurons that express kisspeptin, neurokinin B, and dynorphin (KNDy neurons) in the arcuate (ARC) nucleus of the hypothalamus are thought to be a core component of this GnRH/LH pulse generator activity (124–126). Therefore, it is intriguing that LPS also decreased expression of *Kiss1* and *Kiss1R* mRNA for kisspeptin and the kisspeptin receptor, respectively, in both the ARC and

medial preoptic area (118). Restraint similarly decreased *Kiss1* mRNA in the ARC and both *Kiss1* and *Kiss1R* mRNA in the medial preoptic area, suggesting that both stressors could alter signaling of kisspeptin-mediated pathways. Signaling through $GABA_B$ receptors in the ARC may also be involved in the suppression of LH pulses by restraint (120).

Advances in sensitivity of assays for LH have facilitated detection of LH pulses in mice using repeated tail tip sampling (127–129). In OVX mice without estradiol replacement, restraint stress decreased the frequency of LH pulses and mean LH without changing pulse amplitude (130). Chronic elevation of serum corticosterone via the implantation of subcutaneous pellets two days prior to sampling decreased mean LH and LH-pulse frequency in OVX+E, but not OVX, mice (131). This suggests that, while corticosterone depends on estradiol sensitization to alter LH pulses, restraint stress may also act through additional, non-estradiol-dependent mechanisms. This difference also emphasizes that the totality of the stress response activated by restraint, and contributing to disruption of LH pulses, encompasses more than simply elevating serum corticosterone.

In both mice (132) and rats (133), restraint stress on proestrus that begins 7-9h after lights on and continues for 5-7h disrupts the LH surge. Some restrained rats did not show any evidence of an LH surge during the 8h span of hourly sampling from 5h before to 3h after lights out (14:10h light-dark cycle) and had no evidence of ovulation the following day (133). Others had smaller amplitude surges, with some of these rats ovulating (133). None of the restrained rats had an LH surge at the expected time the following day, indicating that stress did not simply delay the surge by one day (133). In

mice, samples for LH were only collected at a single time point around the time of the expected surge peak (132), making it more likely that this study may have missed surges with altered timing and/or lower amplitude in some mice. Of note, restraint still disrupted the LH surge in CRH knockout mice, indicating that restraint does not depend on CRH signaling to disrupt the LH surge (132).

A 5h acute layered psychosocial stress (ALPS) paradigm consisting of transport to a new cage and room, restraint, and predator odor, also disrupted the LH surge when applied on the morning of proestrus in mice (134). The mechanisms of this effect remain unknown. It does not appear that ALPS reduces estradiol levels, which would remove the signal to switch to positive feedback. Uterine mass, a proxy for estrogen exposure, was unchanged by ALPS (134). Further, in OVX+E mice exhibiting a daily surge (135), ALPS still disrupted this LH surge (134). This disruption may thus reflect changes in the ability of elevated estradiol to induce positive feedback.

Hypothalamic synaptic physiology and the switch to positive feedback

To examine how ALPS could be disrupting the LH surge, it is necessary to first understand the typical physiological changes that accompany the switch to positive feedback. GnRH neurons only express detectable levels of estradiol receptor-β (ER-β) and not ER-α (136). Yet, the LH surge is disrupted in ER-α knockout mice, but not in ER-β knockout mice (137). Despite lacking this critical ER-α receptor, GnRH neurons exhibit clear physiological changes around the time of the surge. Their firing rate increases on the afternoon of proestrus (138), due at least in part to increased neuronal excitability (139). The signals to induce positive feedback are likely conveyed to GnRH

neurons by an estradiol-sensitive, upstream network. GnRH neurons receive an increased frequency of GABAergic postsynaptic currents (PSCs) on the afternoon of proestrous (139). In OVX+E mice that express a daily LH surge, the GnRH neuron firing rate (138), excitability (139), and frequency of GABA PSCs (140) are all increased in the afternoon, suggesting that estradiol is responsible for these changes during proestrous. GABA excites GnRH neurons as these cells maintain a high internal chloride concentration in adults (141). Anatomic (142,143) and functional (144,145) data point to GABA as the main fast synaptic input to GnRH neurons. At the start of this project, it was unknown if ALPS alters the GABAergic input to GnRH neurons; this is assessed in Chapter 2.

A possible source of this GABAergic input is AVPV kisspeptin neurons, which use GABA as a co-transmitter (146,147). AVPV kisspeptin neurons could also be a locus of estradiol feedback, as most of these neurons also express ER-α (146,148). The number of *Kiss1*-expressing cells in the AVPV decreases with OVX and is restored in OVX+E mice; this estradiol sensitivity depends on ER-α (148). Knocking down ER-α specifically in AVPV kisspeptin neurons blunts the LH surge in both proestrous mice and OVX+E mice with a daily surge (149), further solidifying the role of these kisspeptin neurons in the switch to positive feedback and the GnRH/LH surge.

Interactions between early-life and adult stress on reproductive outcomes

For this dissertation, we were interested in how LBN would shape the response to ALPS in adulthood. Studies of LPS in both early-life and adulthood offer an example of how two hits of stress could affect reproductive outcomes such as LH release. Prior to any

treatment in adulthood, the LH interpulse interval of OVX+E rats that received saline or LPS as neonates (PND3+5) were similar (50). LPS exposure in adulthood increased the LH interpulse interval for rats treated with saline as neonates, and the increase was even longer in pups treated with LPS as neonates (50). This indicates that the rats with early-life LPS exposure were more susceptible to the disruption of LH pulses when exposed again in adulthood.

The timing of this LPS early-life stress appears to be important for determining how it shapes the response to a second exposure to LPS in adulthood. In male rats, LPS on PND 15 and PND25, but not on PND10, led to a decrease in LH concentrations when measured 2h later (48). This suggests male rats on PND10 were experiencing a hyporesponsive period for this immunological stressor. Further, when exposed to LPS in adulthood, control rats and those with LPS exposure on PND25 had depressed LH concentrations when measured at a single time point 2h later. In contrast, rats with LPS exposure on PND10 did not exhibit depressed LH concentrations following adult LPS treatment (48), suggesting that LPS exposure on PND10 can reprogram the response to a similar stressor in adulthood. In this case, the early-life LPS exposure appeared to induce a form of resilience to LH suppression upon re-exposure to LPS in adulthood. This highlights the complexities and challenges of trying to predict reproductive consequences to two-hits of stress at different life stages.

Stimulatory effects of stress on the reproductive axis

Though most instances of stress exert inhibitory actions on the reproductive axis, there are substantive examples of stimulatory effects of stress. Approximately one third of

OVX rhesus monkeys exhibited a premature volley of hypothalamic multiunit activity at the start of IV CRH infusion (94). LH-pulse frequency was also increased in some studies of OVX (150) and OVX+E (151) ewes after ICV administration of CRH. LH-pulse amplitude and mean LH were also increased by ICV CRH in orchidectomized rams with or without testosterone replacement (152).

One possible explanation for these variable effects of CRH is that different modes of administration and concentrations could lead to activation of different CRHRs. CRHR1 and CRHR2 have different affinities for CRH; CRHR1 binds CRH with a much higher affinity, meaning that it can be activated by lower concentrations of the peptide than CRHR2 (153). The excitatory and inhibitory effects of CRH may be mediated by CRHR1 and CRHR2, respectively. Lower concentrations of CRH increased the firing rate of GnRH neurons in *ex vivo* slice preparations from OVX+E mice, whereas higher concentrations decreased the firing rate of GnRH neurons (154). This dose-dependent effect is due to activation of distinct receptors, as a CRHR1 agonist also increased firing of GnRH neurons, whereas a CRHR2 agonist decreased firing (154). It is also important to note that CRH only affected the firing rate of GnRH neurons from OVX+E mice, and not from OVX mice without steroid replacement (154), further demonstrating the permissive effect of estradiol in the interactions between the stress and reproductive systems.

Stress-related changes in synaptic physiology

Given our goal of testing if ALPS alters GABAergic input to GnRH neurons, it is helpful to consider if and how stressors alter synaptic physiology in other brain regions. This

overview will be limited to the physiology of parvocellular neurons in the PVN, which include CRH neurons, but there are also documented changes following stress in regions such as the hippocampus and amygdala, as reviewed in Tasker and Joëls (155). In basal conditions, there is tonically active, inhibitory GABAergic input to CRH neurons (155). Restraint stress (60min) or an injection of corticosterone 60min before decapitation both reduced the frequency of GABAergic PSCs recorded in the presence of tetrodotoxin (mini-PSCs, mPSCs) in parvocellular PVN neurons; neither amplitude nor the decay time constant of PSCs were altered by restraint or corticosterone (156). Incubation of hypothalamic slices in corticosterone for 20min similarly decreased mPSC frequency in these cells (156). This indicates that the decreased frequency from the *in vivo* restraint or corticosterone exposure is likely due to local actions of corticosterone in the hypothalamus, and not secondary to peripheral changes or altered activity in non-hypothalamic regions.

The tonic inhibition within the PVN is mediated by $GABA_A$ receptors, as introduction of the $GABA_A$ receptor antagonist bicuculline directly to the PVN results in a rapid increase in plasma corticosterone in control animals (157). Bicuculline does not elevate plasma corticosterone in rats after restraint stress (30min), indicative of changes to the inhibitory tone of GABA following stress. $GABA_A$ receptors are chloride channels, and thus, the outcome following activation of these receptors depends on the membrane potential and the chloride concentration gradient between the intracellular and extracellular environments (158). After 30min of restraint stress, the reversal potential for GABA in parvocellular PVN neurons is depolarized; this effect appears to be dependent on reducing activity of KCC2, a potassium-chloride cotransporter (157).

To test if this depolarization is sufficient to lead to excitation, the researchers used synaptic stimulation of GABAergic inputs at various frequencies while conducting cell-attached recordings in parvocellular PVN neurons. Whereas 10Hz stimulation for 1s did not change the firing frequency in cells from control animals, the firing frequency of cells from stressed animals increased (157). Stimulation at 20Hz decreased firing in control cells, while again increasing firing in stress cells (157). These experiments suggest that GABA might excite parvocellular PVN neurons under certain conditions, but it is also possible that this stimulation protocol released other neuropeptides that could be partially responsible for the observed effect.

These studies are an important demonstration that acute stress exposure can lead to changes in synaptic physiology that persist in *ex vivo* slice preparations up to 5h later. They also demonstrate a known effect of stress to alter GABAergic signaling within the hypothalamus. Thus, if ALPS alters GABAergic input to GnRH neurons on proestrus, we should be able to observe these changes in a similar *ex vivo* preparation.

Dissertation preview

The goal of this dissertation was to assess the independent effects of and interactions between early-life and adult stress on reproductive outcomes. Chapter 2 details the primary experiments that addressed this goal. We test the consequences of early-life stress in the form of LBN from PND4-11 on external markers of sexual maturation in males and females, and on estrous cyclicity in females. Experiments then assess how LBN affects the corticosterone response to ALPS in males and diestrous and proestrous females. The effects and interactions of LBN and ALPS on the proestrous LH surge are

also determined. Finally, electrophysiological experiments test the hypothesis that ALPS decreases the excitatory GABAergic input to GnRH neurons on the afternoon of proestrus. Chapter 3 contextualizes the results of these experiments within the broader literature and proposes future experiments to address the questions generated by this work.

Table 1.1. Summary of reproductive effects of stress studies in non-human primates.

		Immunological stressors	Glucocorticoids	CRH	Psychosocial stressors
Mean GnRH and/or LH	No change				(98)
	Decrease		(88)	(90,91)	(97–99)
GnRH and/or LH amplitude	No change	(95)			(97)
	Decrease				(96)
GnRH and/or LH pulse frequency	Increase			(94)	
	No change				(96)
	Decrease	(95)		(90,91,94)	

Table 1.2. Summary of reproductive effects of stress studies in sheep.

		Immuno-logical stressors	Glucocorticoids	CRH	Psychosocial stressors
Mean GnRH and/or LH	Increase			(151,152)	
	No change			(150,151,159)	(113)
	Decrease	(100)	(104,105,109)		(112–114)
GnRH and/or LH amplitude	Increase		(106,109)	(152)	
	No change		(105,106,109)	(150,151,159)	(113)
	Decrease	(100)	(105,106)		(112–115)
GnRH and/or LH pulse frequency	Increase			(150,151)	
	No change	(100)		(150–152,159)	(113–115)
	Decrease	(100,160)	(104,106,107,109)		(112,113,161)
Pituitary responsiveness to GnRH	No change				(113)
	Decrease	(101)	(105,108)		(113,114,116)
LH surge and/or ovulation incidence	No change	(102)	(110)		(117)
	Decrease	(102,103)	(109,110)	(162)	
LH surge timing	No change	(102)			(161)
	Delay	(102,103)	(106,109,111)		
LH surge amp	No change		(106)		
	Decrease	(103)			

Table 1.3. Summary of reproductive effects of stress studies in rats.

		Immunological stressors	Glucocorticoids	CRH	Psychosocial stressors
Mean GnRH and/or LH	No change			(163)	
	Decrease	(123,164,165)	(166)	(163)	
GnRH and/or LH amplitude	Decrease			(167)	
GnRH and/or LH pulse frequency	No change		(118)	(168)	
	Decrease	(118–121)		(118,168)	(118–122)
Pituitary responsiveness to GnRH	No change			(163)	
	Decrease			(166)	
LH surge and/or ovulation incidence	Decrease	(169)		(163)	(133)
LH surge timing	Delay			(170)	
LH surge amp	Decrease			(170)	(133)

Table 1.4. Summary of reproductive effects of stress studies in mice.

		Glucocorticoids	Psychosocial stressors
Mean GnRH and/or LH	No change	(131)	
	Decrease	(131)	(130)
GnRH and/or LH amplitude	No change	(131)	(130)
GnRH and/or LH pulse frequency	No change	(131)	
	Decrease	(131)	(130)
Pituitary responsiveness to GnRH	No change	(131)	
LH surge and/or ovulation incidence	Decrease	(171)	(132,134)

Chapter 2 Limited Bedding and Nesting Does Not Alter Adult Corticosterone Response to Acute Psychosocial Stress in Male or Female Mice or the Stress-Induced Disruption of the Preovulatory Luteinizing Hormone Surge

Co-authored with Suzanne M. Moenter

Abstract

Early-life stressors can affect the development of the reproductive system and change the responses to adult stress. We tested the hypotheses that limited bedding and nesting (LBN) from postnatal days 4-11 would delay sexual maturation in male and female mice, and would alter the response to an acute, layered, psychosocial stress (ALPS) in adulthood. LBN dams exited the nest more often, but contrary to the hypotheses, the age and mass at vaginal opening, first estrus, and preputial separation were not affected by LBN. Further, basal corticosterone concentrations were similar between males, diestrous females, or proestrous females reared in standard or LBN environments. After exposure to ALPS, serum corticosterone concentrations were also similar between standard and LBN reared offspring. ALPS disrupts the luteinizing hormone (LH) surge in most mice when applied on the morning of proestrus; this effect is not changed by LBN. To test if ALPS disrupts the LH surge by blunting the observed increase in excitatory GABAergic input to gonadotropin-releasing hormone (GnRH) neurons on the afternoon of proestrus, whole-cell voltage-clamp recordings were conducted following ALPS treatment. The frequency of GABAergic postsynaptic currents (PSCs) in GnRH neurons was not altered by LBN, ALPS, or their interaction.

This study suggests LBN did not confer either susceptibility or resilience to ALPS and leaves open the possibilities that ALPS acts at other neuronal populations upstream of GnRH neurons, changes the response of GnRH neurons to input, or alters pituitary responsiveness to GnRH.

Significance Statement

The stress and reproductive neuroendocrine systems interact, and early-life stress has reproductive consequences in humans. This study in mice rejected the hypotheses that an early-life stress, limited bedding and nesting (LBN), would delay sexual maturation and alter the response to an acute, layered, psychosocial stress (ALPS) in adulthood. ALPS disrupts the proestrous luteinizing hormone (LH) surge, which is critical for ovulation; this disruption is not altered by LBN. To assess a possible mechanism for this disruption, we conducted electrophysiological recording of gonadotropin-releasing hormone neurons to test if ALPS reduces excitatory GABAergic input to these cells. The frequency of GABAergic input was similar among groups, suggesting that LBN and ALPS act elsewhere in the broader neuroendocrine network controlling reproduction.

Introduction

The neuroendocrine systems regulating stress and reproduction are important for organisms to respond to their environments and ensure the continuation of the species. Gonadotropin-releasing hormone (GnRH) neurons in the hypothalamus integrate many inputs and serve as the final common central output to the downstream reproductive axis. GnRH is released in a pulsatile manner and acts on the pituitary to stimulate the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (10), which

activate gametogenesis and steroidogenesis. In males and during most of the female reproductive cycle, sex steroids exert negative feedback to reduce overall GnRH and LH concentrations. Sustained elevated estradiol concentrations in the preovulatory period (proestrus in rodents), exerts positive feedback to induce prolonged surges of GnRH and LH release (17–20). The LH surge triggers ovulation (21). The organization of the neuroendocrine stress axis is similar, with corticotropin-releasing hormone (CRH) release from hypothalamic neurons stimulating secretion of adrenocorticotropic hormone (ACTH) from the pituitary, and the adrenal cortex producing glucocorticoids that provide negative feedback at the brain and pituitary (27).

Neuroendocrine axes interact with one another. In humans, stress has clinical effects on the reproductive system with social consequences (172). Perceived stress in adulthood can reduce likelihood of both natural conception and pregnancy via assisted reproductive technology (26,81,82). Early-life stress can disrupt reproductive development, though whether stress delays or advances puberty appears to depend on the type and timing of stressor (30–33). In humans, it is challenging to disentangle the independent effects of early-life and adult stress on reproduction from each other and from other factors, and to understand how a history of early-life stress affects the response to adult stress. These questions can be better addressed using animal models.

A common model for early-life stress in rodents is the limited bedding and nesting (LBN) paradigm in which dams and pups are moved to a low-resource environment for several days; this alters the way the dam interacts with the pup without ongoing investigator interference (38,75). Effects of LBN on reproductive outcomes have been mixed in both

mice and rats. LBN delayed the age at vaginal opening, an external indicator of puberty, in some studies (56,173), advanced it in another (174), and had no effect in others (57,175). Some studies found a delay in preputial separation in males (57,173,174). There are many models of adult stress including psychosocial (e.g., restraint), metabolic (e.g., hypoglycemia) and immune (e.g., endotoxin) (176–178). In adult mice, exposure to an acute, layered, psychosocial stress (ALPS) paradigm on the morning of proestrus disrupts the preovulatory (proestrous) LH surge in most mice (134). The mechanisms by which ALPS leads to this disruption are not known, but do not include disrupting the preovulatory estradiol rise. With regard to possible mechanisms, the rate of GABAergic transmission to GnRH neurons (which is excitatory in these cells, DeFazio et al., 2002) increases on the afternoon of proestrus (139) and around the onset of the estradiol-induced LH surge (140). The increase in GABAergic postsynaptic currents (PSCs) could account for the increased activity in GnRH neurons during the surge. Stressors alter GABAergic signaling within other hypothalamic regions. For example, restraint stress reduced the frequency of GABA transmission in parvocellular neurons in the paraventricular nucleus (156). A reduction in GABAergic input to GnRH neurons following ALPS could help explain the disruption to the LH surge by this psychosocial stressor.

Early-life stress can affect the response to adult stressors, though whether animals are more susceptible or resilient depends on the type of stressors and the outcomes measured (179). In the present study, we investigated the effect of LBN on reproductive maturation and adult response to stress in male and female mice. We also tested the hypothesis that ALPS disrupts the LH surge by reducing the excitatory input to GnRH

neurons on the afternoon of proestrus and determined if LBN altered ability of ALPS to disrupt the LH surge.

Materials and Methods

Animals

All animal procedures were approved by the University of Michigan Institutional Animal Care and Use Committee. Mice had *ad libitum* access to water and food; breeders were provided Teklad 2919 chow (Inotiv) through weaning. After weaning, mice were maintained on Teklad 2916 (Inotiv). The vivarium followed a 14/10h light-dark cycle (lights on at 3AM EST); at times, dim red light (<10 lux) was used overnight. A male GnRH-GFP (Tg(Gnrh1-EGFP)51Sumo MGI:6158457, C57Bl6/J background) mouse expressing GFP under control of the GnRH promoter (180) was housed with one or two female CBA mice (Strain #000656; The Jackson Laboratory) in cages containing 550-650 mL of corn cob bedding (Bed-o'Cobs ¼, The Andersons). Female body mass was measured on the day of pairing with the male. Females were examined for vaginal plugs for up to 5d after pairing and their body mass measured again around hypothetical gestational day 12-14. An increased body mass (>110% of initial mass) was used as an initial confirmation of pregnancy, at which point females were moved to individual cages with 550-650 mL bedding, and a Nestlet (Ancare) and plastic igloo (Bio-Serv) for enrichment.

Experimental design

This study assessed the independent effects of and interactions between early-life and adult stress on reproduction. Pilot studies were conducted to examine timing of LBN

treatment and effects of litter size. Based on these, offspring from the first litter of a dam were studied, and litter size was normalized to 5-8 pups per mouse as small litters had early vaginal opening whereas large litters were delayed as has been observed (58). Dams and litters were assigned to either standard (STD) housing or LBN treatment as an early-life stress. Body mass of individual offspring was monitored after early-life treatment through adulthood. To determine if LBN affected external markers of reproductive maturation, offspring were monitored for preputial separation (male) or vaginal opening and first appearance of estrus, as indicated by cornification of the vaginal epithelium (female) (181). Estrous cycles of female offspring in adulthood were monitored to test if cycles were disrupted by LBN.

To evaluate if LBN alters the response to an adult stressor, adult offspring were assigned to either control (CON) or ALPS treatment, resulting in four experimental groups (STD-CON, STD-ALPS, LBN-CON, LBN-ALPS). Block assignment within litters and minimization strategies were used to balance adult treatments. Serum corticosterone concentration was assessed in males and in diestrous and proestrous females to determine if cycle stage altered baseline corticosterone concentration or corticosterone rise in response to stress. In proestrous females, we also assessed if a history of LBN affected the disruptions of the LH surge by ALPS. To investigate the mechanisms by which ALPS disrupts the surge, GABAergic transmission to GnRH neurons was monitored using whole-cell voltage-clamp around the time of the typical LH surge.

Limited bedding and nesting (LBN) paradigm

LBN was used to model early life stress. STD cages contained 550-650mL of bedding and one full 5x5 cm square of Nestlet material. LBN cages contained ~100 mL of bedding, enough to cover the floor of the cage with a single layer, and a 2.5 x 5 cm piece of Nestlet. LBN cages were fitted with a wire mesh platform ~1.5cm above the cage floor (allFENZ 23-Gauge Hardware Cloth, Home Depot). Food was provided in a small container placed on the floor of the cage to prevent pups crawling into the food hopper from the raised platform, and to permit unobstructed video monitoring of dam behavior. Food was replenished daily.

Females were monitored for births daily before lights off beginning 19d after observation of vaginal plug or after pairing with a male if no plug was noted. The day of birth was designated postnatal day (PND) 0. Pups (CBB6/F1 hybrids) from litters born within one day of each other were cross-fostered if needed to standardize litter sizes to 5-8 pups by PND2. The STD or LBN treatment period began on PND4. From PND4 through PND11, animals were undisturbed in their respective treatment cages apart from transferring for video monitoring and daily replenishment of food and water. On the morning of PND11, a tail blood sample was collected from the dam for assessment of serum corticosterone concentration, then all dams and litters were transferred to clean cages containing 550-650 mL of bedding and a 5 x 5 cm Nestlet until weaning at PND21. All offspring were weaned with same-sex littermates into standard cages with the addition of a plastic igloo; the igloo was removed on PND28.

Dam behavior monitoring

Continuous video monitoring occurred for either 24h from the morning of PND5 to the morning of PND6, 48h from the morning of PND4 to PND6, or for the duration of the early-life treatment from PND4-11. The number of exits made by the dam from the nest were manually counted for 1h periods beginning at Zeitgeber times (ZT) 1 (4AM EST), ZT15 (6PM EST), and ZT19 (10PM EST). The average number of exits and amount of time off the nest per hour was calculated for each dam. Recordings were conducted using ffmpeg (version 4.3.1) on the following computers: MacBook Air (Mid 2009, running OS X El Capitan, Version 10.11.6, with a 2.13 GHz Intel Core 2 Duo), MacBook Pro (Mid 2012, running macOS Catalina, Version 10.15.7, with a 2.5 GHz Dual-Core Intel Core i5), MacBook Pro (Early 2015, running macOS Catalina, Version 10.15.7, with a 2.7 GHz Dual-Core Intel Core i5), Mac mini (2018, running macOS Sonoma, Version 14.0, with a 3 GHz 6-Core Intel Core i5), and a MacBook Pro (Late 2013, running macOS Mojave, Version 10.14.6, with a 2.3 GHz Intel Core i7).

Body mass

Dam mass was recorded on PND4 (start of paradigm), PND11 (end of paradigm), and PND21 (pup weaning). Before placing pups into the new cage at the start of the paradigm, the average mass for each litter was recorded to identify any potential outliers. At the end of the paradigm, on the morning of PND11, pups were ear-marked and identified with Sharpie markings on the tail to permit individual tracking of each pup. Pup mass was recorded daily through PND24 and then weekly through PND70 and as indicated below.

Offspring maturation

Beginning at weaning, offspring were checked daily for preputial separation or vaginal opening (182,183). Body mass was recorded on the day of preputial separation/vaginal opening. After vaginal opening, female mice underwent daily vaginal lavage to identify the day of first estrus based on cornification of vaginal epithelium; body mass was recorded on the day of first estrus. Anogenital distance (AGD) was measured with digital calipers (Marathon) for three consecutive days during the tenth postnatal week and averaged for each animal.

Adult female estrous cycles

To study the effect of LBN on estrous cycles, vaginal lavages were monitored daily from PND70-90 (184–186). Number of cycles (defined as the number of days in proestrus preceded by diestrus or estrus per 21-day monitoring period), mean cycle length (days between successive proestrous stages), and the percentage of days in diestrus, estrus, and proestrus are reported.

Acute, layered, psychosocial stress (ALPS) paradigm

Animals exposed to adult stress were habituated for at least two weeks beforehand to tail and general handling. Female mice were studied on either the first day of diestrus or on proestrus, as determined by vaginal cytology and confirmed by uterine mass (diestrus <100mg; proestrus >125mg) measured the same day. An acute, layered, psychosocial stress (ALPS) paradigm that disrupts the proestrous LH surge in most females was used (134); both male (>PND84) and female (≥PND90) mice were studied. At 0h (6.5h after lights on), a tail blood sample (~30µL) was collected and the serum

stored for assessment of corticosterone. In females, an additional 6μL of whole blood was obtained for LH measurement. Control mice were only removed from their home cage for tail blood sampling at times equivalent to sampling in stressed mice and remained in the vivarium. ALPS mice were placed individually into a new cage and transferred to a new room. At 1h, the mice were placed in a restraint tube (BrainTree Scientific, flat-bottom restrainer small or Tailveiner-150 restrainer). At 3h, restrained mice were exposed to a component of red fox (*Vulpes vulpes*) urine as a predator odor (2,3,5-trimethyl-3-thiazoline,TMT; ~6nmol; Contech Enterprises, Victoria, BC, Canada). Tail blood samples were collected at the end of the paradigm (5h, 2:30pm EST, 2.5h before lights out) for corticosterone, and LH in females. For females in diestrus, an additional tail blood sample for LH was collected at the time of lights out (5pm EST). For those in proestrus, additional tail blood samples to monitor the effect of LBN and/or ALPS on the LH surge were taken hourly from 3pm to 7pm EST, unless noted. Animals were considered to exhibit an LH surge if any PM value was greater than 3.8ng/ml. This threshold of 3.8ng/mL was determined from the mean+3SD of LH concentrations measured on the morning of proestrus at 0h.

Body mass was recorded at the start of ALPS and just before euthanasia. Males were transported to the laboratory at the end of the ALPS paradigm (2:30p EST). The mass of the adrenal glands, testes, and seminal vesicles was recorded and normalized to PM body mass. Diestrous females were transported to the laboratory after the LH sample at lights out, and proestrous females used for LH surge sampling were transported after the last sample 2h after lights out. Adrenal and uterine masses were recorded in females.

Corticosterone administration

To mimic serum corticosterone concentrations induced by ALPS, standard-reared males from our main colony were fed corticosterone in Nutella, a design based on ongoing studies in females in which 2mg/kg oral corticosterone at 0h, 1h, and 3h (times corresponding to the ALPS transitions) mimics the serum corticosterone pattern observed during the ALPS paradigm (187). To habituate animals to this feeding paradigm, cage mates were transferred to a holding cage and one mouse was left in the home cage with Nutella on a Petri dish for up to 5min. Mice were habituated daily for at least a week prior to the experiment. On the day of the experiment, corticosterone (2mg/kg) or 36% DMSO vehicle in Nutella (60mg Nutella mixture for a 30g mouse, range 56.2-75.2mg) was administered at 0h (9:30am EST, 6.5h after lights on), 1h, and 3h. Tail blood samples were collected as described above at 0h, 1h, 2h, 3h, 4h, and 5h after Nutella administration. Only one mouse per cage was sampled on a given day, as preliminary experiments determined that transfer to the holding cage while a cage mate consumed Nutella increased serum corticosterone concentration. Body and tissue masses were recorded for these mice as described for the ALPS paradigm.

Corticosterone enzyme immunoassay

Serum corticosterone concentrations were determined in duplicate samples diluted 1:100 by enzyme immunoassay (Arbor Assays, DetectX Corticosterone Kit, K014). Standard curves from 78.1pg/mL to 5000pg/mL or 39.0pg/mL to 10000pg/mL were run on each plate. Intraassay %CVs for standards ranged from 3-6%; functional sensitivity, defined as the lowest standard with a CV <20%, was 39.0pg/mL. The reportable range was 3.9ng/mL to 1000ng/mL.

Ultra-sensitive LH assay

At the time of tail blood collection, 6μL of whole blood was mixed with 54μL of assay buffer (0.2% BSA – 0.05% Tween 20 – PBS, pH 7.5) and immediately placed on ice for up to 3h then stored at -20°C. Samples were assayed by Center for Research in Reproduction at the University of Virginia with the Ultra-Sensitive Mouse & Rat LH ELISA method (129). The capture monoclonal antibody (anti-bovine LH beta subunit, 518B7) was provided by Janet Roser, University of California. The detection polyclonal antibody (rabbit LH antiserum, AFP240580Rb) was provided by the National Hormone and Peptide Program (NHPP). HRP-conjugated polyclonal antibody (goat anti-rabbit) was purchased from DakoCytomation (Glostrup, Denmark; D048701-2). Mouse LH reference prep (AFP5306A; NHPP) was used as the assay standard. The Limit of Quantitation (Functional Sensitivity) is defined as the lowest concentration that demonstrates accuracy within 20% of expected values and intra-assay coefficient of variation (%CV) <20% and was determined by serial dilutions of a defined sample pool. Intraassay %CV is 2.2%. Interassay %CVs were 7.3% (Low QC, 0.13 ng/mL), 5.0% (Medium QC, 0.8 ng/mL) and 6.5% (High QC, 2.3 ng/mL). Functional sensitivity was 0.016 ng/mL, and the reportable range is 0.016 ng/mL to 4.0 ng/mL. Samples were diluted 1:10, making the reportable range 0.16 ng/mL to 40 ng/mL.

Electrophysiology

A subset of the adult proestrous females was used to characterize the effect of LBN and/or ALPS on GABAergic transmission to GnRH neurons. The mouse was transported to the laboratory between 3:00-3:30PM EST (1.5-2h before lights out), and body mass recorded. All solutions were bubbled with 95% O_2/5% CO_2 for at least

15 min before tissue exposure and throughout the procedures; chemicals were purchased from Sigma-Aldrich unless noted. The brain was rapidly removed and placed in ice-cold sucrose saline solution containing the following in mM: 250 sucrose, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 3.8 $MgCl_2$. Coronal brain slices (300 μM) containing the preoptic area and GnRH neurons were prepared in the sucrose saline solution with a vibratome (VT1200S, Leica Biosystems). Slices were incubated at room temperature for 30 min in a 50%-50% mixture of the sucrose saline and artificial cerebrospinal fluid (ACSF) containing the following in mM: 135 NaCl, 3.5 KCl, 26 $NaHCO_3$, 10 D-glucose, 1.25 Na_2HPO_4, 1.2 $MgSO_4$, and 2.5 $CaCl_2$ (pH 7.4). Slices were then incubated for 0.5 to 5h in 100% ACSF before being transferred to the recording chamber mounted to an Olympus BX51WI upright fluorescent microscope.

Slices in the recording chamber were perfused (3-5mL/min) with ACSF via a MINIPULS 3 peristaltic pump (Gilson). GABAergic PSCs were isolated by blocking ionotropic glutamate receptors with CNQX (10 μM) and D-APV (20 μM). Solution temperature was maintained between 29–32°C with an inline heating system (Warner Instrument Corporation). Individual GFP-positive GnRH neurons were visualized using infrared differential interference contrast and brief illumination with fluorescence microscopy. The recording pipette was filled with a high-chloride internal solution containing (in mM): 140 KCl, 10 HEPES, 5 EGTA, 0.1 $CaCl_2$, 4 MgATP, and 0.4 NaGTP. A high-resistance (>1GΩ) seal was made between the cell membrane and the pipette, and then the whole-cell configuration achieved. The cell was held at -65 mV in voltage-clamp mode, and recording quality was monitored by averaging the response to 16 hyperpolarizing

voltage steps (5mV, 20ms, acquisition 100kHz, filter 10kHz). GABAergic PSCs were recorded during 2-3 min series (acquisition 10kHz, filter 5kHz).

Custom routines in Igor Pro (WaveMetrics) were used to detect PSCs, which were manually confirmed. The frequency of PSCs (# events / recording duration) was determined for each cell. For each event, the interevent interval was calculated, defined as the backwards interval from the time of that event's peak to the time of peak for the previous event. The cumulative probability distributions of interevent interval for each treatment group were also calculated. The true interevent interval for the first event in a recording is unknown and thus not included in calculations of interevent interval. For events with an interevent interval of at least 200ms, the amplitude (absolute value of the difference between the peak and baseline) was determined, and cumulative probabilities of amplitudes calculated for each group; this analysis included first events preceded by >200ms of recording time). Isolated events (>200ms interval in both the forwards and backwards direction between adjacent event peaks) were selected and averaged by cell. These averaged traces were used to estimate the decay time from 80% to 20% of the peak for each cell.

Statistical analysis

Statistical analysis was conducted in R (188); statistical packages used included rstatix (189), lme4 (190), lmerTest (191), lspline (192), afex (193), emmeans (194), and nparLD (195). Plots were made with the packages ggplot2 (196) and cowplot (197). Tables were made with the flextable package (198).

Linear mixed models (LMMs) were used to analyze these data as these models can account for the dependencies among data attributable to experimental design and permit missing data (199). For example, litter was included as a random effect for outcomes measured in offspring, as mice within a litter are not fully independent from one another, violating assumptions of more traditional tests such as ANOVAs. The type of test for each outcome measure and associated figure are in Table 2.1. Type III tests with effects coding were used, as recommended by Singmann and Kellen (199). The Kenward-Roger approximation was used for estimation of degrees of freedom for linear mixed models. *Post hoc* comparisons were made using pairwise tests of estimated marginal means (emmeans). For multiple pairwise comparisons, p values were adjusted using Holm's method; confidence intervals could only be adjusted using the more conservative Bonferroni method with this package in R. The pairwise comparisons for all tests are in Table 2.2.

Residual and Q-Q (Quantile-Quantile) plots were used to check the assumptions of models, alternative models were selected when available if assumptions were not met, as reported in Table 2.1. Nonparametric longitudinal analysis has been used to assess mouse behavioral data across time (195,200) and was used to analyze the number of exits that the dams made from the nest over time. Because the ultra-sensitive LH assay can report a maximum concentration of 40ng/mL and many of our samples in proestrous mice exceeded this concentration, data for this parameter were not normally distributed and an accurate estimate of the effect of stress on absolute LH concentrations was precluded. We instead focused on the binary outcome of whether or not the mouse exhibited an LH surge (at least one measurement ≥3.8ng/mL) and fit

these data with a generalized linear mixed-effects model for the binomial logistic regression family. The sample sizes and the low variance in the adult control groups, because of the high likelihood of observing a surge in those groups, precluded the model from adequately estimating the interaction term of early-life treatment (LBN) and adult treatment (ALPS). Thus, the formula was simplified to consider only the independent main effects of each stressor.

The number of PSCs was fit with a generalized linear mixed-effects model for the negative binomial family to handle count data with meaningful zeros (no observed PSCs during recording period). To assess if early-life or adult treatment affected PSC properties (interval and amplitude), the distributions from all four treatment groups were compared with the Anderson-Darling test using the kSamples package in R (201). If the test comparing all four distributions was significant, *post hoc* Anderson-Darling tests comparing the (a) STD-CON and STD-ALPS, (b) LBN-CON and LBN-ALPS, (c) STD-CON and LBN-CON, and (d) STD-ALPS and LBN-ALPS groups were conducted and p-values were adjusted for multiple comparisons. To help with interpretation of the comparison of these distributions, a bootstrapping approach was used to estimate the 95% confidence interval of the mean of each treatment group and the difference in means for the four comparisons described just above (a-d). This approach, inspired by Ho et al. (202), was necessitated by the non-normality of the distributions and adapted using custom code to account for the experimental design. Briefly, the dataset was resampled 5000 times; for each resampling iteration, the mean of each treatment group was calculated, as was the difference in the means for the comparisons described above (a-d). The average of these group means and differences in means were

calculated for the 5000 iterations. The boundaries for the 95% confidence intervals, or percentile intervals, were found by ranking the group's mean estimates for all 5000 iterations and selecting the 2.5th percentile and the 97.5th percentile as the lower and upper bounds of the interval, respectively; the same process was applied to obtain a confidence interval for the differences in means.

Code accessibility

The PSC detection and analysis code used is freely available online at https://gitlab.com/um-mip/coding-project/. The ffmpeg and R analysis code is freely available online at https://github.com/gibson-amandag/LBN. Analyses were conducted on a Lenovo Yoga 9, 11th Gen Intel Core i7, running Windows 11 Home.

Results

LBN dams exited the nest more frequently

LBN was applied from PND4-11 (Figure 2.1A, numbers in Table 2.1.1). LBN dams had a higher body mass than STD dams at PND4, 11, and 21 (Figure 2.1B, Table 2.1.2, STD: n = 25, LBN: n = 24, Table 2.2, row 1: $p = 0.033$). Regardless of treatment, dams gained body mass during the paradigm (Table 2.2, row 2: $p < 0.001$) and decreased body mass between PND11 and 21 (Table 2.2, row 3: $p = 0.007$). There were no differences in morning serum corticosterone concentration between STD and LBN dams on PND11, indicating LBN did not chronically elevate this hormone in dams (Figure 2.1C, STD: n = 24, LBN: n = 24, $t_{46} = -1.67$, $p = 0.102$; difference (STD-LBN) = -9.08; 95% CI = [-20.04, 1.88]; Cohen's d = -0.48). This may indicate the LBN phenotype is milder in CBA dams than in other strains. Dam behavior was captured on video during

the paradigm. The number of exits that each dam made from the nest was scored for one-hour periods beginning at ZT1, 15, and 19 then averaged for each postnatal day (STD: n = 19-25, LBN: n = 19-24). LBN dams had more exits than STD dams throughout the paradigm (Figure 2.1D, warmer colors indicate dams with more exits over time, Table 2.1.2, $p < 0.001$). Interestingly, there was no difference in the percentage of time that STD and LBN dams spent on the nest (Figure 2.1E, Table 2.1.2, $p = 0.156$). Together, these observations suggest that LBN dams have more fragmented interactions with the pups.

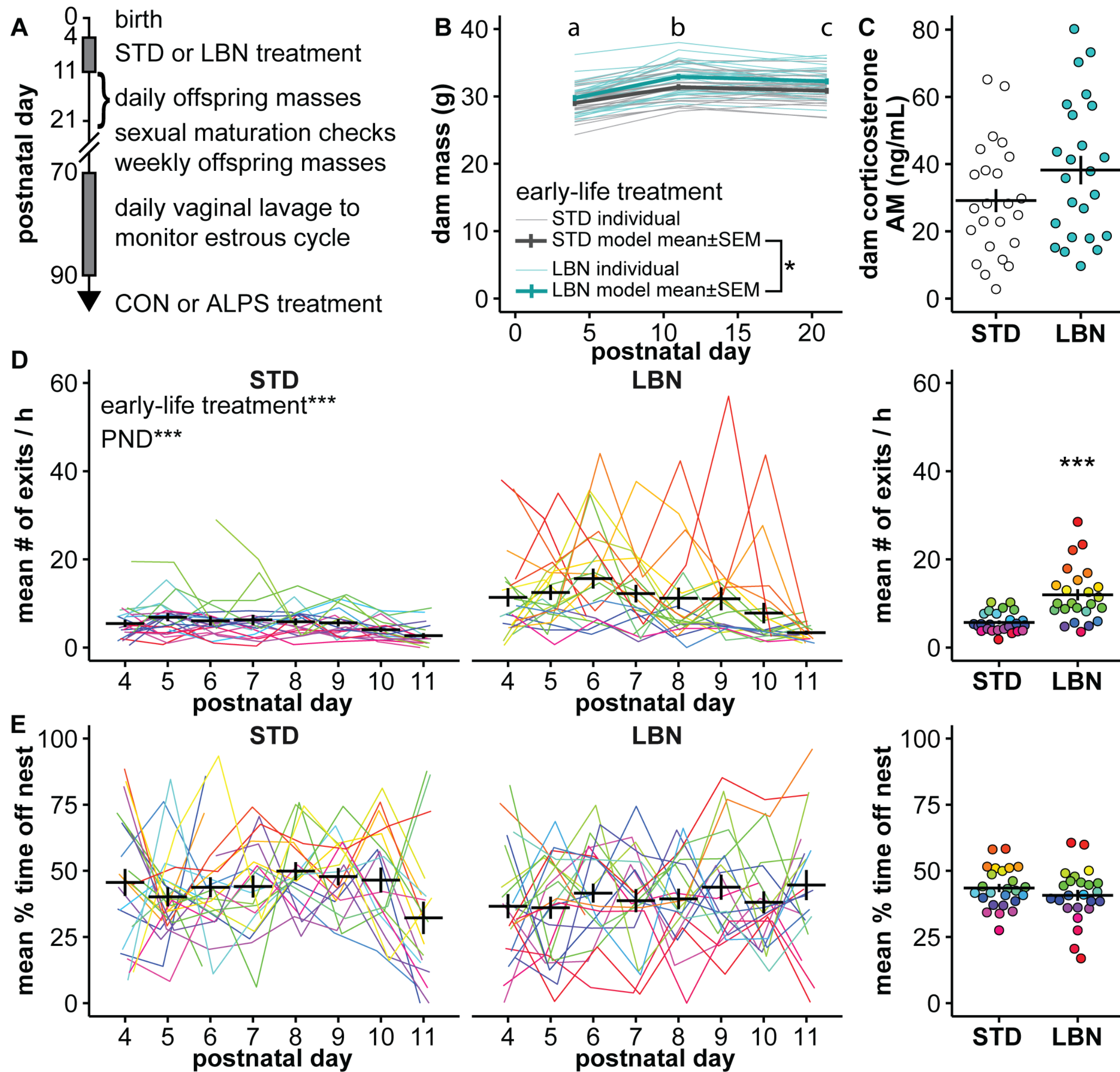

Figure 2.1. The LBN paradigm altered dam behavior. **A**. Experimental timeline. **B**. Dam mass before and after the paradigm and at weaning on PND21. Thin lines are individual dams, LMM model mean±SEM shown in thick lines. Letters (a-c) indicate that dam mass, combined across treatment groups, differed on each postnatal day ($p < 0.01$). **C**. Individual values and mean±SEM serum corticosterone from the dams at the end of the paradigm on the morning of PND11. **D**. (left) The number of exits averaged by PND for individual dams are shown by colored lines; warmer colors indicate more exits, mean±SEM number of nest exits versus PND is in black. (right) Individual dam averages and mean±SEM number of nest exits; color is consistent with left graph. **E**. (left) The percentage of time spent off nest averaged by PND for individual dam are shown by colored lines; warmer colors indicated more time off the nest, mean±SEM percentage of time off the nest versus PND is in black. (right) Individual dam averages and mean±SEM percentage of time off nest; color is consistent with left graph. Some error bars are obscured by mean line. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Numbers in Table 2.1.1. Full statistical model results are in Table 2.1.2. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; CON, adult control treatment; ALPS, acute layered psychosocial stress; PND, postnatal day.

LBN affected pup mass

Prior to treatment on PND4, there were no differences in offspring body mass between the litters that would receive STD (mean±SEM: 2.8g±0.06, 25 litters) and LBN (2.9g±0.06, 24 litters) treatment (Figure 2.2A, $t_{47} = -1.18$, $p = 0.245$; difference (STD-LBN) = -0.10; 95% CI = [-0.27, 0.07]; Cohen's d = -0.34). After treatment on PND11, LBN offspring were smaller than STD offspring (Figure 2.2B, Table 2.2.1, Table 2.2, row 5, $p = 0.006$; STD: 74 females and 80 males; LBN: 74 females and 58 males). Offspring sex did not affect mass at PND11 ($p = 0.616$) or interact with treatment ($p = 0.124$); the overall litter averages are thus displayed in Figure 2.2B. This demonstrates that mass gain during the treatment window was slower in LBN offspring, consistent with prior studies of this early-life stressor.

We continued recording offspring mass into adulthood to test if there was an effect of rearing conditions on subsequent growth, creating separate linear models of growth for female and male offspring. LBN treatment did not alter body mass growth in females (Figure 2.2C, Table 2.2.2, STD: post-weaning: 24 litters and 74 mice; LBN: post-weaning: 22 litters and 73 mice). In contrast in males, LBN treatment altered the overall pattern of growth (Figure 2.2C, Table 2.2.2, $p < 0.001$, STD: post-weaning: 19 litters and 63 mice; LBN: post-weaning 14 litters and 42 mice). To understand how LBN changed the trajectory of growth, we conducted *post hoc* comparisons of body mass at discrete days throughout development (PND11, 21, 35, 56, and 72). The masses were not different through PND35 (PND11, Table 2.2, row 6: $p = 0.129$; PND 21, Table 2.2, row 7: $p = 0.129$; PND 35, Table 2.2, row 8: $p = 0.121$). As males transitioned from adolescence to adulthood, the LBN mice appeared to gain mass more slowly, and by

PND56, the LBN mice were about 1g smaller than STD mice (Table 2.2, row 9: p = 0.045) with the difference persisting into the end of the observation period at PND72 (Table 2.2, row 10: p = 0.024).

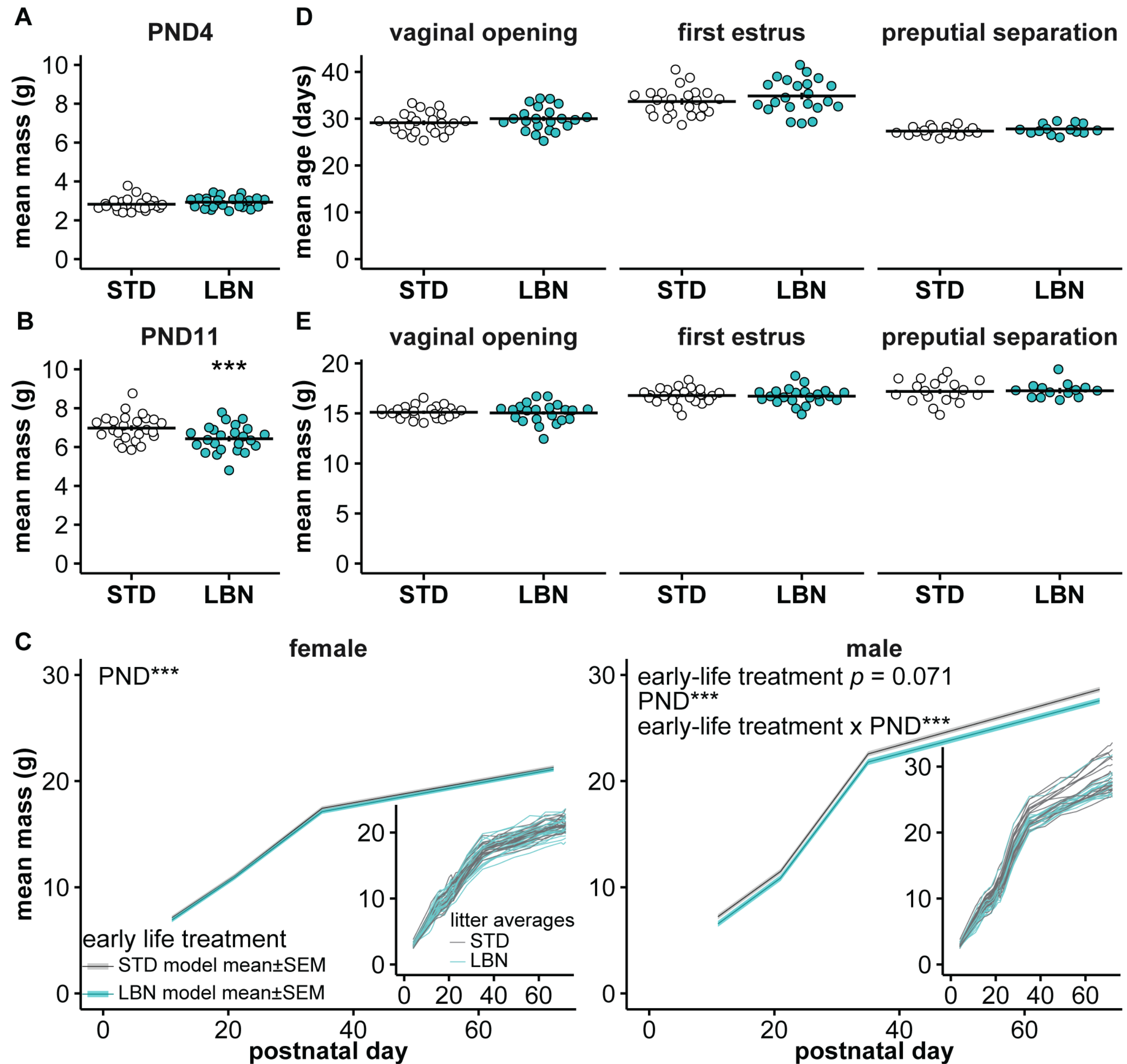

Figure 2.2. LBN decreased PND11 body mass and lowered body mass in adult males but did not affect reproductive maturation.**A**. Mean litter values and mean±SEM for PND4 mass. **B**. Mean litter values (both sexes) and model mean±SEM for PND11 mass. **C**. Statistical model mean±SEM for the body mass of the female (left) and male (right) offspring. The average mass of each litter is plotted in the insets. **D-E**. Mean litter values and model mean±SEM for age (**D**) at vaginal opening (left), first estrus (center), and preputial separation (right); for mass (**E**) at vaginal opening (left), first estrus (center) and preputial separation (right). Some error bars are obscured by mean line. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Full statistical model results are in Tables 2.2.1 (mass at PND11), 2.2.2 (mass from PND11-72), and 2.2.3 (maturation). Abbreviations: STD, standard-reared; LBN, limited bedding and nesting.

LBN did not alter reproductive maturation or estrous cycles

To assess if LBN altered external markers of reproductive maturation, the age and mass at vaginal opening and first estrus were monitored in females (STD: 24 litters and 74 mice; LBN: 22 litters and 73 mice, unless otherwise noted below); age and mass at preputial separation were monitored in males (STD: 19 litters and 63 mice; LBN: 14 litters and 41 mice). LBN did not affect age at vaginal opening (Figure 2.2C, Table 2.2.3, $p = 0.217$) or first estrus ($p = 0.221$), and there were no differences in body mass at these milestones (Figure 2.2D, Table 2.2.3; vaginal opening: $p = 0.754$; first estrus $p = 0.758$, STD: 23 litters and 70 mice, LBN: 22 litters and 73 mice). Similarly, the age (Figure 2.2C, Table 2.2.3, $p = 0.177$) and mass (Figure 2.2D, Table 2.2.3, $p = 0.846$) at preputial separation were not affected by LBN. Adult anogenital distance was not affected by LBN in either sex (model mean±SEM (mm): female STD: 6.4±0.08; female LBN: 6.3±0.09; male STD: 16.8±0.09; male LBN: 16.6±0.1; Table 2.2.1, $p = 0.257$), but the typical increased AGD in males vs females was observed (Table 2.2.1, Table 2.2, row 11: $p < 0.001$). To test if LBN altered estrous cycles, daily vaginal lavages were obtained from PND70-90 (STD: 23 litters and 73 mice; LBN: 22 litters and 73 mice). Figure 2.3A shows representative estrous cycles from both groups. LBN had no effect on the number of estrous cycles (Figure 2.3B, Table 2.3.1, $p = 0.359$) or features of the cycle including the length (Figure 2.3C, Table 2.3.1, $p = 0.457$), or the percentage of days spent in each stage (Figure 2.3D, Table 2.3.1, $p = 0.865$). Together, these results indicate that LBN did not disrupt reproductive maturation or estrous cyclicity.

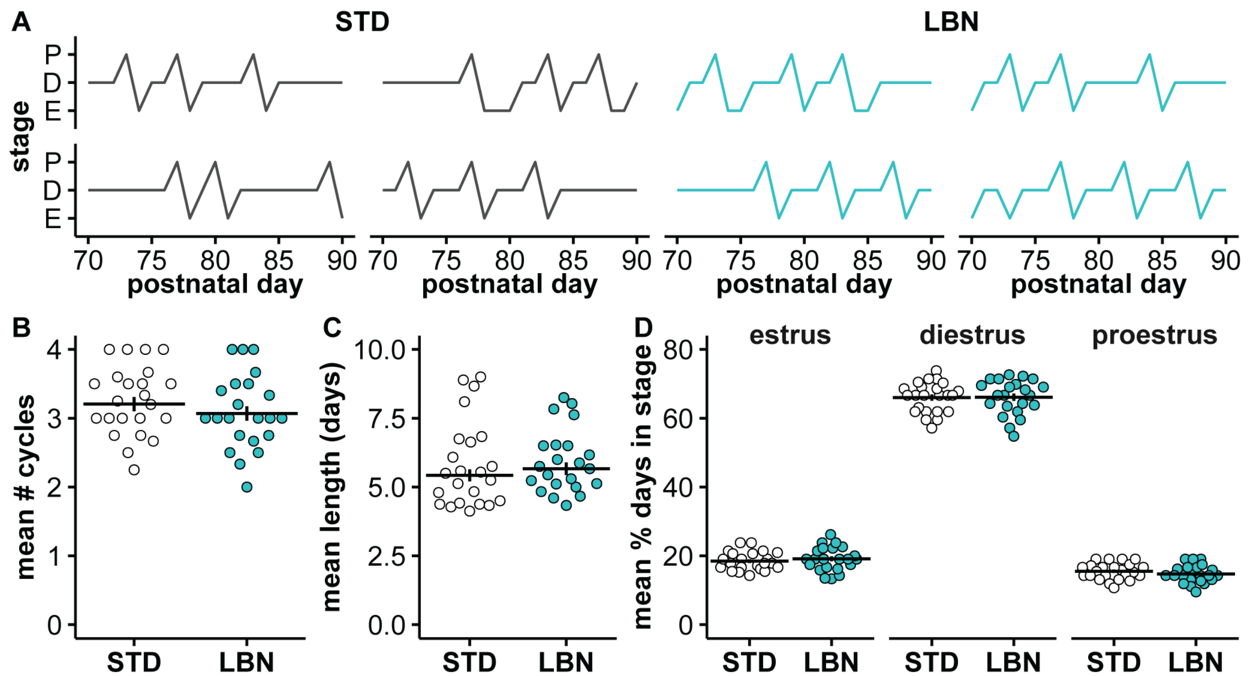

Figure 2.3. LBN did not alter estrous cyclicity from PND70-90. **A**. Representative individual estrous cycle traces for STD (left) and LBN (right) offspring, proestrus (P), estrus (E), or diestrus (D). **B**. Number of cycles averaged for female littermates; model mean±SEM. **C**. Mean cycle length averaged for female littermates; model mean±SEM. **D**. Mean percent of days spent in each cycle stage for female littermates; data mean±SEM; no model value is available as Chi-square test was used for analysis of percentage values. Full statistical model results are in Table 2.3.1. Some error bars obscured by mean line. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; PND, postnatal day.

Early-life stress did not alter the corticosterone response to adult stress

To determine if early-life stress alters the serum corticosterone response to adult stress, STD and LBN mice were exposed to an acute, layered, psychosocial stress (ALPS) paradigm (134) or remained in non-stressed, home cage control (CON) conditions (numbers in Table 2.4.1). There were no effects of early-life stress at any point in either sex or either cycle stage in females (Table 2.4.2), thus results are combined in Figure 2.4. Baseline corticosterone levels were the same in CON and ALPS males (Table 2.2, row 12: p = 0.883) and diestrous (Table 2.2, row 16: p = 0.826) and proestrous (Table 2.2, row 18: p = 0.387) females. Baseline corticosterone levels were elevated in

proestrous relative to diestrous mice that received ALPS (Table 2.2, row 25: $p < 0.001$). In all three groups, CON mice exhibited the typical diurnal increase in corticosterone (male: Table 2.2, row 14: $p < 0.001$; diestrus: Table 2.2, row 20: $p < 0.001$, proestrus: Table 2.2, row 22: $p < 0.001$). Similarly, all three groups had a similar response to ALPS treatment, post-paradigm corticosterone concentrations being 2- to 3-fold greater in ALPS than CON mice (male: Table 2.2, row 13: $p < 0.001$; diestrus: Table 2.2, row 17: $p < 0.001$, proestrus: Table 2.2, row 19: $p < 0.001$). These results indicate that early-life stress in the form of LBN treatment from PND4-11 did not alter this neuroendocrine response to a series of psychosocial stressors in adulthood in either males or females.

Body mass was monitored before and after treatment (males: Figure 2.4.1, Tables 2.4.3 and Table 2.4.4; females: Figure 2.4.2, Tables 2.4.5 and 2.4.6). Consistent with the weekly monitoring of body mass in early adulthood, LBN males were smaller than STD males at the start of the experiment (Figure 2.4.1A, Table 2.4.4, Table 2.2, row 28: $p = 0.037$) and LBN did not affect initial body mass in females (Figure 2.4.2A, Table 2.4.6). ALPS animals of both sexes lost a greater percentage of body mass during treatment (males: Figure 2.4.1B, Table 2.4.4, Table 2.2, row 30: $p < 0.001$, females: Figure 2.4.2B, Table 2.4.6, Table 2.2, row 39: $p < 0.001$), likely attributable in part to no access to food or water during the last four hours of stress treatment. By chance, morning body mass of ALPS males was greater than CON males (Figure 2.4.1A, Table 2.4.4, Table 2.2, row 29: $p = 0.008$), complicating interpretation of these observations, but males had apparent changes following ALPS in the normalized mass of seminal vesicles (Figure 2.4.1F, Table 2.4.4, Table 2.2, row 34: $p = 0.048$) and mass of the testes (absolute: Figure 2.4.1G, Table 2.4.4, Table 2.2, row 36: $p = 0.003$; normalized: Figure 2.4.1H,

Table 2.4.4, Table 2.2, row 37: $p = 0.021$). Females did not exhibit changes in either uterine or adrenal mass following ALPS (Figure 2.4.2, Table 2.4.6). Small changes in organ masses in males and females associated with LBN treatment are in Table 2.4.4, Table 2.4.6, and Table 2.2 (rows 31, 33, 35, and 40).

To test if corticosterone could reproduce the effects of ALPS on organ masses in males, additional mice were fed corticosterone or vehicle (Figure 2.4.3, Tables 2.4.7 and 2.4.8). Corticosterone decreased testicular mass (Table 2.4.7, absolute: Figure 2.4.3H, Table 2.2, row 51: $p = 0.010$, normalized: Figure 2.4.3I, Table 2.2, row 52: $p = 0.085$), suggesting this parameter may be sensitive to stress.

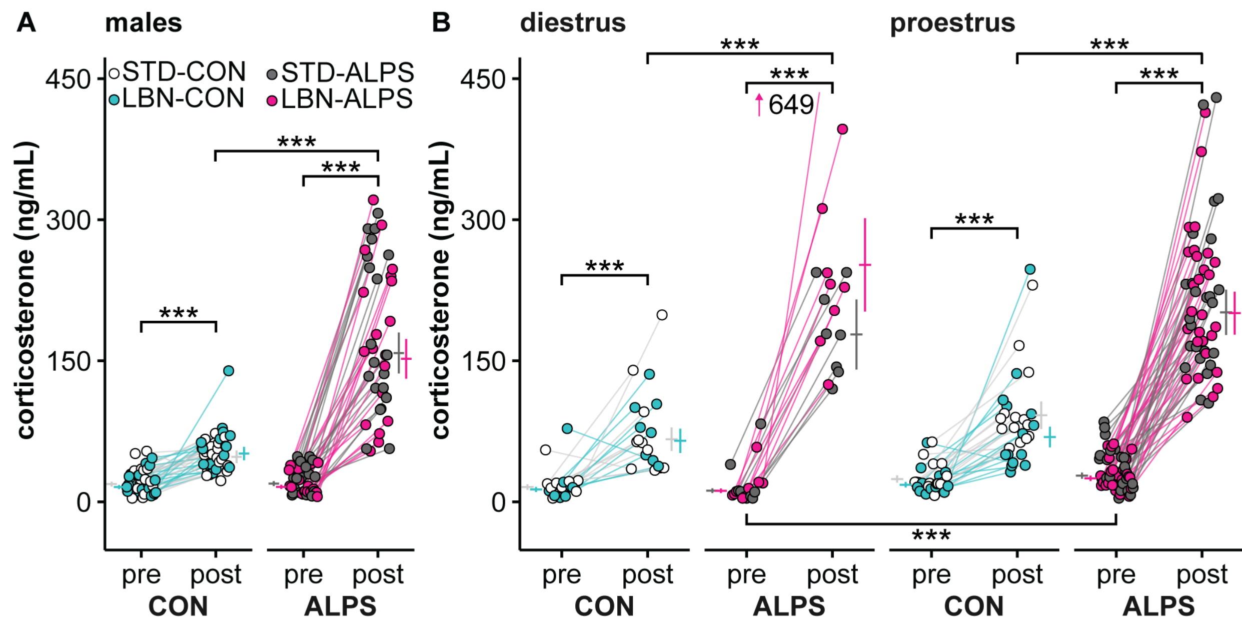

Figure 2.4. LBN does not change the corticosterone response to ALPS. Individual values and model mean±SEM (adjacent horizontal lines and vertical error bars) for pre- and post-treatment serum corticosterone concentrations in males (**A**) and females (**B**) (diestrus left, proestrus right). Early-life treatment groups are plotted together as there were no effects of LBN treatment on serum corticosterone concentrations at any point. Numbers are in Table 2.4.1. Results from the full statistical models are in Table 2.4.2. Additional data regarding tissue masses and the effect of corticosterone on masses are in Figures 2.4.1 (male tissue masses), 2.4.2 (female tissue masses), 2.4.3 (male corticosterone administration) and Tables 2.4.3 to 2.4.8. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; CON, adult control; ALPS, acute, layered, psychosocial stress in adulthood. *** p<0.001.

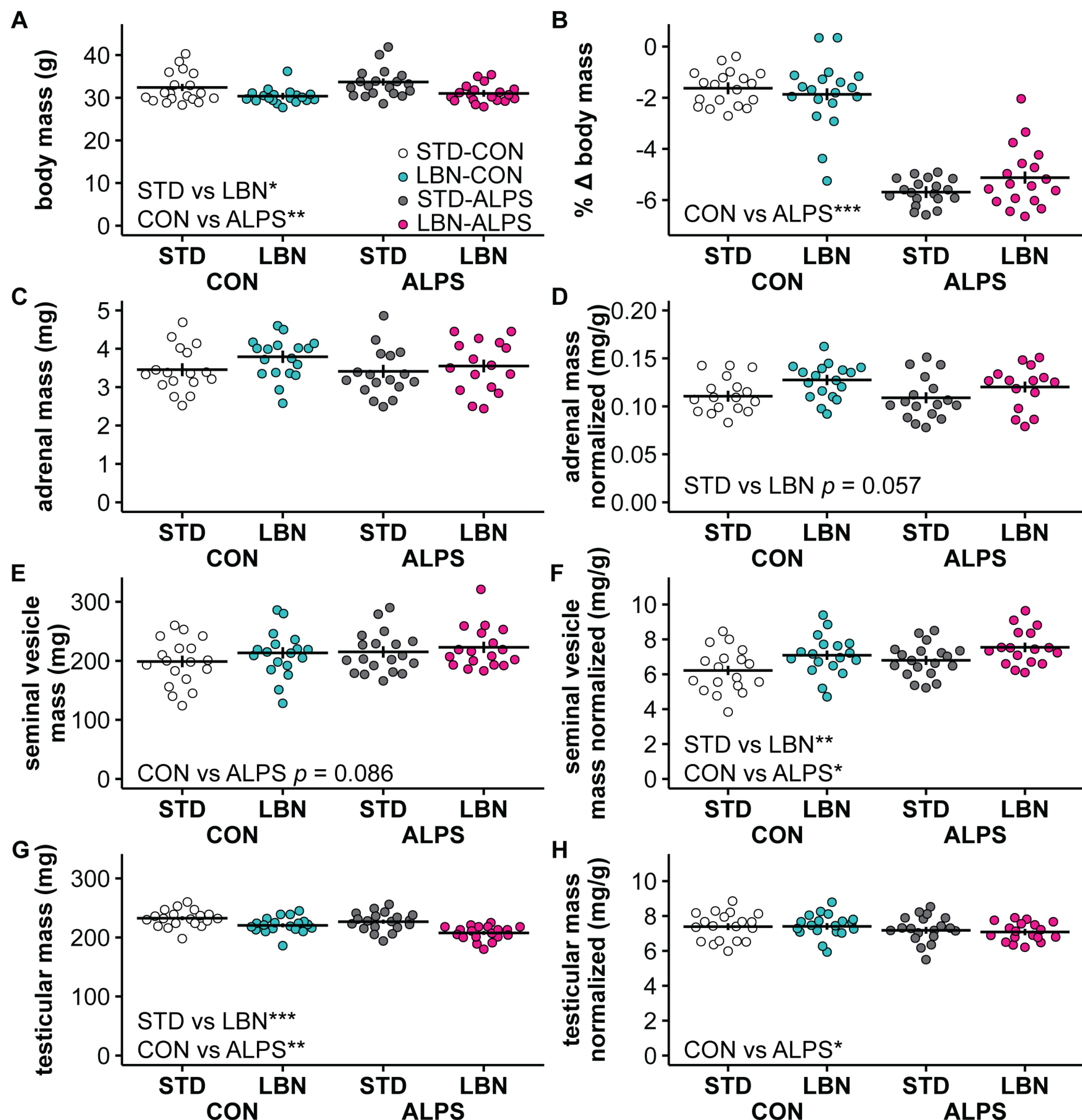

Figure 2.4.1. The ALPS paradigm caused small changes in tissue mass in males. Individual values and model mean±SEM for **A**. morning body mass; **B**. percent change in body mass after adult treatment; **C**. adrenal mass; **D**. normalized adrenal mass; **E**. seminal vesicle mass; **F**. normalized seminal vesicle mass; **G**. testicular mass; and **H**. normalized testicular mass. Some error bars obscured by mean lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Numbers are in Table 2.4.3. Results from the full statistical models are in Table 2.4.4. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; CON, adult control; ALPS, acute, layered, psychosocial stress in adulthood.

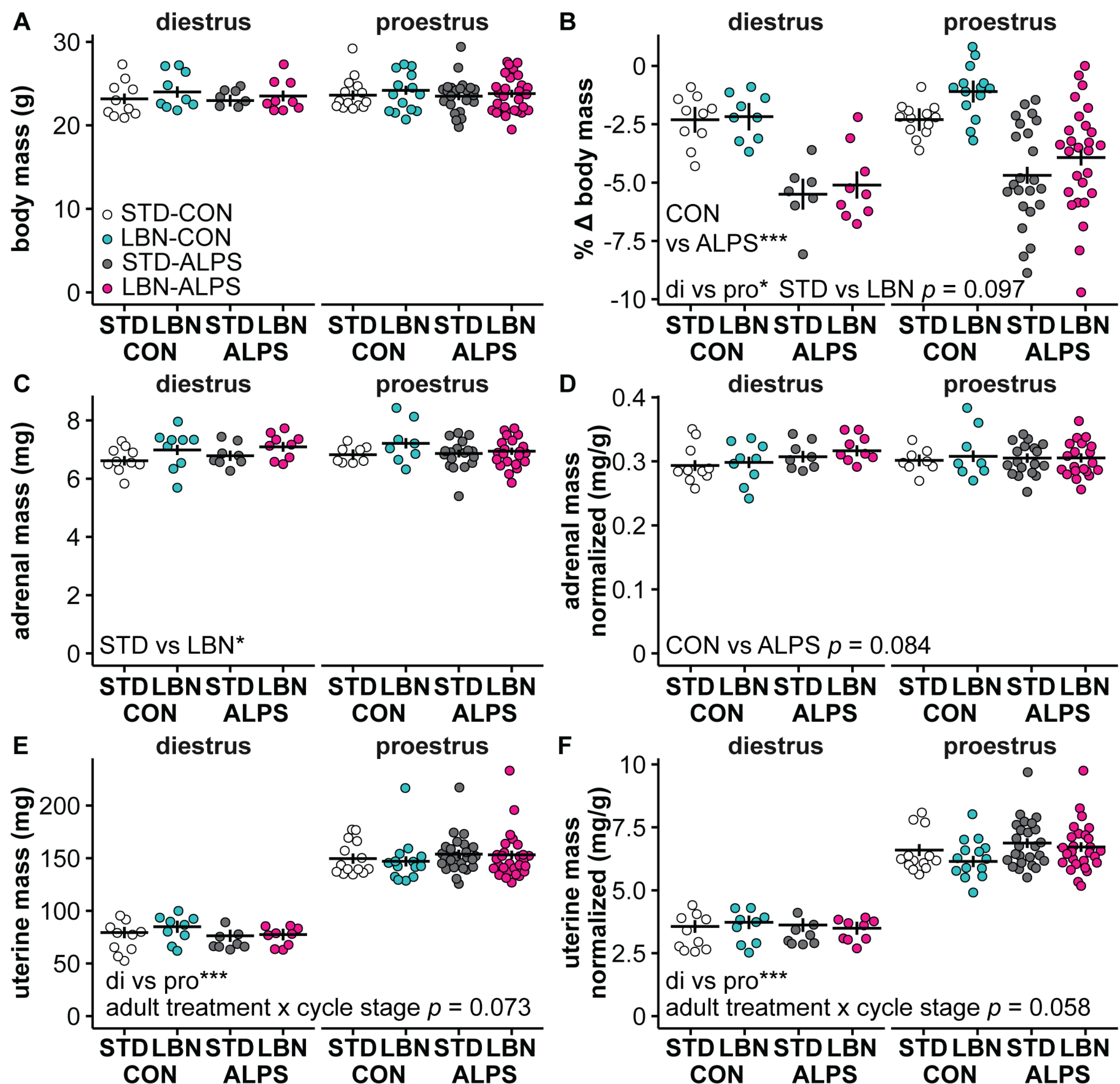

Figure 2.4.2. LBN and ALPS cause limited changes in tissue masses in females. Individual values and model mean±SEM for **A**. morning body mass; **B**. percent change in body mass after adult treatment. **C**. adrenal mass; **D**. normalized adrenal mass; **E**. uterine mass; **F**. normalized uterine mass in diestrous (left) and proestrous (right) females. Some error bars obscured by mean lines. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Numbers are in Table 2.4.7. Results for the full statistical models are in Table 2.4.8. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; CON, adult control; ALPS, acute, layered, psychosocial stress in adulthood.

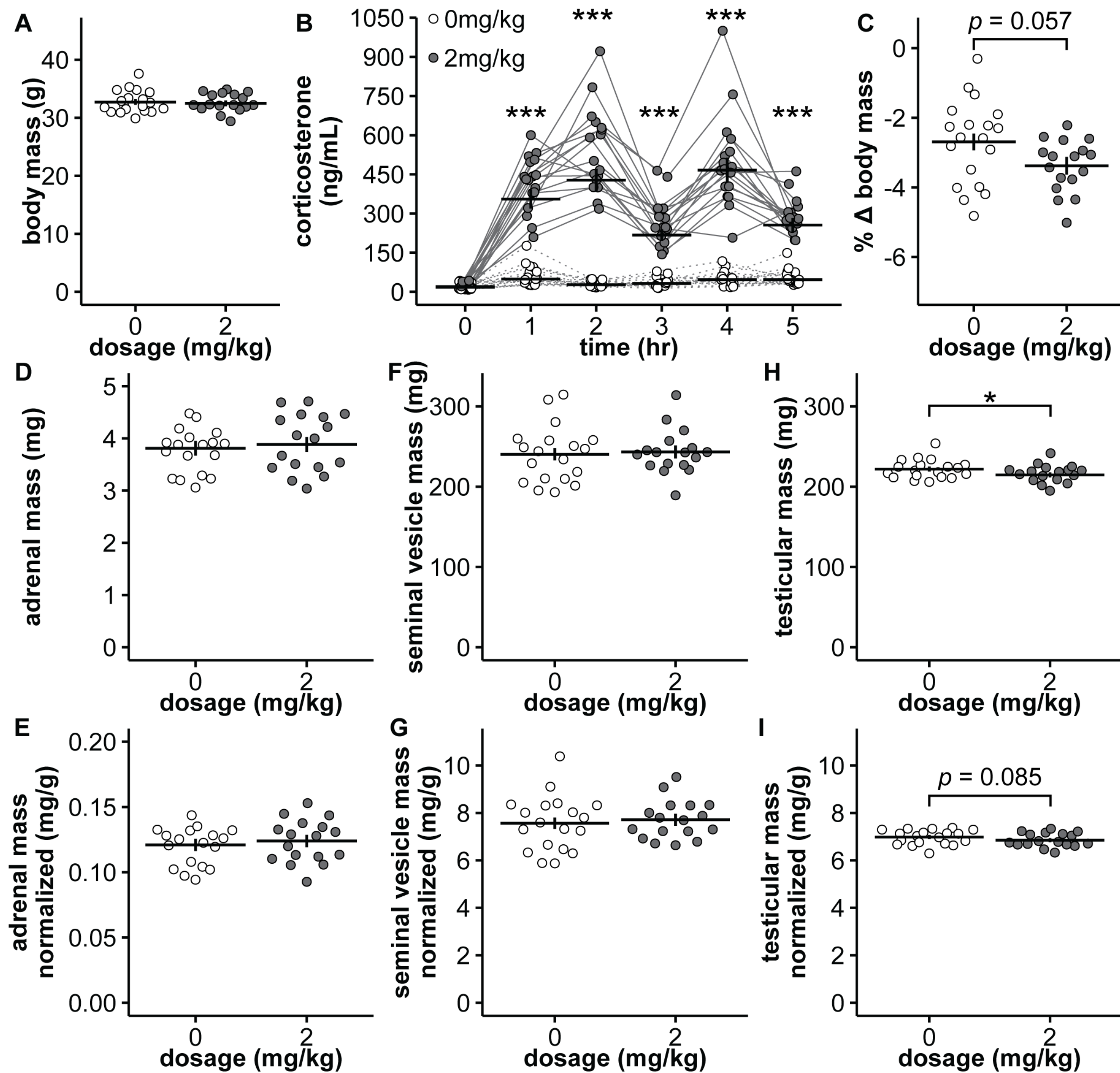

Figure 2.4.3. Acute elevation of serum corticosterone decreases testicular mass in males. Individual and model mean±SEM for **A**. morning body mass; **B**. serum corticosterone concentrations; comparisons between 0 and 2 mg/kg treatment at each hour; **C**. percent change in body mass after adult treatment. **D**. adrenal mass; **E**. normalized adrenal mass; **F**. seminal vesicle mass; **G**. normalized seminal vesicle mass; **H**. testicular mass; and **I**. normalized testicular mass. Some error bars are obscured by mean line. Vehicle (0mg/kg): 11 litters and 19 mice, except for adrenal mass with 18 mice; corticosterone (2mg/kg): 11 litters and 17 mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Results for the full statistical models are in Tables 2.4.5 and 2.4.6.

ALPS decreased afternoon LH in proestrous mice, LBN had no additional effect

Samples for LH collected at the end of the ALPS paradigm and at lights out from diestrous mice were averaged (STD-CON: 9 litters and 10 mice; STD-ALPS: 8 litters and 8 mice; LBN-CON: 7 litters and 9 mice; LBN-ALPS: 9 litters and 9 mice). Neither early-life stress (p = 0.687) nor adult stress (p = 0.067) had an effect on mean PM LH concentrations on diestrus (Figure 2.5A, Table 2.5.1); though the effect of adult stress approached the level set for significance. We tested if LBN alters the ability of ALPS to disrupt the proestrous LH surge (134). (STD-CON: 7 litters and 8 mice; STD-ALPS: 11 litters and 16 mice; LBN-CON: 8 litters and 8 mice; STD-ALPS: 14 litters and 19 mice). The maximum observed LH, the proportion of mice with an LH surge, and individual LH concentration profiles are in Figure 2.5B-D, respectively. Adult stress decreased the proportion of proestrous mice with an LH surge (logistic regression, $X^2 = 26.12$, $p < 0.001$) but exposure to early-life stress did not change the likelihood of observing an LH surge ($X^2 < 0.01$, $p = 0.991$, Figure 2.5C).

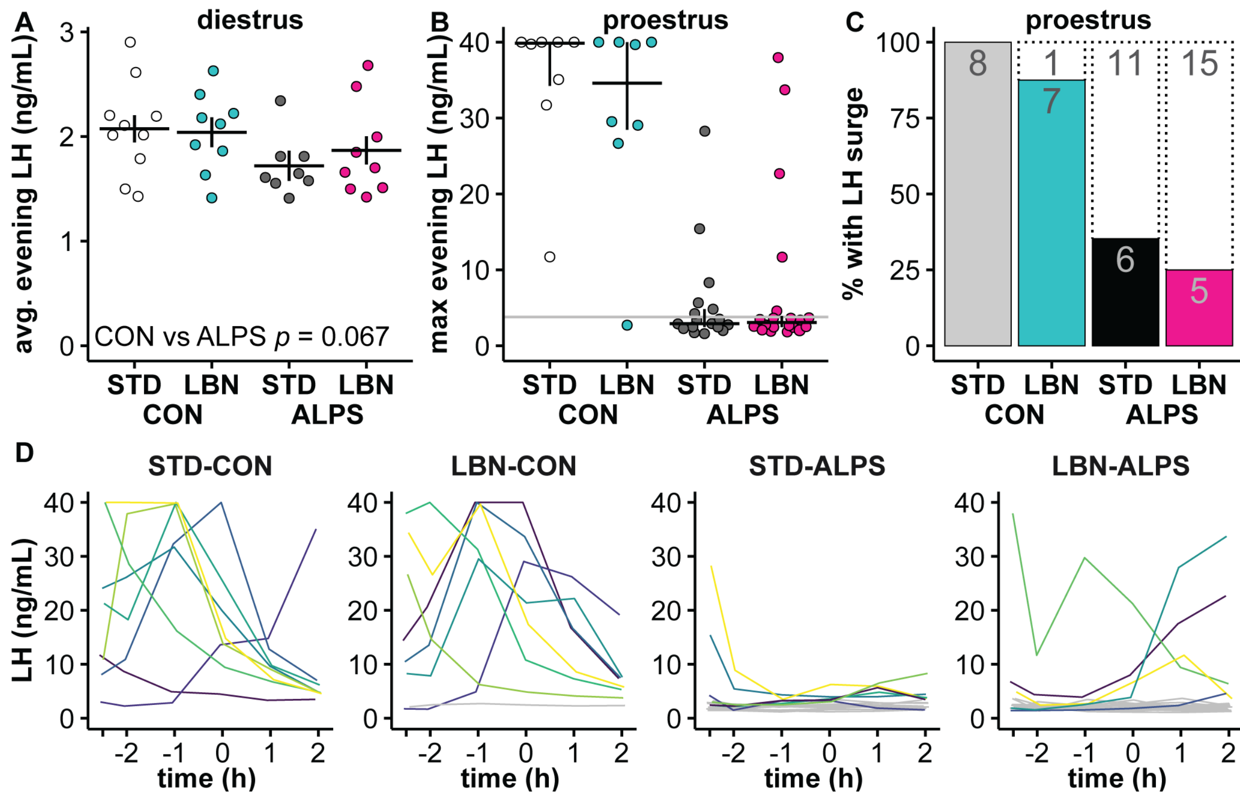

Figure 2.5. The LH surge is disrupted by adult stress. **A**. Individual values and model mean±SEM for the average LH concentrations on diestrus. **B**. Individual values and median±interquartile range of maximum evening LH for proestrous mice. Grey line at 3.8ng/mL is the cutoff for an LH surge. **C.** Percentage of proestrous mice with a maximum LH concentration >3.8ng/mL (filled bars; numbers are counts per result). **D**. Individual LH profiles for proestrous mice in each treatment group sampled hourly until 2h after lights out; time is relative to lights out. Grey lines show mice with no LH concentrations above 3.8ng/mL. Results from the full statistical model of diestrous concentrations are in Table 2.5.1. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; CON, adult control; ALPS, acute, layered, psychosocial stress in adulthood.

Neither LBN nor ALPS reduced the frequency of GABA PSCs in GnRH neurons

The frequency of GABA PSCs in GnRH neurons increases around the time of the LH surge on proestrus (139). We thus tested the hypothesis that ALPS decreases the frequency of these PSCs (STD-CON: 14 cells; STD-ALPS 15 cells; LBN-CON: 15 cells; LBN-ALPS: 14 cells; 5 litters and 6 mice in all groups). There were no differences in the passive properties or recording quality among treatment groups (Figure 2.6A-D, Table 2.6.1). Representative PSC recordings from neurons in each group are in Figure 2.7A;

the average PSC from each group is in Figure 2.7B. Neither LBN nor ALPS altered the frequency of GABA PSCs (Figure 2.7B, Table 2.7.1). Shifts in PSC patterns can occur within datasets with the same mean; however, when averaged by cell, the interevent interval of GABA PSCs is also similar among groups (Figure 2.7D, Table 2.6.1, n as above except LBN-ALPS n=13 as one cell did not have PSCs). There were no differences in mean amplitude (Figure 2.7E) or decay time (Figure 2.7F, Table 2.6.1). In contrast, the cumulative distribution of interevent intervals for all events is shifted towards longer intervals in the ALPS groups compared to the CON groups (Figure 2.7G, Table 2.7.2, pairwise AD tests, STD-CON vs STD-ALPS: $p < 0.001$; LBN-CON vs LBN-ALPS: $p < 0.001$).

Because of the large number of events in each group, the Anderson-Darling test could be identifying significant differences in the distribution that are not biologically relevant. To determine the effect size of these changes, we used bootstrapping to estimate 95% confidence intervals for the differences in means between groups. The mean interevent interval for events from STD-ALPS mice was estimated to be 0.78s (95% CI: [0.65, 0.92]) longer than for events from STD-CON mice; in LBN mice, ALPS lengthened mean interevent interval by about 0.32s (95% CI: [0.19, 0.46]). This suggests that ALPS alters the excitatory input to GnRH neurons on the evening of proestrous. We interpret these results with caution, however, as three control cells with high PSC frequencies contribute a disproportionate number of short interevent intervals.

The amplitude distribution for the STD-ALPS groups was shifted towards larger events (Figure 8H, Table 2.7.2, pairwise AD tests, STD-CON vs STD-ALPS, $p = 0.006$, STD-ALPS vs LBN-ALPS, $p < 0.001$). In LBN mice, ALPS did not shift the amplitude

distribution (Table 2.7.2, pairwise AD tests, LBN-CON vs LBN-ALPS, $p = 0.711$). The mean amplitude of the STD-ALPS group was about 3.6pA larger (95% CI: [1.36, 5.81]) than the mean of the STD-CON group and about 5.2pA larger (95% CI: [3.09, 7.34]) than the mean of the LBN-ALPS group, as estimated with bootstrapping.

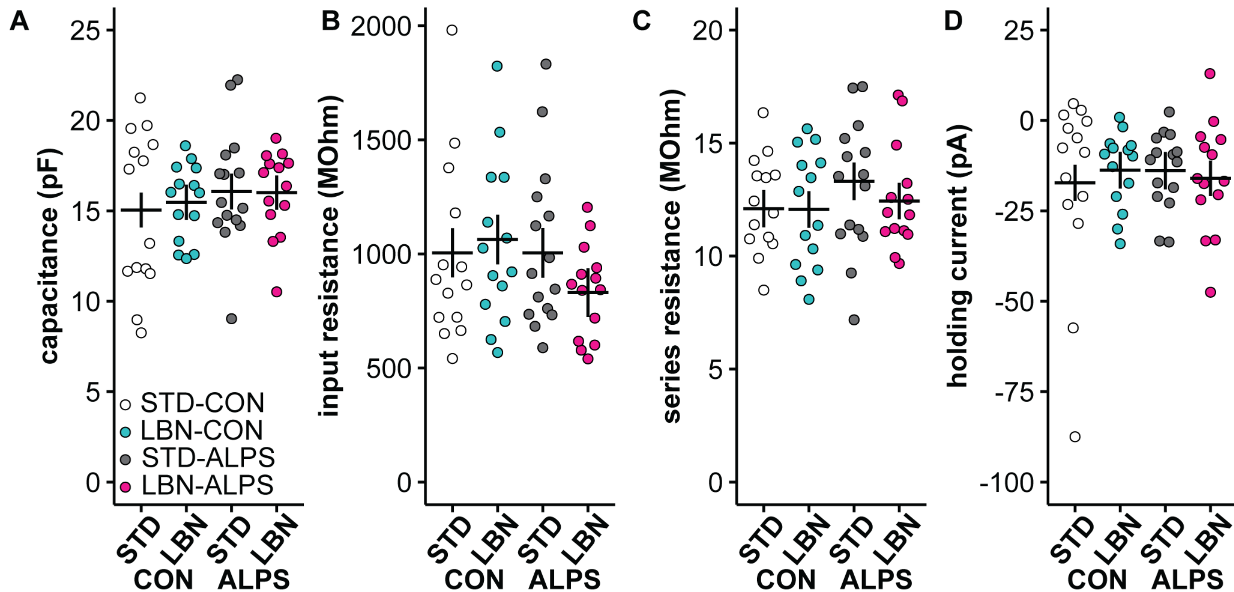

Figure 2.6. Recording quality and passive properties of GnRH neurons were similar among groups. **A-D**. Individual cell values and model mean±SEM for **A**. capacitance, **B**. input resistance, **C**. series resistance, **D**. holding current. Results for the full statistical model are in Table 2.6.1. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; CON, adult control; ALPS, acute, layered, psychosocial stress in adulthood.

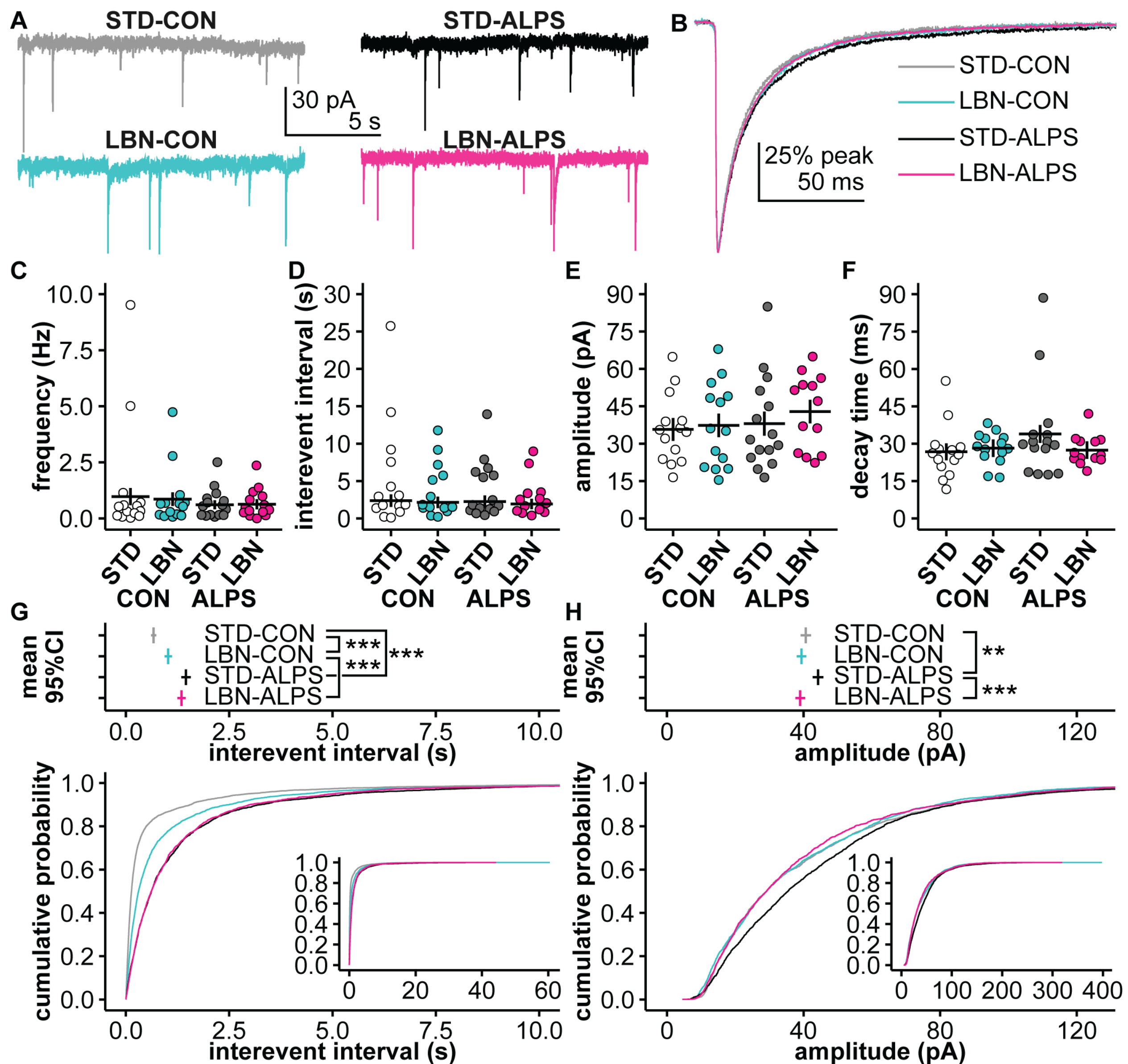

Figure 2.7. Stress treatments did not alter overall GABA PSC frequency, but ALPS may lengthen the interevent interval in GnRH neurons. **A**. Representative 15s traces (Box 9 smoothed) near the median frequency and amplitude from a GnRH neuron in each group. **B**. Normalized average PSC for each treatment group. **C-F**. Individual cell values and model mean±SEM for **C**. PSC frequency (# events/duration), **D**. mean interevent interval, **E**. mean amplitude, and **F**. decay time from 80% to 20% of peak calculated from the cell's normalized average trace. **G-H**. Distribution of **G**. interevent interval and **H**. amplitude. (top) Bootstrapped mean estimates with 95% confidence interval for each group. (bottom) Cumulative probability distribution plots for each group. Inset plots show the full range of the distribution. Results for the full statistical models are in Tables 2.6.1, 2.7.1, and 2.7.2. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, from bootstrapped results. Abbreviations: STD, standard-reared; LBN, limited bedding and nesting; CON, adult control; ALPS, acute, layered, psychosocial stress in adulthood; PSC, postsynaptic current.

Discussion

The stress and reproductive neuroendocrine systems interact, and early-life stress has reproductive consequences in humans. We tested the hypotheses that early-life stress in the form of LBN would delay sexual maturation and alter the response to subsequent stress exposure in adulthood in mice. We rejected these hypotheses, finding that LBN did not delay external indicators of sexual maturation in males or females. Further, the corticosterone response to adult psychosocial stress was not altered by LBN in either sex. On proestrus, adult stress disrupts the LH surge, but this is not affected by a history of LBN. In contrast to our hypothesis that ALPS disrupts the LH surge by decreasing the frequency of excitatory GABA PSCs in GnRH neurons, these currents were not appreciably altered by either adult psychosocial stress or limited bedding and nesting. This suggests that the disruption of the LH surge by adult stress is not attributable to changes in the GABAergic input to GnRH neurons.

The LBN paradigm was chosen as the model for early-life stress because animal behavior is minimally disrupted by ongoing investigator interference (38). Over its implementation in several labs, LBN effectiveness has been evaluated in three main ways: dam behavior, pup mass at the end of the paradigm and/or dam stress parameters. In both rats (39–41) and mice (42,75), maternal care is fragmented by LBN treatment, leading to more transitions between behavioral states and more exits from the nest. This fragmentation of maternal care was confirmed in CBA dams in the present study. On the last day of treatment, mice and rat offspring of LBN dams exhibit elevated basal corticosterone concentrations (39,72–75) and increased adrenal masses in rat pups (39,72). Because the aforementioned outcomes require terminal studies in

neonates, monitoring of body mass is a common proxy measure of the impact of LBN treatment in offspring, with LBN pups being smaller than STD pups after the paradigm (39,56,57,72,73,173,175,203,204). We similarly observed a lower pup mass at the end of the LBN paradigm in both male and female CBB6/F1 hybrid offspring, suggesting that the LBN paradigm was effectively implemented as an early-life stressor in our laboratory.

Strain can impact dam behavioral responses to LBN and the effect on offspring mass in mice (205), and there are indications that the LBN treatment may induce a milder phenotype in CBA dams and their CBB6/F1 hybrid offspring in the present study. Morning plasma corticosterone concentrations were elevated in rat dams at the end of the paradigm on PND9 (40); serum corticosterone was not, however, elevated on PND11 in CBA dams. Adaptation to psychosocial stressors resulting in decreased glucocorticoid output has been observed in mice (134) and ewes (117). Perhaps such acclimation occurs more quickly in CBA dams than in other species or strains, which could contribute to milder outcomes in their pups. The PND11 difference in body mass resolved quickly in female offspring, and there were no further impacts of LBN treatment on body mass growth through adulthood the females we studied. In contrast, LBN males had mildly slowed growth that was evident later in adulthood. This suggests there may also be a difference in susceptibility to LBN treatment between the female and male offspring.

The effects of LBN on reproductive maturation vary across studies (56,57,173–175). In the present study, we did not observe differences in body mass near the time of puberty or changes in the age at reproductive maturation, in part because we normalized litter

sizes to provide more consistent nutrition. Some of the variations in the literature may be related to age of LBN exposure, and/or subtle protocol differences such as the type of bedding or nesting material, the wire platform material, or vivarium conditions, along with the species and strain of animals. The variability in body mass may also underlie some of the variability in vaginal opening outcomes, as a decrease in body mass is known to delay vaginal opening (58–60,60–62). Together, these studies point to the importance of considering possible confounding and interacting factors when assessing the effect of early-life stress on body mass and reproductive maturation.

A lack of effect of LBN on the estrous cycle is a more consistent observation across studies in both mice (56) and rats (174,175), and was confirmed in the present study. Estrous cycles also remain unchanged following maternal separation as an early life stress (206,207). Although the observation of typical adult estrous cyclicity does not preclude other underlying changes in reproductive physiology (208), the ability of animals exposed to early life stress to establish cyclicity is an indication that aspects of the reproductive system can recover from developmental perturbations caused by this treatment.

Our findings that LBN did not alter adult basal corticosterone concentrations in the morning or afternoon are consistent with the observations of others (39,57,173,175). The original study of LBN in mice did, however, observe persistently elevated basal corticosterone concentrations in 4-7mo-old males (75). In one study, adult LBN rats responded similarly to standard-reared rats when exposed to a 1h restraint stress (175). The latter is consistent with our finding that adult LBN mice exhibited similar corticosterone profiles to standard-reared mice in response to a 5h, layered stress

paradigm, ALPS, which included restraint. This suggests that any transient changes in the neuroendocrine stress response following perinatal LBN exposure were normalized by subsequent rearing and weaning into standard housing conditions.

The primary motivation for this work was to study the reproductive consequences of LBN, including how it affects responses to ALPS exposure. In diestrous mice, the ALPS effect to reduce mean LH concentrations approached the value accepted for significance. This may reflect stress suppression (130) of the pulsatile LH release typical of this stage (127), but the infrequent sampling in the present study is not designed to assess pulse parameters. The ALPS paradigm was developed in the context of understanding the effects of acute stress exposure on the sustained preovulatory increase in LH concentration that occurs on the afternoon of proestrus. As reported (134), ALPS initiated on the morning of proestrus disrupts the LH surge in most mice. We hypothesized LBN exposure would alter the effect of ALPS on the LH surge, but LBN had no additional effect, suggesting that the paradigm studied for early-life stress did not confer either resilience or susceptibility to the adult stress studied for this parameter. There are several potential explanations for this finding. First, LBN may be milder than other perinatal stressors, such as lipopolysaccharide exposure (50), that have lasting effects on the reproductive consequences of adult stress. Second, the preweaning return to standard housing conditions may have also buffered the effects of early resource limitations. Third, work by Peña et al. (179) demonstrated the challenges of trying to predict if experiencing one stressor will lead to susceptibility or resilience to a subsequent stressor by comparing the impact of early-life stressors on the behavioral responses to 10 days of chronic social defeat. Most pertinent to the work presented

here, maternal separation with reduced bedding from PND2-12 did not affect post-defeat behavior, whereas the same treatment from PND10-17 increased susceptibility (179), a difference which the authors attributed to the transition out of the stress hyporesponsive period for pups who experienced the paradigm later in development (63). The lack of effect of LBN from PND4-11 could thus be reflective of pups experiencing the paradigm during the stress hyporesponsive period.

The mechanisms by which ALPS disrupts the LH surge, both in terms of the components of the stress response and the site of action within the reproductive axis, remain unknown. GnRH neurons from proestrous mice receive a higher frequency of GABAergic input, which is excitatory in these cells (141), in the evening than in the morning, consistent with the switch from negative to positive feedback and the timing of the LH surge (139). We thus tested the hypothesis that GABA input to GnRH neurons is diminished by ALPS. The frequency of GABAergic PSCs in GnRH neurons was not altered by stress, nor was the mean interevent interval by cell. The distribution of all interevent intervals was shifted towards longer intervals in cells from ALPS mice, but this appears to be primarily related to three control cells with a high frequency of PSCs. It is possible that one mechanism by which ALPS ultimately disrupts the LH surge is by reducing the incidence of GnRH neurons receiving a high frequency of GABAergic input, but the current study is not powered to assess this. The observation that the amplitude distribution of PSCs from STD-ALPS mice is shifted towards larger events runs counter to the hypothesis that this stressor reduces the efficacy of GABA input to GnRH neurons. The magnitudes of these observed changes in event distributions are small, and near our limit of detection for differentiating signal and noise in these

electrophysiological recordings. Amplitude did not differ when comparing the mean values from each cell; thus, the subtle shifts in the cumulative probability distributions of events may also reflect some bias towards the amplitude of PSCs from cells with more events included in the analysis.

One possible caveat to this work is that brain slice preparation could sever key neuronal networks that may be critical for the disruption of reproductive output following ALPS, or that other *in vivo* changes attributable to early-life or adult stress do not persist in this configuration. In this regard, both acute and chronic stressors induced measurable changes in synaptic physiology of the hypothalamic paraventricular nucleus in brain slices (209). The increase in GABA PSC frequency in GnRH neurons on the evening of proestrus occurs concurrently with the expected time of the LH surge, but the experimental design precludes the ability to directly correlate the properties of PSCs to the incidence of the LH surge in that animal; in this regard, uterine mass in animals used for PSC recordings were consistent with proestrus (Figure 2.4.2). The source of the increased GABAergic transmission to GnRH neurons during the LH surge is not known and has been postulated to be the suprachiastmatic nucleus (140) or the anteroventral periventricular kisspeptin neurons (208). Although GABAergic transmission was not altered by ALPS or LBN, it is possible that peptidergic modulators from these, or other, populations are altered in a manner that disrupts the LH surge.

While persistent effects of LBN were not observed into adulthood in this study, care must be taken not to over-extrapolate these findings as demonstrating that early-life psychosocial and environmental manipulations are of no consequence. To allow rigorous control of conditions, this study considered a single type of early-life stress

during one week of development, after which time animals were returned to standard housing conditions. Altering the timing, type, or duration of the stressor may lead to different outcomes. Indeed, a recent study found persistent reproductive effects following three weeks of postweaning social isolation, including on vaginal opening, estrous cycles, and activity of hypothalamic neurons (210), indicating that housing conditions during certain developmental periods can lead to changes that persist into adulthood. The observations of the present study direct future attention to the effects of both early-life and adult stress on the broader neuroendocrine network controlling reproduction, including upstream neuronal populations and pituitary gonadotropes.

Table 2.1. Type of statistical test used to analyze each outcome. Linear mixed models (LMM) were used where appropriate for the data structure and experimental design. [@]indicates that postnatal day (PND) was treated as a factor variable. For the analysis of dam behavior with a non-parametric longitudinal test, the nparLD package in R was used, with the F1 LD F1 Model. The subject variable was each dam, early-life treatment (standard or limited bedding and nesting cage) was the between-subject factor ('whole-plot' factor), and PND was the within-subject factor ('sub-plot' repeated factor). [^]indicates that linear splines at PND21 and 35 for the models of offspring mass allow the model to change the slope of the line for the segments between PND11-21, from 21-35, and from 35-72. Generalized linear mixed models (GLMM) were fit with either logistic regression or negative binomial (NB) families.

Fig	outcome	test	formula
2.1B	dam mass	LMM	mass ~ early-life treatment * PND[@] + (1|dam)
2.1C	dam corticosterone	t-test	corticosterone ~ early-life treatment
2.1D-E	# of nest exits	non-parametric longitudinal F1 LD F1	# of nest exits ~ early-life treatment * PND[@]
2.1F-G	% of time off nest	non-parametric longitudinal F1 LD F1	% off nest ~ early-life treatment * PND[@]
2.2A	PND4 offspring mass *litter average*	t-test	mass ~ early-life treatment
2.2B	PND11 offspring mass *individual pups*	LMM	mass ~ early-life treatment * sex + (1|dam)
2.2C	offspring mass *males and females run separately*	LMM with splines	mass ~ early-life treatment * PND[^] + (1 | dam) + (1 | mouse)
2.2D	age at vaginal opening	LMM	age ~ early-life treatment + (1|dam)
	age at first estrus		
	age at preputial separation		
2.2E	mass at vaginal opening	LMM	mass ~ early-life treatment + (1|dam)
	mass at first estrus		

	mass at preputial separation		
text	anogenital distance	LMM	anogenital distance ~ early-life treatment * sex + (1|dam)
2.3B	# of estrous cycles	LMM	# of estrous cycles ~ early-life treatment + (1|dam)
2.3C	cycle length	LMM	$\log_{10}$(cycle length) ~ early-life treatment + (1|dam)
2.3D	% days in stage	X^2 test	Distribution of days spent in diestrus, proestrus, and estrus by early-life treatment
2.4A	male serum corticosterone	LMM	$\log_{10}$(cort) ~ early-life treatment * adult treatment * time + (1 | mouse) + (1 | dam)
2.4B	female serum corticosterone	LMM	$\log_{10}$(cort) ~ cycle stage * early-life treatment * adult treatment * time + (1 | mouse) + (1 | dam)
2.4.1A	body mass	LMM	outcome ~ early-life treatment * adult treatment + (1 | dam)
2.4.1B	% change in body mass		
2.4.1C	adrenal mass		
2.4.1D	adrenal mass normalized to body mass		
2.4.1E	seminal vesicle mass		
2.4.1F	seminal vesicle mass normalized to body mass		
2.4.1G	testicular mass		
2.4.1H	testicular mass normalized to body mass		
2.4.2A	body mass	LMM	outcome ~ early-life treatment * adult treatment * cycle stage + (1 | dam)
2.4.2B	% change body mass		

2.4.2C	adrenal mass		
2.4.2D	adrenal mass normalized to body mass		
2.4.2E	uterine mass		
2.4.2F	uterine mass normalized to body mass		
2.4.3B	serum corticosterone	LMM	$\log_{10}$(cort) ~ dosage * time + (1 | mouse) + (1 | dam)
2.4.3A	body mass	LMM	outcome ~ dosage + (1 | dam)
2.4.3C	% change in body mass		
2.4.3D	adrenal mass		
2.4.3E	adrenal mass normalized to body mass		
2.4.3F	seminal vesicle mass		
2.4.3G	seminal vesicle mass normalized to body mass		
2.4.3H	testicular mass		
2.4.3I	testicular mass normalized to body mass		
2.5F	average LH, diestrous mice	LMM	avg LH ~ early-life treatment * adult treatment + (1 | dam)
2.5C	proportion with LH surge, proestrous	GLMM – logistic regression	surged ~ early-life treatment + adult treatment + (1 | dam)
2.6A	capacitance	LMM	outcome ~ early-life treatment * adult treatment + (1|mouse) + (1|dam)
2.6B	input resistance		
2.6C	series resistance		
2.6D	holding current		

<table>
<tr><td>2.7C</td><td>PSC frequency</td><td>GLMM – negative binomial</td><td># of events in 4 min ~ early-life treatment * adult treatment + (1|mouse) (1|dam)</td></tr>
<tr><td>2.7D</td><td>mean interevent interval</td><td>LMM</td><td>$\log_{10}$(interval) ~ early-life treatment * adult treatment + (1|mouse) + (1|dam)</td></tr>
<tr><td>2.7E</td><td>mean relative amplitude</td><td rowspan="2">LMM</td><td rowspan="2">outcome ~ early-life treatment * adult treatment + (1|mouse) + (1|dam)</td></tr>
<tr><td>2.7F</td><td>decay time</td></tr>
<tr><td>2.7G</td><td>distribution of interevent interval</td><td colspan="2" rowspan="2">Anderson-Darling test and bootstrapping. See “Statistical analysis” section of methods</td></tr>
<tr><td>2.7H</td><td>distribution of amplitude</td></tr>
</table>

Table 2.2. Statistical table for pairwise comparisons for post-hoc tests. Holm's method for p-value adjustment was used for multiple comparisons. Confidence intervals were adjusted using the more conservative Bonferroni method. The statistical test used for each outcome is in Table 2.1.

fig	outcome	group level	contrast	estimate	95% CI	SEM	df	t	p	row
2.1B	dam mass		STD - LBN	-1.23	[-2.36, -0.10]	0.562	47.0	-2.20	0.033	1
			PND4 - PND11	-2.78	[-3.33, -2.22]	0.227	94.0	-12.21	<0.001	2
			PND4 - PND21	-2.15	[-2.70, -1.59]	0.227	94.0	-9.45	<0.001	3
			PND11 - PND21	0.63	[0.07, 1.18]	0.227	94.0	2.76	0.007	4
2.2B	PND11 mass		STD - LBN	0.55	[0.17, 0.94]	0.192	47.0	2.87	0.006	5
2.2C	male mass	PND 11	STD - LBN	0.67	[-0.28, 1.63]	0.355	43.2	1.90	0.129	6
		PND 21	STD - LBN	0.62	[-0.33, 1.57]	0.353	41.9	1.76	0.129	7
		PND 35	STD - LBN	0.77	[-0.21, 1.74]	0.364	47.5	2.11	0.121	8
		PND 56	STD - LBN	0.93	[-0.02, 1.88]	0.352	41.7	2.66	0.045	9
		PND 72	STD - LBN	1.06	[0.10, 2.03]	0.358	44.5	2.97	0.024	10
	anogenital distance		female - male	-10.35	[-10.48, -10.23]	0.065	239.8	-160.51	<0.001	11
2.4A	male serum corticosterone	pre	CON / ALPS	0.98	[0.71, 1.35]	0.125	121.7	-0.15	0.883	12
		post	CON / ALPS	0.32	[0.23, 0.44]	0.041	122.2	-8.91	<0.001	13
		CON	pre / post	0.35	[0.25, 0.47]	0.042	74.1	-8.68	<0.001	14
		ALPS	pre / post	0.11	[0.08, 0.15]	0.014	73.2	-18.04	<0.001	15

fig	outcome	group level	contrast	estimate	95% CI	SEM	df	t	p	row
2.4B	female serum corticosterone	di pre	CON / ALPS	1.21	[0.69, 2.13]	0.237	200.2	0.97	0.826	16
		di post	CON / ALPS	0.31	[0.18, 0.55]	0.061	200.2	-5.98	<0.001	17
		pro pre	CON / ALPS	0.79	[0.53, 1.18]	0.110	215.8	-1.67	0.387	18
		pro post	CON / ALPS	0.40	[0.27, 0.59]	0.055	215.8	-6.70	<0.001	19
		di CON	pre / post	0.22	[0.13, 0.38]	0.041	109.0	-8.08	<0.001	20
		di ALPS	pre / post	0.06	[0.03, 0.10]	0.011	109.0	-14.47	<0.001	21
		pro CON	pre / post	0.26	[0.17, 0.41]	0.041	109.0	-8.62	<0.001	22
		pro ALPS	pre / post	0.13	[0.09, 0.18]	0.015	109.0	-18.00	<0.001	23
		pre CON	diestrus / proestrus	0.68	[0.41, 1.14]	0.120	213.5	-2.16	0.160	24
		pre ALPS	diestrus / proestrus	0.45	[0.28, 0.72]	0.074	215.5	-4.85	<0.001	25
		post CON	diestrus / proestrus	0.83	[0.50, 1.37]	0.145	213.5	-1.09	0.826	26
		post ALPS	diestrus / proestrus	1.05	[0.65, 1.70]	0.174	215.5	0.31	0.826	27
2.4.1A	body mass		STD - LBN	2.37	[0.16, 4.59]	1.067	21.9	2.22	0.037	28
			CON - ALPS	-0.97	[-1.68, -0.26]	0.353	50.7	-2.75	0.008	29
2.4.1B	% change body mass		CON - ALPS	3.66	[3.20, 4.11]	0.229	56.7	16.00	<0.001	30
2.4.1D	adrenal mass normalized to body mass		STD - LBN	-0.01	[-0.03, 0.00]	0.007	21.2	-2.01	0.057	31

fig	outcome	group level	contrast	estimate	95% CI	SEM	df	t	p	row
2.4.1E	seminal vesicle mass		CON - ALPS	-12.78	[-27.44, 1.88]	7.295	49.4	-1.75	0.086	32
2.4.1F	seminal vesicle mass normalized to body mass		STD - LBN	-0.81	[-1.39, -0.23]	0.278	20.6	-2.91	0.008	33
			CON - ALPS	-0.51	[-1.02, -0.01]	0.253	52.4	-2.03	0.048	34
2.4.1G	testes mass		STD - LBN	15.52	[8.10, 22.94]	3.565	20.8	4.35	<0.001	35
			CON - ALPS	9.36	[3.25, 15.46]	3.046	55.0	3.07	0.003	36
	testes mass normalized to body mass		CON - ALPS	0.27	[0.04, 0.49]	0.113	50.6	2.37	0.021	37
2.4.2B	% change body mass		diestrus - proestrus	-0.77	[-1.49, -0.04]	0.365	103.9	-2.10	0.038	38
			CON - ALPS	2.83	[2.11, 3.55]	0.363	93.6	7.81	<0.001	39
2.4.2C	adrenal mass		STD - LBN	-0.29	[-0.57, 0.00]	0.140	30.6	-2.05	0.049	40
2.4.2D	adrenal mass normalized to body mass		CON - ALPS	-0.01	[-0.02, 0.00]	0.005	60.8	-1.76	0.084	41
2.4.2E	uterine mass		diestrus - proestrus	-71.38	[-77.31, -65.46]	2.981	85.5	-23.95	<0.001	42
2.4.2F	uterine mass normalized to body mass		diestrus - proestrus	-2.98	[-3.26, -2.71]	0.138	84.4	-21.62	<0.001	43
2.4.3B	male cort admin serum corticosterone	0hr	0mg/kg / 2mg/kg	1.12	[0.78, 1.61]	0.151	172.7	0.86	0.393	44
		1hr	0mg/kg / 2mg/kg	0.14	[0.10, 0.20]	0.019	172.7	-14.72	<0.001	45
		2hr	0mg/kg / 2mg/kg	0.06	[0.04, 0.09]	0.008	172.7	-20.57	<0.001	46

fig	outcome	group level	contrast	estimate	95% CI	SEM	df	t	p	row
		3hr	0mg/kg / 2mg/kg	0.15	[0.10, 0.21]	0.020	172.7	-14.34	<0.001	47
		4hr	0mg/kg / 2mg/kg	0.10	[0.07, 0.14]	0.013	172.7	-17.18	<0.001	48
		5hr	0mg/kg / 2mg/kg	0.18	[0.13, 0.26]	0.024	172.7	-12.70	<0.001	49
2.4.3C	% change body mass		0mg/kg - 2mg/kg	0.68	[-0.02, 1.39]	0.344	27.5	1.99	0.057	50
2.4.3H	testes mass		0mg/kg - 2mg/kg	7.28	[1.90, 12.67]	2.613	24.5	2.79	0.010	51
2.4.3I	testes mass normalized to body mass		0mg/kg - 2mg/kg	0.13	[-0.02, 0.28]	0.073	24.5	1.80	0.085	52

Table 2.1.1. Number of dams in each group for studies in Figure 2.1. Dam behavior includes values for number of nest exits per hour and percentage of time spent off the nest.

		# of dam measurements on postnatal day								
variable	treatment	4	5	6	7	8	9	10	11	21
dam mass	STD	25							25	25
	LBN	24							24	24
dam corticosterone	STD								24	
	LBN								24	
dam behavior	STD	23	24	25	19	19	19	19	19	
	LBN	22	23	24	20	20	20	20	19	

Table 2.1.2. Statistics for tests of dam mass and dam behavior over time. Linear mixed model and pairwise comparisons of the dam mass on PND4, 11, and 21 was fit with the equation mass ~ early-life treatment * PND@ + (1 | dam). Postnatal day (PND) was treated as a factor variable. Dam behavior parameters were analyzed with a nonparametric longitudinal model using the nparLD package in R, with the F1 LD F1 Model. The subject variable was each dam, early-life treatment (STD or LBN cage) was the between-subject factor ('whole-plot' factor), and PND was the within-subject factor ('sub-plot' repeated factor).

	early-life treatment			PND			early-life treatment * PND		
feature	F	df	p	F	df	p	F	df	p
dam mass	4.83	1, 47.0	0.033	82.02	2, 94.0	<0.001	1.69	2, 94.0	0.189
# of exits	19.49	1.0	<0.001	12.33	5.7	<0.001	1.11	5.7	0.356
% off nest	2.01	1.0	0.156	0.90	5.6	0.490	1.52	5.6	0.171

Table 2.2.1. Linear mixed models of offspring mass on PND11 and anogenital distance. Equations were outcome ~ early-life treatment * sex + (1 | dam). Early-life treatment is STD vs LBN rearing. Sex is males vs females.

	early-life treatment			sex			early-life treatment * sex		
feature	F	df	p	F	df	p	F	df	p
PND11 mass	8.26	1, 47.0	0.006	0.25	1, 249.7	0.616	2.38	1, 249.7	0.124
anogenital distance	1.32	1, 46.5	0.257	25763.19	1, 239.8	<0.001	0.01	1, 239.8	0.940

Table 2.2.2. Linear mixed model of the offspring mass from PND11-72 fit with the equation mass ~ early-life treatment * PND@ + (1 | dam) + (1 | mouse). Early-life treatment is STD vs LBN rearing. PND@: Linear splines at PND21 and 35 allow the model to change the slope of the line for the segments between PND11-21, from 21-35, and from 35-72. Male and female offspring were fit with separate models.

	females			males		
variable	F	df	p	F	df	p
early-life treatment	0.78	1, 76.8	0.381	3.37	1, 69.4	0.071
PND@	59283.03	3, 3229.4	<0.001	68522.70	3, 2619.4	<0.001
early-life treatment * PND@	0.85	3, 3229.4	0.466	6.84	3, 2619.4	<0.001

Table 2.2.3. Linear mixed models for maturation with the equation maturation feature ~ early-life treatment + (1 | dam). Early-life treatment is STD vs LBN rearing. Models were fit for age and for mass at vaginal opening, first estrus, and preputial separation.

	effect of early-life treatment					
	age			mass		
	F	df	p	F	df	p
vaginal opening	1.57	1, 43.6	0.217	0.10	1, 41.2	0.754
first estrus	1.54	1, 43.7	0.221	0.10	1, 41.4	0.758
preputial separation	1.91	1, 30.9	0.177	0.04	1, 30.7	0.846

Table 2.3.1. Statistics for estrous cycles from postnatal days 70-90. The number of cycles was fit with the linear mixed model equation # of cycles ~ early-life treatment + (1 | dam). The log of the mean cycle length in days was fit with equation $\log_{10}$(cycle length) ~ early-life treatment + (1 | dam). Early-life treatment is STD vs LBN rearing. The number of days spent in each cycle stage was assessed with a Chi-squared test (n = 3066).

	# cycles			mean cycle length			Cycle stage distribution		
variable	F	df	p	F	df	p	Chi-sq	df	p
early-life treatment	0.86	1, 37.3	0.359	0.56	1, 37.2	0.457	0.29	2	0.865

Table 2.4.1. Number of litters and mice with serum corticosterone measurements before and after adult treatment.

		STD				LBN			
		CON		ALPS		CON		ALPS	
	time	litters	mice	litters	mice	litters	mice	litters	mice
male	pre	11	20	11	20	13	19	13	19
	post	11	19	11	20	13	19	13	19
diestrus	pre	9	10	8	8	7	9	9	9
	post	9	10	8	8	7	9	9	9
proestrus	pre	11	14	17	25	12	14	19	28
	post	11	14	17	25	12	14	19	28

Table 2.4.2. Statistics for serum corticosterone in male and female offspring. Data from males were fit with the linear mixed model equation log_{10}(cort) ~ early-life treatment * adult treatment * time + (1 | mouse) + (1 | dam). Data from females were fit with the linear mixed model equation log_{10}(cort) ~ cycle stage * early-life treatment * adult treatment * time + (1 | mouse) + (1 | dam). Cycle stage is diestrus vs proestrus; early-life treatment is STD vs LBN rearing; adult treatment is CON vs ALPS; time is pre (0h) vs post (5h).

	males			females		
variable	F	df	p	F	df	p
cycle stage				23.85	1, 216.3	<0.001
early-life treatment	1.60	1, 44.0	0.213	1.36	1, 102.0	0.246
adult treatment	0.02	1, 121.7	0.883	0.03	1, 200.2	0.867
time	356.09	1, 73.6	<0.001	539.59	1, 109.0	<0.001
cycle stage * early-life treatment				0.18	1, 216.3	0.672
cycle stage * adult treatment				3.06	1, 212.1	0.081
early-life treatment * adult treatment	0.01	1, 121.7	0.943	0.53	1, 200.2	0.468
cycle stage * time				9.65	1, 109.0	0.002
early-life treatment * time	1.39	1, 73.6	0.241	0.83	1, 109.0	0.364
adult treatment * time	42.80	1, 73.6	<0.001	37.79	1, 109.0	<0.001
cycle stage * early-life treatment * adult treatment				0.00	1, 212.1	0.975
cycle stage * early-life treatment * time				0.38	1, 109.0	0.539
cycle stage * adult treatment * time				3.95	1, 109.0	0.049
early-life treatment * adult treatment * time	0.07	1, 73.6	0.791	0.22	1, 109.0	0.643
cycle stage * early-life treatment * adult treatment * time				0.02	1, 109.0	0.878

Table 2.4.3. Number of litters and male mice with mass measurements on the day of adult treatment. Lower numbers for some tissue masses are attributable to loss of or damage to tissue at dissection.

	STD				LBN			
	CON		ALPS		CON		ALPS	
feature	litters	mice	litters	mice	litters	mice	litters	mice
AM body mass (g)	11	19	11	19	13	19	13	19
% change body mass	11	19	11	19	13	19	13	18
adrenal mass (mg)	10	17	10	17	12	18	11	16
adrenal mass normalized to PM mass (mg/g)	10	17	10	17	12	18	11	15
seminal vesicle mass (mg)	11	18	11	19	13	18	13	18
seminal vesicle mass normalized to PM mass (mg/g)	11	18	11	19	13	18	13	17
testicular mass (mg)	11	19	11	19	13	19	13	19
testicular mass normalized to PM mass (mg/g)	11	19	11	19	13	19	13	18

Table 2.4.4. Statistics from linear mixed models of male masses on day of adult treatment. Data were fit with the formula feature ~ early-life treatment * adult treatment + (1 | dam). Early-life treatment is STD vs LBN rearing; adult treatment is CON vs ALPS.

	Early-life treatment			adult treatment			early-life treatment * adult treatment		
feature	F	df	p	F	df	p	F	df	p
AM body mass (g)	4.95	1, 21.9	0.037	7.57	1, 50.7	0.008	0.78	1, 50.7	0.381
% change body mass	0.50	1, 20.4	0.489	255.89	1, 56.7	<0.001	3.07	1, 56.7	0.085
adrenal mass (mg)	1.30	1, 21.2	0.267	1.80	1, 46.0	0.186	0.87	1, 46.0	0.356
adrenal mass normalized to PM mass (mg/g)	4.06	1, 21.2	0.057	1.44	1, 45.6	0.237	0.59	1, 45.6	0.448
seminal vesicle mass (mg)	0.95	1, 21.4	0.341	3.07	1, 49.4	0.086	0.22	1, 49.4	0.643
seminal vesicle mass normalized to PM mass (mg/g)	8.47	1, 20.6	0.008	4.12	1, 52.4	0.048	0.06	1, 52.4	0.804
testicular mass (mg)	18.95	1, 20.8	<0.001	9.43	1, 55.0	0.003	1.35	1, 55.0	0.250
testicular mass normalized to PM mass (mg/g)	0.03	1, 21.7	0.862	5.63	1, 50.6	0.021	0.25	1, 50.6	0.622

Table 2.4.5. Number of litters and female mice with mass measurements on the day of adult treatment. Adrenal masses were not collected from females used for electrophysiology studies (Figures 2.6.7). Lower numbers for some tissue masses are attributable to loss of or damage to tissue at dissection.

		STD				LBN			
		CON		ALPS		CON		ALPS	
cycle stage	feature	litters	mice	litters	mice	litters	mice	litters	mice
diestrus	AM body mass (g)	9	10	7	7	7	9	9	9
	% change body mass	9	10	7	7	7	9	9	9
	adrenal mass (mg)	9	10	8	8	7	9	9	9
	adrenal mass normalized to PM mass (mg/g)	9	10	8	8	7	9	9	9
	uterine mass (mg)	9	10	8	8	7	9	9	9
	uterine mass normalized to PM mass (mg/g)	9	10	8	8	7	9	9	9
proestrus	AM body mass (g)	11	14	15	23	12	14	19	28
	% change body mass	10	13	15	23	12	14	18	27
	adrenal mass (mg)	7	8	12	18	8	8	14	21
	adrenal mass normalized to PM mass (mg/g)	7	8	12	18	8	8	13	20
	uterine mass (mg)	11	14	17	25	12	14	19	28
	uterine mass normalized to PM mass (mg/g)	10	13	17	25	12	14	18	27

Table 2.4.6. Statistics from linear mixed models of female masses on day of adult treatment. Data were fit with the formula feature ~ early-life treatment * adult treatment * cycle stage + (1 | dam). Early-life treatment is STD vs LBN rearing; adult treatment is CON vs ALPS; cycle stage is diestrus vs proestrus.

feature	variable	F	df	p
AM body mass (g)	early-life treatment	0.96	1, 43.5	0.333
	adult treatment	0.78	1, 77.9	0.380
	cycle stage	1.02	1, 86.0	0.315
	early-life treatment * adult treatment	0.16	1, 77.9	0.689
	early-life treatment * cycle stage	0.11	1, 86.0	0.744
	adult treatment * cycle stage	0.02	1, 83.1	0.896
	early-life treatment * adult treatment * cycle stage	0.00	1, 83.1	0.987
% change body mass	early-life treatment	2.91	1, 34.0	0.097
	adult treatment	60.96	1, 93.6	<0.001
	cycle stage	4.41	1, 103.9	0.038
	early-life treatment * adult treatment	0.02	1, 93.6	0.899
	early-life treatment * cycle stage	0.96	1, 103.9	0.330
	adult treatment * cycle stage	0.38	1, 101.7	0.539
	early-life treatment * adult treatment * cycle stage	0.23	1, 101.7	0.631
adrenal mass (mg)	early-life treatment	4.20	1, 30.6	0.049
	adult treatment	0.01	1, 68.6	0.913
	cycle stage	0.76	1, 67.9	0.386
	early-life treatment * adult treatment	0.82	1, 68.6	0.368
	early-life treatment * cycle stage	0.25	1, 67.9	0.617
	adult treatment * cycle stage	1.40	1, 76.3	0.241
	early-life treatment * adult treatment * cycle stage	0.32	1, 76.3	0.575

feature	variable	F	df	p
adrenal mass normalized to PM mass (mg/g)	early-life treatment	0.37	1, 32.5	0.547
	adult treatment	3.08	1, 60.8	0.084
	cycle stage	0.05	1, 61.7	0.831
	early-life treatment * adult treatment	0.01	1, 60.8	0.926
	early-life treatment * cycle stage	0.15	1, 61.7	0.696
	adult treatment * cycle stage	2.40	1, 66.9	0.126
	early-life treatment * adult treatment * cycle stage	0.28	1, 66.9	0.601
uterine mass (mg)	early-life treatment	0.03	1, 43.6	0.871
	adult treatment	0.00	1, 77.2	0.995
	cycle stage	573.41	1, 85.5	<0.001
	early-life treatment * adult treatment	0.05	1, 77.2	0.819
	early-life treatment * cycle stage	0.69	1, 85.5	0.407
	adult treatment * cycle stage	3.30	1, 83.6	0.073
	early-life treatment * adult treatment * cycle stage	0.28	1, 83.6	0.595
uterine mass normalized to PM mass (mg/g)	early-life treatment	0.34	1, 43.3	0.565
	adult treatment	1.81	1, 74.9	0.183
	cycle stage	467.39	1, 84.4	<0.001
	early-life treatment * adult treatment	0.00	1, 74.9	0.993
	early-life treatment * cycle stage	1.39	1, 84.4	0.242
	adult treatment * cycle stage	3.70	1, 81.0	0.058
	early-life treatment * adult treatment * cycle stage	1.21	1, 81.0	0.274

Table 2.4.7. Statistics from linear mixed models of male masses on day of vehicle (0mg/kg) or corticosterone (2mg/kg) treatment. Data were fit with the formula feature ~ dosage + (1 | dam).

feature	Dosage		
	F	df	p
AM body mass (g)	0.28	1, 24.5	0.602
% change body mass	3.96	1, 27.5	0.057
adrenal mass (mg)	0.55	1, 23.4	0.466
adrenal mass normalized to PM mass (mg/g)	0.70	1, 23.5	0.410
seminal vesicle mass (mg)	0.07	1, 27.5	0.790
seminal vesicle mass normalized to PM mass (mg/g)	0.18	1, 27.5	0.674
testicular mass (mg)	7.77	1, 24.5	0.010
testicular mass normalized to PM mass (mg/g)	3.22	1, 24.5	0.085

Table 2.4.8. Statistics for serum corticosterone in males with vehicle or corticosterone administration. Data were fit with the linear mixed model equation log_{10}(cort) ~ dosage * time + (1 | mouse) + (1 | dam). Dosage is 0mg/kg vs 2mg/kg; time compares 0h, 1h, 2h, 3h, 4h, and 5h.

variable	F	df	p
dosage	432.21	1, 36.4	<0.001
time	178.35	5, 245.0	<0.001
dosage * time	75.13	5, 245.0	<0.001

Table 2.5.1. Statistics from linear mixed models of average LH in diestrous mice on day of adult treatment. Data were fit with the formula average LH ~ early-life treatment * adult treatment + (1 | dam). Early-life treatment is STD vs LBN treatment; adult treatment is CON vs ALPS treatment.

variable	F	df	p
early-life treatment	0.17	1, 16.4	0.687
adult treatment	3.69	1, 23.1	0.067
early-life treatment * adult treatment	0.44	1, 23.1	0.515

Table 2.6.1. Statistics from linear mixed models of electrophysiology properties on day of adult treatment. The mean value for each cell was calculated, and data were fit with the formula feature ~ early-life treatment * adult treatment + (1 | dam) + (1 | mouse). Early-life treatment is STD vs LBN rearing; adult treatment is CON vs ALPS.

	early-life treatment			adult treatment			early-life treatment * adult treatment		
feature	F	df	p	F	df	p	F	df	p
capacitance (pF)	0.03	1, 8.1	0.863	0.77	1, 9.4	0.401	0.07	1, 9.4	0.791
input resistance (MOhm)	0.23	1, 8.4	0.641	1.52	1, 9.5	0.248	1.50	1, 9.5	0.250
series resistance (MOhm)	0.30	1, 7.8	0.601	0.97	1, 11.3	0.346	0.27	1, 11.3	0.613
holding current (pA)	0.02	1, 7.8	0.898	0.01	1, 9.2	0.909	0.35	1, 9.2	0.566
interevent interval (pA)	0.12	1, 7.8	0.740	0.05	1, 9.2	0.820	0.00	1, 9.2	0.946
amplitude (pA)	0.45	1, 7.0	0.524	0.72	1, 8.8	0.420	0.12	1, 8.8	0.737
decay time (ms)	0.50	1, 7.0	0.503	0.85	1, 8.8	0.381	1.31	1, 8.8	0.283

Table 2.7.1. Statistics for number of postsynaptic current (PSC) events per 240s in GnRH neurons on the day of adult treatment. As the frequency data were skewed right and included zeros, a generalized linear mixed effects negative binomial model was used. Data were fit with the model equation # events per 240s ~ early-life treatment * adult treatment + (1 | mouse) + (1 | dam). The joint_tests function of the emmeans package was used to obtain these p-value estimates from the model. Early-life treatment is STD vs LBN rearing; adult treatment is CON vs ALPS.

variable	F ratio	df	Chi-sq	p
early-life treatment	0.013	1, Inf	0.013	0.910
adult treatment	1.476	1, Inf	1.476	0.224
early-life treatment * adult treatment	0.067	1, Inf	0.067	0.796

Table 2.7.2. Pairwise comparisons of distributions of interevent interval and amplitude for PSCs recorded in GnRH neurons. The Anderson-Darling criterion (AD), the standardized test statistic (T AD), and asymptotic p-value were calculated with the kSamples package (201). Bootstrapping was used to estimate the mean difference. The confidence interval is not adjusted for multiple comparisons. The p-values for both tests were adjusted using Holm's method for multiple comparisons.

		# PSCs		Anderson-Darling			bootstrap		
variable	comparison	group 1	group 2	AD	T AD	p	mean diff	95% CI	p
interevent interval (ms)	STD-CON vs STD-ALPS	4,597	2,324	531.5	696.91	<0.001	0.78	[0.65, 0.92]	<0.001
	STD-CON vs LBN-CON	4,597	3,032	214.5	280.48	<0.001	0.35	[0.25, 0.46]	<0.001
	LBN-CON vs LBN-ALPS	3,032	2,217	104.0	135.40	<0.001	0.32	[0.19, 0.46]	<0.001
	STD-ALPS vs LBN-ALPS	2,324	2,217	0.5	-0.65	0.744	-0.11	[-0.27, 0.05]	0.177
amplitude (pA)	STD-CON vs STD-ALPS	1,787	1,849	15.5	19.02	<0.001	3.60	[1.36, 5.81]	0.006
	STD-CON vs LBN-CON	1,787	1,908	3.8	3.62	0.023	-1.23	[-3.36, 0.80]	0.498
	LBN-CON vs LBN-ALPS	1,908	1,759	2.3	1.69	0.065	-0.39	[-2.42, 1.66]	0.711
	STD-ALPS vs LBN-ALPS	1,849	1,759	21.1	26.37	<0.001	-5.22	[-7.34, -3.09]	<0.001

Chapter 3 Conclusion

This dissertation examined the implications of early-life stress on sexual maturation, estrous cycles, and the response to adult stress. LBN from PND4-11 did not alter the age or mass at vaginal opening, first estrus, or preputial separation. Further, estrous cycles were not disrupted by LBN. The basal corticosterone concentrations in both the morning and afternoon were similar for STD and LBN-reared mice. LBN did not alter the corticosterone response to ALPS. In both STD and LBN proestrous females, ALPS disrupted the LH surge in a majority of mice. We tested the hypothesis that ALPS reduces the frequency of GABAergic PSCs in GnRH neurons, but the results did not support this.

LBN did not disrupt reproductive maturation

In the time since the project was developed, a few additional studies have explored the consequences of LBN on sexual maturation. As with prior studies, there is variability in the outcomes. LBN from PND2-9 in Long Evans rats did not change the age at vaginal opening (175), whereas a modified LBN paradigm without the wire mesh advanced vaginal opening in this strain (174). In mice, a group found that LBN from PND2-9 delayed vaginal opening (173), contrasting with their own earlier finding in which LBN did not affect vaginal opening (57). While these studies were investigating two different genetic knockouts, these effects were observed in wildtype littermates, and both lines had been backcrossed to C57Bl/6 mice.

In addition to considering the genetic background and strain of the pups, it is also likely that the effects of LBN are sensitive to the strain of the dam. In the present studies, we used wildtype CBA dams. These dams were bred with males on a C57Bl/6J background resulting in F1 CBA/C57Bl/6 hybrid pups. In early pilot studies, we compared the effects of the paradigm when using CBA or C57Bl/6J dams from PND2-9. Anecdotally, CBA dams are thought to provide better maternal care than C57Bl/6J dams, but how CBA dams would react to LBN treatment was unknown. There was greater pup loss during the paradigm in litters from C57Bl/6J dams than from CBA dams, and some of the C57Bl/6J litters also required additional nutritional support or delayed weaning due to extremely low body mass. These early results led us to continue the studies using only CBA dams from PND4-11, with the goal of disentangling delays in reproductive maturation inherent to the stress exposure from delays that are secondary to reductions in body mass.

We were able to verify that the LBN paradigm had the expected effect on maternal behavior with CBA dams. LBN dams made more exits from the nest, without changing the amount of time that they spent on the nest, indicative of fragmented maternal care. The lack of substantive differences in pup body mass after the paradigm and around the time of weaning suggests that the effect of LBN may have been milder in pups reared by CBA dams. At least at this milder level, there did not appear to be independent effects of LBN on the age at reproductive maturation in males or females.

STD and LBN mice had similar corticosterone responses to ALPS

LBN did not change the corticosterone response to ALPS in males, diestrous females, or proestrous females. This is consistent with a study in both male and female Long Evans rats in which STD and LBN exhibited similar corticosterone concentrations at the end of a 60min restraint stress and 30min after cessation of the stressor (175). However, in response to a shorter restraint stress of only 10min, LBN female mice in another study exhibited blunted corticosterone release after the cessation of the stressor (79). In the present study, corticosterone was only measured at the end of the 5h stress. Therefore, we are unable to determine if LBN mice had different patterns of release at the onset of the different stressors, or if the termination of the corticosterone release following the stressors was altered.

Timing of limited bedding and nesting paradigm

LBN is typically only applied for one week early in the postnatal period, often overlapping with the stress hyporesponsive period. This design is particularly useful for assessing the consequences of early-life stress on critical windows of development. However, this design does limit the ability to translate this paradigm broadly to human experiences. This week-long span of rodent development roughly corresponds to late fetal development through the first couple years of life for a human infant (65). Thus, LBN may best correspond to situations in which the maternal-infant dyad experiences low resources very early in life, but then had adequate resources throughout the rest of the child’s life. This early disruption would likely have many developmental consequences, but it is a distinct situation from that of individuals who were reared throughout their childhood and adolescence in a low-resource environment. With this in

mind, I would expect that extended exposure to the LBN environment through weaning would lead to distinct consequences for the development of the reproductive system, and for the response to subsequent stressors like ALPS in adulthood.

Diurnal and sex steroid effects on corticosterone release

In rodents, serum corticosterone concentrations exhibit a diurnal pattern; concentrations are low early in the light phase and then rise throughout the light phase reaching a peak around the time of lights out (211–213). This matches our observation that adult control mice (males, diestrous and proestrous females) all had a rise in corticosterone concentrations from the morning pre-treatment to the afternoon post-treatment samples. The 5h stress paradigm, ALPS, further increased serum corticosterone in all groups, demonstrating its effective activation of the neuroendocrine stress response.

Corticosterone levels at baseline and in response to stressors are thought to vary with the estrous cycle and between sexes in rodents; for review see (214). Females typically exhibit higher basal levels and a larger corticosterone response to stress than males (212,213,215,216). The pattern of corticosterone release, as demonstrated by the onset of corticosterone release during a stressor, and persistence of elevated corticosterone after cessation of the stressor, also are impacted by sex. When considering the impact of the estrous cycle, the prevailing consensus is that proestrous females have higher basal corticosterone levels and a more pronounced stress response than diestrous females, who tend to exhibit patterns more similar to males (214). This is attributed to the role of estradiol in potentiating the stress axis; estradiol replacement alone

increases corticosterone release during and following a 20min restraint stress in OVX females relative to estradiol and progesterone combined or a vehicle control (217).

Proestrous females should have higher morning estradiol concentrations than diestrous females, and we observed that proestrous mice had elevated morning serum corticosterone concentration consistent with the hypothesis above. In contrast, there were no differences in afternoon corticosterone concentrations between these stages for either control or ALPS-exposed mice. While this lack of difference conflicts with the general hypothesis in the field, there have also been many studies that failed to detect a basal difference in corticosterone between proestrous and diestrous females (215,217–219). Estradiol and progesterone levels change throughout the day in both diestrous and proestrous females, and it is possible that the magnitudes of the differences in estradiol and progesterone between these two groups are smaller in the afternoon than in the morning. However, it is difficult to compare these hormone levels between groups with certainty; for example, the onset of the rise in progesterone as the rodent transitions from proestrus to estrus is likely to depend on the individual timing of the LH surge. Technical limitations related to the quantity of serum necessary to detect these steroids preclude the ability to attain data about estradiol and progesterone levels across the full cycle in individual animals.

We also did not observe corticosterone differences between proestrous and diestrous females after the stress paradigm. A limitation to the design of the study is that animals were only sampled at the beginning and end of the stress paradigm. It is thus possible that proestrous and diestrous mice may have had distinct patterns of corticosterone release during the paradigm, or that the termination of the corticosterone response after

cessation of stress could have differed. Furthermore, this study did not assess non-neuroendocrine components of the stress response that may have differed between cycle stages.

Does ALPS suppress LH pulses?

As described in detail in Chapter 1, many psychosocial stressors, including restraint, suppress aspects of LH pulses (such as mean LH, LH-pulse amplitude, and/or LH-pulse frequency) in non-human primates (96–99), sheep (112–116), and rodents (118–122,130). This dissertation project was not designed to directly test pulsatile LH release. Yet, the trend toward decreased mean LH in diestrous females with limited sampling at the end of the ALPS paradigm and 2.5h later at lights out suggests that ALPS could alter pulsatile release. Future studies could be specifically designed to assess how the layered stressors in the ALPS paradigm affect LH pulses in both females and males.

Mechanisms of ALPS-induced disruption of the LH surge

This study replicated the previous finding (134) that ALPS disrupts the LH surge in most animals, but many questions remain about the mechanism of this disruption.

Hypothalamus

As described in Chapter 1, AVPV kisspeptin neurons may be a primary source of increased GABAergic input to GnRH neurons on the afternoon of proestrus, and given their role in mediating estradiol positive feedback, we predicted that GABAergic input to GnRH neurons would be altered by ALPS. The results of this study indicate that a change in GABAergic PSC frequency or properties of GABA PSCs cannot easily account for the disruption of the LH surge following ALPS. An important caveat to this

work is that, due to the timing of the stress, slices were prepared around or after the time of the LH surge peak in many mice. While the frequency of GABA PSCs is elevated in coronal slices prepared from proestrous mice at similar times (139), in OVX+E mice the frequency is only elevated in coronal slices prepared around the onset of the surge and not in coronal slices at the peak of the surge (140). The design of this dissertation may have precluded detection of any changes in GABA PSC frequency induced by ALPS around the time of the onset of the surge, which could have disrupted surge initiation. Further, this study does not exclude a possible role for AVPV kisspeptin neurons in mediating ALPS-induced disruptions. We have yet to test if kisspeptin release by AVPV kisspeptin neurons is altered after ALPS, nor have we assessed if ALPS alters how GnRH neurons respond to kisspeptin.

Even though ALPS did not decrease the frequency of GABAergic input to GnRH neurons, it could be changing the activity of these cells by changing their excitability. Future experiments could use whole-cell current-clamp recordings to test the membrane response of GnRH neurons to controlled current injections. The number of action potentials generated at each current step is an indication of the excitability of GnRH neurons and could be compared between stress groups. The shape of the resulting action potentials could also point to potential underlying changes in voltage-dependent ion channels that could be altered by stress.

Additional studies could directly test if ALPS decreases the firing activity of GnRH neurons on the afternoon of proestrous, such as by conducting long-term extracellular recordings of these cells (220,221). If GnRH neurons have reduced excitability after stress or receive increased inhibitory input, this could translate to a decreased firing

frequency or pattern of activity that ultimately may decrease GnRH release in the median eminence. However, if the activity of GnRH neurons is not altered by ALPS, this could point to gonadotropes within the pituitary, as opposed to central effects within the hypothalamus, as the primary site of disruption to the LH surge.

Pituitary

Studies *in vivo* could test if ALPS alters the response to exogenous kisspeptin and/or GnRH administration, as measured by LH release (24,149). If LH release after exogenous GnRH is lower in ALPS than CON mice, this could indicate that pituitary gonadotropes are less responsive to GnRH after stress. A reduction in LH release after kisspeptin administration could indicate changes at either the level of GnRH neurons or the pituitary, and these results would need to be contextualized with the experiment described above to test how the firing frequency of GnRH neurons changes in response to bath application of kisspeptin after stress.

Actions of corticosterone

To test the hypothesis that corticosterone is sufficient to disrupt the LH surge and ovulation, I worked with an undergraduate student, Bo Dong, to develop a protocol for feeding mice Nutella to increase serum corticosterone concentrations. We chose this route of oral administration as pilot studies within the lab had demonstrated that even with habituation to injection procedures, mice still had elevated corticosterone in response to saline injections. After a series of additional pilot studies with Bo, we determined that a dosage of 2mg/kg corticosterone administered at times corresponding

to the stressors during ALPS leads to a similar serum corticosterone profile as ALPS (Figure 2.3.1A).

In the proestrous mice studied, 2mg/kg corticosterone administration elevated afternoon serum corticosterone relative to vehicle treatment (Figure 2.3.1B, linear mixed model, dosage * time: $F_{(1,24)} = 22.60$, $p < 0.001$; emmeans *post hoc*, 0mg/kg vs. 2mg/kg at post treatment time: $t_{46.1} = -2.80$, $p = 0.007$). As evident in the individual LH profiles (Figure 2.3.1C), most mice in each group exhibited an LH surge during the sampling window (Figure 2.3.1D). There was not a clear relationship between serum corticosterone concentrations and exhibiting an LH surge (mice without a surge are colored magenta in Figure 2.3.1B and D). We also checked for the presence of ovulated oocytes in the oviduct the following morning; all mice with a surge above the threshold of 3.8ng/mL ovulated and none below ovulated. Contrary to our hypothesis, vehicle and corticosterone-treated mice had similar incidences of observed LH surges and ovulation (Figure 2.3.1E, $X^2 = 0.04$, $p = 0.848$). This suggests that corticosterone is not the primary driver of the disruptive effects of ALPS on the LH surge.

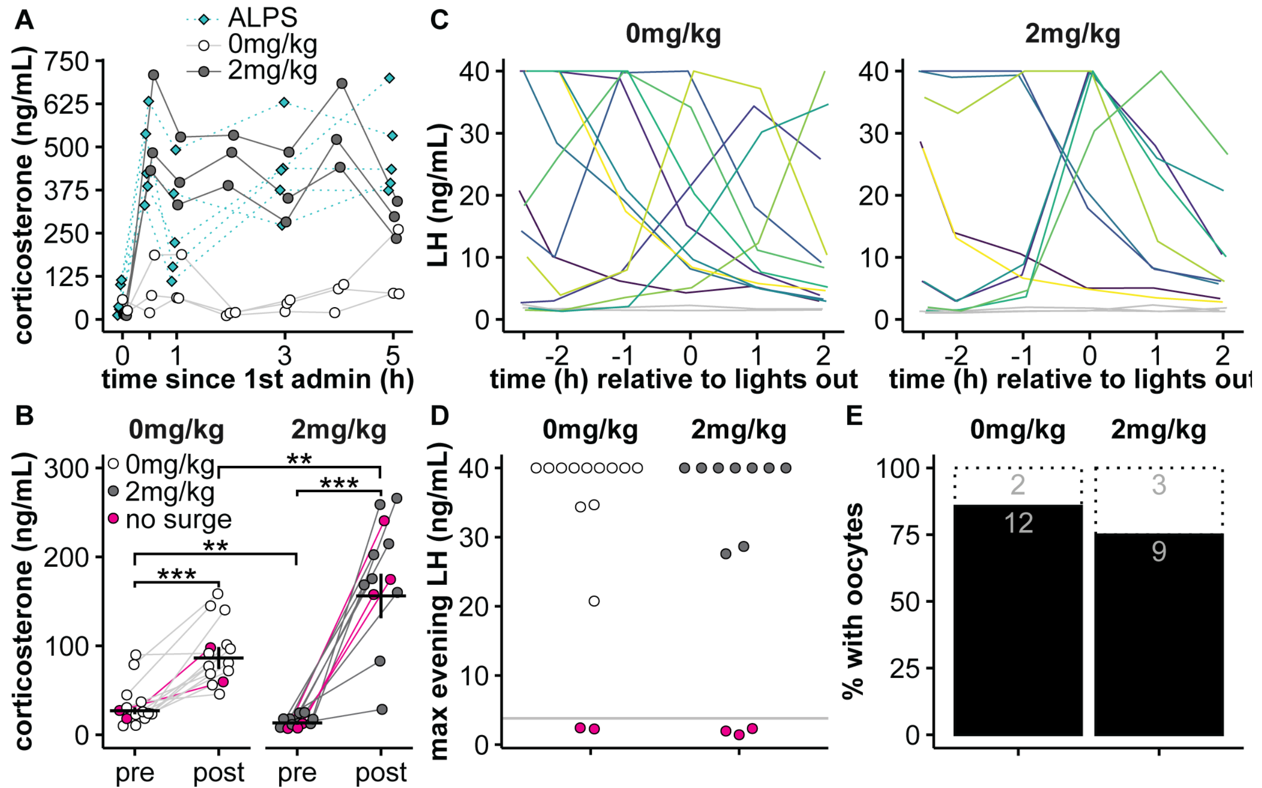

Figure 2.3.1. Corticosterone administration on proestrus does not disrupt the LH surge or ovulation. **A**. Individual serum corticosterone after 0mg/kg or 2mg/kg repeated oral administration of corticosterone (0h, 1h, 3h) or ALPS treatment for female mice in various cycle stages. **B**. Individual and linear mixed model mean±SEM for serum corticosterone concentrations after 0mg/kg or 2mg/kg repeated oral administration of corticosterone in proestrous mice. Note the difference in the y-axis scale between **A** and **B**. Magenta dots in **B** and **D** are mice that did not exhibit an LH surge. **C**. Individual LH profiles for proestrous mice in each treatment group sampled hourly until 2h after lights out; time is relative to lights out. Grey lines show mice with no LH concentrations above 3.8ng/mL. **D**. Individual values of maximum evening LH. Grey line at 3.8ng/mL is the cutoff for an LH surge. **E.** Percentage of mice with oocytes the following morning (filled bars; numbers are counts per result). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$
Abbreviations: LH, luteinizing hormone; ALPS, acute, layered, psychosocial stress in adulthood

Activation of CRH neurons

CRH was thought to be another candidate to mediate the effects of ALPS on the reproductive axis. In the PVN, CRH neurons are part of the neuroendocrine aspect of the stress response (27); other regions also help regulate related processes including behavioral responses to stress (222,223). CRH disrupts LH pulses in rodents (163,168), non-human primates (90,91,94), and humans (224). In OVX rats, the suppression of mean LH by CRH is similar in adrenal-intact and adrenalectomized rats (163). This suggests that there are independent effects of CRH beyond solely elevating serum corticosterone, though this does not exclude the possibility that a rise of ACTH could be involved. CRH antagonists ameliorate the LH-inhibiting effects of stress induced by foot shock (225), restraint (119,122), and LPS (119) in rats. Chemogenetic activation of PVN CRH neurons reduced the frequency of LH pulses in OVX mice (226), indicating these cells can affect GnRH neuron output. PVN lesions do not, however, prevent stress-induced disruption in LH (227), suggesting a role for extra-PVN CRH or other neurons.

CRH neuron activation during ALPS

Given these relationships, we hypothesized that ALPS disrupts the LH surge by increasing CRH neuron output, which directly or indirectly interrupts estradiol-induced increases in GnRH neuron output and thus the LH surge. I mentored an undergraduate student, Jesse Lange, on a project to determine if ALPS activates CRH neurons, including in regions outside the PVN. We planned to assess expression of cFos, an immediate early gene which is activated by stress (228,229), in CRH neurons. CRH neurons were identified by GFP expression in CRH-Cre mice crossed with an L10a-

GFP reporter line (230). Control or ALPS proestrous females were perfused at 1.5h, 2.5h, and 5h after the start of the paradigm to assess CRH neuron activation at different stages of the stress paradigm.

Jesse worked to modify a protocol for the use of immunofluorescence to detect the co-expression of cFos and GFP. He identified CRH neurons in the PVN that expressed cFos following ALPS, but systematic study of the activation of CRH neurons in various regions throughout the brain remains to be conducted. We anticipate that the results of this study could help direct future optogenetic experiments investigating the effects of endogenous CRH circuits on the reproductive neuroendocrine axis.

Optogenetic activation of CRH neurons

As described, CRH alters activity of GnRH neurons in brain slices in a dose- and estradiol-dependent manner (154). This provides important proof-of-principle premise for optogenetic studies of the effect of CRH neuron activation on GnRH and AVPV kisspeptin neurons. Yet, how CRH actions are integrated with other mediators *in vivo*, including co-transmitters arising from CRH neurons, is not known. To fill these gaps, it is important to understand the endogenous circuits by which CRH neurons interact with components of the reproductive neuroendocrine system. Thus, an important future direction for this work is to use channelrhodopsin-assisted circuit mapping (CRACM) to determine if activation of CRH terminals leads to evoked currents in GnRH or AVPV kisspeptin neurons. These studies would also help address the current debate on the existence of direct synaptic connections between CRH and GnRH neurons (231–235). Protocols could be designed to alter the frequency and duration of activation to bias the

release of neuropeptides, in contrast to fast-synaptic transmitters (236), and test if this sustained activation of CRH neurons alters the activity of GnRH or AVPV kisspeptin neurons.

In the initial design of these experiments, we had planned to use the Ai167(TIT2L-ChrimsonR-tdT-ICL-tTA2, Allen Brain Institute) (237) mouse line to express the opsin ChrimsonR in all CRH neurons. ChrimsonR is a red-shifted excitatory opsin with fast kinetics, a high peak current, and high fidelity that can be activated with lower intensity light (237,238). Given the wide distribution of CRH neurons throughout the brain, we chose to begin with mouse-line expression of ChrimsonR in all CRH neurons to first test if there were *any* effects of their activation on GnRH and/or kisspeptin neurons. If this approach suggested the presence of connections, we would then use a viral approach with stereotaxic injections to target expression of ChrimsonR in specific populations of CRH neurons and isolate the source of these connections.

However, as I began pilot experiments to verify that CRH neurons fired action potentials in response to red-light stimulation, I noted that the expression pattern of tdTomato/ChrimsonR$^+$ cells did not match the expected distribution for CRH neurons. I compared the distribution of CRH$^+$ neurons in similar sections from the Ai167 (ChrimsonR) line, which uses the TIGRE2.0 approach for Cre-dependent expression (237) and the L10a line, which inserts the floxed stop and GFP gene at the more traditional Rosa26 locus (230). The reduction in CRH$^+$ cells identified in sections from the Ai167 line was particularly notable in the bed nucleus of the stria terminalis, where there should be a relatively high density of CRH neurons (230,239). Upon further review of the literature, other examples of a lower density of expression when using the

Ai167 mouse line compared to a viral-based approach or a reporter line using the Rosa26 locus were identified (240). Thus, these experiments will need to use viral approaches targeting specific CRH populations moving forward. As mentioned above, the selection of these initial targets could be guided by the CRH-cFos studies. Populations of CRH neurons that are activated by ALPS are perhaps more likely to be among those that may have reproductive consequences.

Chemogenetic activation of CRH neurons

As another approach to testing the hypothesis that activation of CRH neurons disrupts the LH surge and ovulation, other researchers in the lab are presently testing chemogenetic activation of designer receptors exclusively activated by designer drugs (DREADDs) that express a modified G_q-receptor in CRH neurons. Contrary to the hypotheses, preliminary results indicate that activation of CRH neurons is not sufficient to disrupt the LH surge or ovulation. Though activity of CRH neurons in isolation does not appear to disrupt the LH surge, it is possible that these neurons are still involved in the broader spectrum of changes that occur during the response to layered stress.

Other components of the stress response

Multiple lines of evidence, including experiments in progress within the lab that are not presented here, are converging on the conclusion that the disruptive effects of ALPS on the LH surge cannot be fully explained by the action of hormones involved in the neuroendocrine stress axis. Therefore, this work must expand to more broadly consider systemic changes that accompany the stress response. This could include sympathetic pathways involving adrenergic or noradrenergic signaling. Many of the effects of adult

stress presented in Chapter 1 were reversed by naloxone, an opioid receptor antagonist, indicating a potential role for endogenous opioid peptides in mediating the effects of ALPS on the LH surge. Other members of the laboratory plan to directly test the hypothesis that naloxone prevents ALPS-induced disruption of the LH surge in upcoming experiments.

Summary

This dissertation project assessed the reproductive consequences of early-life and adult stress. Despite rejecting the hypotheses that LBN would delay reproductive maturation and alter the response to adult stress, these studies contribute to a growing, albeit complex, body of literature testing the effects of LBN at different times and across different species and strains. This project also excluded a possible mechanism – reducing excitatory GABAergic input to GnRH neurons – by which adult stress could disrupt the LH surge in mice, helping to direct future research towards alternative possibilities.

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