Effects of early life adversity on male reproductive behavior and the medial preoptic area transcriptome

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Abstract

Early life adversity can alter reproductive development in humans, changing the timing of pubertal onset and sexual activity. One common form of early adversity is limited access to resources. This adversity can be modeled in rats using the limited bedding/nesting model (LBN), in which dams and pups are placed in a low resource environment from postnatal days 2-9. Our laboratory previously found that adult male rats raised in LBN conditions have elevated levels of plasma estradiol compared to control males. In females, LBN had no effect on plasma hormone levels, pubertal timing, or estrous cycle duration. Estradiol mediates male reproductive behaviors. Thus, here we compared reproductive behaviors in adult males exposed to LBN vs. control housing. LBN males acquired the suite of reproductive behaviors (mounts, intromissions, and ejaculations) more quickly than their control counterparts over 3 weeks of testing. However, there was no effect of LBN in males on puberty onset or masculinization of certain brain regions, suggesting LBN effects on estradiol and reproductive behaviors manifest after puberty. In male and female rats, we next used RNA sequencing to characterize LBN-induced transcriptional changes in the medial preoptic area (mPOA), which underlies male reproductive behaviors. LBN produced sex-specific alterations in gene expression, with many transcripts showing changes in opposite directions. Many transcripts altered by LBN in males are regulated by estradiol, linking hormonal changes to molecular changes in the mPOA. Pathway analysis revealed that LBN induced changes in neurosignaling and immune signaling in males and females, respectively. Collectively, these studies reveal novel neurobiological mechanisms by which early life adversity can alter reproductive strategies.

Millions of people experience some form of early life adversity (ELA)[1]. Stressful experiences early in life are tied to a variety of negative health outcomes, including alterations in gonadal hormone levels and reproductive milestones[2-4]. ELA comes in many different forms, and the severity and type of stress experienced can affect outcomes observed. One prevalent form of ELA is limited access to resources, such as growing up in a low socioeconomic status (SES) household. Given the number of people affected by low SES early in life and its lasting impact[5,6], more research is needed to understand the mechanisms by which low resources alter reproductive behaviors and development. In rats, the limited bedding/nesting (LBN) manipulation, where insufficient nesting materials are provided for dams and pups from postnatal day (PND) 2-9[7,8], is used to model early resource scarcity. LBN induces stress in the dams and alters maternal care, which in turn produces stress in the pups. Our laboratory and others have shown that LBN dams engage in more pup-directed behaviors and fewer self-care behaviors compared to their control counterparts[4,9]. While this increase in care given to pups may appear beneficial, these behaviors are not in line with the type of maternal care given by healthy dams living in an enriched environment with ample resources, and likely reflects a hypervigilant state in the dams[10,11].

We have previously found LBN increases plasma estradiol levels in adult males, but not females, and has no effect on hormone-mediated endpoints in females, including the timing of puberty onset and estrous cycle duration[4]. More research is necessary to investigate how these lasting LBN-induced changes in estradiol affect hormone-dependent endpoints in males. One important motivated behavior that is modulated by estradiol in males is sex behavior. Sex behavior is crucial for species survival. There is a high prevalence of sexual dysfunction in human populations, including premature ejaculation, sexual desire disorder, and

anorgasmia[12,13]. Additionally, the brain circuitry underlying sexual motivation overlaps with circuitry involved in other disorders characterized by changes in motivation, including major depression and substance use disorder[14,15]. Thus, it is important to study the mechanisms by which early adversity alters sex behavior. Understanding how sex behaviors are impacted by the environment may also help improve strategies for reproductive healthcare.

The medial preoptic area (mPOA) is sensitive to the effects of estradiol and crucial for male sex behavior. During copulation, the mPOA integrates sensory input and projects to motor regions critical for physical behavior and the mesocorticolimbic system to mediate reward and motivation[16,17]. The current study investigates the impact of LBN on male sex behavior in adulthood. To gain further understanding of how LBN might impact gene expression in the mPOA to alter these behaviors, we also ran RNAsequencing (RNAseq) on the mPOA of adult males and females.

An additional goal of this research was to determine if LBN altered developmental endpoints affected by hormones, including brain sexual differentiation and the onset of puberty. To assess brain sexual differentiation, we evaluated the number of neurons in a subregion of the mPOA called the sexually dimorphic nucleus (SDN/POA). While the overall mPOA is not sexually dimorphic in size, the SDN/POA contains more neurons in adult male rodents compared to females[18-20]. If LBN males had high levels of estradiol in the perinatal sensitive period for brain masculinization (which overlaps the LBN model)[21], they would have a hypermasculinized SDN/POA. Additionally, the timing of preputial separation was recorded in LBN and control males to determine whether pubertal onset was changed by LBN. Together, these studies better characterize the impact of LBN-induced changes in gonadal hormones in male rats.

Materials and Methods

Animals and Early Life Manipulations

Our implementation of the LBN model includes breeding Long Evans rats in-house (to prevent shipping pregnant dams which is a stressor[22]), using the standard PND2-9 time length[23], and the inclusion of a metal (to ensure easy cage cleaning) rather than mesh grate for restricting bedding[4]. Control housing includes ample bedding, two cotton nestlets, and one enrichment tube. LBN housing contains a metal grate to prevent access to bedding, one paper towel for nesting, and no enrichment. Details on housing conditions are in Supplemental Methods. All experiments were approved by and in accordance with guidelines implemented by Temple University's Institutional Animal Care and Use Committee and the National Institutes of Health guidelines.

Preputial Separation

The PND of preputial separation was recorded in LBN and control males as a measure of pubertal onset. Beginning at weaning on PND21, males were checked for preputial separation daily as described[24]. The skin around the penis was gently pushed back. Preputial separation was recorded if the preputial skin slid back easily to reveal the glans penis.

Sex Behavior Assay

Adult male offspring from control and LBN conditions were tested in a sex behavior assay for 3 sessions over the course of 3 consecutive weeks. All males were virgins between PND80-120 at the start of testing. This age range was selected for consistency with previous reports[25]. In each session, males were placed alone in a clean cage. After 5min of habituation, a novel, hormonally primed stimulus female was added to the cage. Each session was 30min long and was video recorded (GoPro Hero 5) for later behavioral scoring. All video data was

scored using DOSBox v0.74-3 by raters blind to experimental conditions and was subsequently analyzed using a behavioral observation program[26]. Males were scored for both number and latency of mounts, intromissions, and ejaculations. Males that did not engage in any sexual behaviors were dropped from analysis (n=1). All behavioral testing took place between 2-5pm. All stimulus females were retired breeders who were ovariectomized and then hormonally primed as details in Supplemental Materials.

Immunohistochemistry (IHC)

Calbindin-D28K (Cb) was used as a marker for the sexual differentiation of the SDN/POA because it is sensitive to early hormone exposure and allows for a more precise visualization of the number of SDN/POA neurons than traditional methods that label all cell bodies, such as a Nissl stain[20,21]. Calbindin IHC and analysis was performed as detailed in Supplemental Methods to gauge whether LBN affected sexual differentiation of the brain. For details on statistical analysis for behavior and IHC, see Supplemental Methods.

RNA Sequencing and Analysis

A separate cohort of adult (PND80-120) male and female, LBN and control housing rats was used for RNA-seq. This age range is consistent with male rats at the time of the first sex behavior test. Rats were sacrificed by rapid decapitation and their brains were flash frozen in 2-methyl butane and stored at -80C until sample collection. Tissue punches (n=6 per group) were taken of the whole mPOA rather than only the SDN/POA as other nuclei within the mPOA are involved in reproductive behaviors[27]. The whole mPOA is not sexually dimorphic in size between males and females, so a 2.0mm punch was used across sex. Details on RNA extraction, sequencing, and analysis are in Supplemental Methods.

Results

LBN Effects on Latency to Engage in Male Sexual Behavior

Experimental design for animals used in the sex behavior assay is shown in Figure 1A. We analyzed the effect of housing condition on the latency of males to mount, intromit, and ejaculate (control n=11, LBN n=12). Mauchly's test of sphericity was significant for both mounts [$\chi2(2)=7.09$, p=.029] and intromissions [$\chi2(2)=7.19$, p=.027], so Greenhouse-Geisser estimates were used for correction (ε =.73 for both measures). There were significant main effects of week such that the latency to mount [F(1.45, 23.25)=6.16, p=.012, $\eta p2$=.28] and intromit [F(1.47, 24.96)=8.35, p=.004, $\eta p2$=.33] decreased over the course of the three weeks (Figure 1B,E). There was no main effect of housing condition on latency to mount [F(1,17)=0.751, p=0.398] or latency to intromit [F(1,18)=1.385, p=0.255]. There was also no week by housing condition interactions for latency to mount [F(2,34)=0.073, p=0.930] or latency to intromit [F(2,36)=0.528, p=0.594].

Planned comparison analyses showed that LBN males had a shorter latency to mount on week 2 [t(19)=2.15, p=.044, d=.92], but there were no significant differences on week 1 or week 3. Intromissions were not significantly different between LBN and control groups for week 2 or week 3. However, the latency to intromit reached significance on week 1 such that LBN males were quicker than controls [t(11.36)=2.18, p=.051, d=.93] when Welch's t-test was used to correct for the violation of homogeneity of variance (Levene's test: [F(1, 20)=21.22, p<.001]). Given our *a priori* predictions, planned comparisons were justified[28], however Bonferroni corrected p-values are provided in Supplementary Table 1.

There was no difference between control and LBN males in terms of ejaculation latency during week 1 [U=59, z=-0.44, p=.695, r= -.09] or week 3 [U=56, z=-0.62, p=.566, r= -.13],

LBN males showed a significantly shorter latency to first ejaculation than control males during week 2 [U=29, z=-2.30, p=.023, r= -.48] (Figure 1H).

The Effect of LBN on the Frequency of Male Sex Behaviors

Both LBN and control males increased the number of mounts, intromissions, and ejaculations over the course of the 3 sessions (Figure 1C, F, I), but there was no difference between LBN and control rats. The full statistics are in Supplementary Statistics. The latency data suggested that week 2 was the timepoint was when effects of LBN were most pronounced, so we conducted a more refined analysis on week 2 count data by assessing counts across three, 10 min blocks. We found a significant block by housing condition interaction for the number of intromissions [F(2,42)=5.80, p=.006]. LBN males exhibited more intromissions during only the first 10 min block [t(21)=-2.493, p=0.021], indicating an initial facilitation of this behavior in the LBN group. There was no interaction between housing condition and block for either mounts [F(2,42)=1.873, p=0.166] or ejaculations [F(2,42)=1.373, p=0.264] during week 2.

LBN Does Not Affect Number of Calbindin Cells in the SDN/POA or Pubertal Onset in Males

Experimental designs for rats used for calbindin immunohistochemistry or preputial separation observation are depicted in Figure 2A. Representative images of calbindin immunoreactivity in the SDN/POA of LBN and control males and females are in Figure 2B (control male n=11; LBN male n=10; control female n=8; LBN female n=8). Males exhibited significantly more cb-ir cells in the SDN/POA than females [F(1,32)=65.689, p<.001] (Figure 2C), which is consistent with prior reports [20,21]. There was no main effect of housing condition on the number of cb-ir cells and no significant sex by housing condition interaction.

The average PND of preputial separation did not differ between control (n=22) and LBN (n=29) males [t(49)=-1.43, p=.160] (Figure 2D). The timing of preputial separation was plotted

using survival curves and analyzed using the Log-rank (Mantel-Cox) test, but no differences were found [$X^2(1)=1.069$, p=.301] (Figure 2E). Rats from both treatment groups displayed preputial separation by PND40.

LBN Alters Gene Transcription in the mPOA of Male and Female Rats

To investigate molecular signatures in the mPOA that may underlie LBN-induced changes in behavior, we collected a punch of the bilateral mPOA from behaviorally naïve male and female rats (n=6 per group; Figure 3A). Tissue was processed for library preparations and RNA sequencing. We first used rank-rank hypergeometric overlap (RRHO) analysis to compare overall gene expression patterns in males and females induced by LBN. This analysis included all genes that were differentially expressed, including gene changes not reaching the threshold of statistical significance, and thus facilitated the agnostic comparison of gene expression patterns after LBN in males and females. There was little overlap in genes similarly up- or down-regulated by LBN in males compared to females (Figure 3B; cool colors in top right and bottom left quadrants). Instead, we found that LBN induced unique gene transcription changes in males and females. Many genes that were upregulated in females were downregulated in males (Figure 3B; hot spots in top left quadrant) and *vice versa* (Figure 3B; hot spots in bottom right quadrant).

We next narrowed down our analysis to genes showing a significant difference in expression between control and LBN and found 176 differentially expressed genes (DEGs) in males and 212 DEGs in females (see full list of DEGs in Supplementary Table 2). These gene expression changes were largely sex-specific, with only 15 common genes altered by LBN across sexes (Figure 3C). While there was very little overlap in specific DEGs between males and females, heatmaps sorted by fold change of LBN DEGs revealed similar proportions of up- and down-regulated genes: in males, 46.0% of identified DEGs were downregulated following

LBN while in females, 51.9% of DEGs were downregulated (Fig. 3D). Thus, LBN induces gene transcription changes in unique sets of genes in males and females, with similar levels of up- and down-regulation. Of the 15 common DEGs between males and females, only 7 were regulated in the same direction across sexes while the remaining 8 were changed in opposite directions (Figure 3E). We next examined whether baseline sex differences in gene expression – comparing control males to control females were affected in the LBN condition (comparing LBN males to LBN females) and whether any of these DEGs were also present in the LBN versus controls comparison for either sex (Figure 3F). Of the 20 genes that were present in both sex comparisons (controls and LBN), 13 were differences that were maintained and 7 were reversed (Figure 3G). LBN caused a loss sex difference for some of the DEGs identified in the control male versus control female comparison. These genes were categorized as "feminized" when LBN drove their expression to be similar to control female expression levels, and "masculinized" when LBN drove expression to be similar to control male control levels (Figure 3G).

KEGG and Wikipathways pathway analyses examined the biological processes altered by LBN (see full list of pathways in Supplementary Table 2). Top gene ontology (GO) terms are displayed in Figure 3H&I. We focused on pathways involved in neurotransmission, neurosignaling, and the immune system as these pathways were most relevant to changes in brain function [29]. Largely different sets of DEGs were regulated within this network (*Prkcb*, *Rac1*, and *Hspb1* in males; *Akt1*, *Atf1*, *Prkcb*, *Stat3*, and *Jak1* in females, **Table 1**), indicating unique transcriptional profiles following LBN in males and females. Markers of astrocytes and endothelial cells such as *Nr1d1 and nr1h2* also showed sex-specific patterns in their changes in expression in response to LBN (**Table 1**).

Discussion

Early life adversity is linked to a variety of developmental and hormone-regulated changes in humans[2,3,30], yet the mechanisms by which these changes occur are poorly understood. Previously, we found that LBN increases plasma estradiol levels in adult male rats[4]. In this study, we show that LBN also alters reproductive behavior in adult males. LBN males exhibited shorter latencies to mount, intromit, and ejaculate at early, but not late, timepoints in the sex behavior assay, which suggests that LBN facilitates the acquisition of male sex behavior. In contrast, LBN did not alter the sexual differentiation of the SDN/POA or the timing of puberty, suggesting LBN does not impact earlies stages of development. To begin to identify mechanisms by which LBN can alter reproductive behavior, we assessed gene transcription in the adult mPOA. RNA sequencing provides and agnostic genome-wide profile of the molecular correlates of early life adversity but constitutes only the first step in delineating the functional pathways and networks that contribute to the endophenotypes of early life adversity. Transcription changes were found to be largely sex-specific, which was expected due to the high level of sexual dimorphism in the mPOA. KEGG and Wikipathways analyses identified several pathways involved in cell signaling that were changed by LBN in males, pointing to possible mechanisms by which ELA has lasting impacts on mPOA functions, such as reproductive behavior.

LBN Enhances Acquisition of Male Sex Behavior

The rat male reproductive behavior assay uses three sessions to allow initially virgin males to gain efficiency in mounts, intromissions, and ejaculations over time. In addition to assessing the time course for acquiring these behaviors, this assay can be used to distinguish motivational vs. consummatory behaviors[31,32]. Here we assessed how LBN altered male

reproductive behaviors. Although we did not find a global increase in all sex behaviors at all timepoints induced by LBN, we did find effects that indicate LBN causes facilitated acquisition of motivational and consummatory male reproductive behaviors. Reduced latencies to initiate copulation (i.e., reduced latencies to mount and intromit) have been interpreted as evidence of enhanced sexual motivation[25,27,33]. In our study, both control and LBN males showed reduced latencies in mounts and intromissions over the three weeks, consistent with the expected increase in motivation that occurs with experience. However, in the early testing sessions, LBN males were quicker than controls to mount (week 2) and intromit (week 1), suggesting that LBN males are initially more motivated than controls for a sexually receptive female. Reduced latencies to ejaculate as well as increased numbers of sex behaviors are considered evidence of enhancements in consummatory behaviors[33,34]. LBN and control males engaged in more consummatory behaviors during week 3 than week 1 with no differences between groups during those weeks. However, in week 2, LBN males were quicker to ejaculate and had a greater number of intromissions during the first 10min of the session than controls, indicating that LBN enhances the consummatory aspects of sex behavior during this week. We interpret this combination of findings to indicate that LBN males more quickly become proficient at consummatory behaviors than control males. The most robust effect of LBN on male sex behavior in the current study was the LBN-induced reduction in ejaculation latency during week 2 of testing. Ejaculation is the behavior most directly tied to successful fertilization and reproduction. Thus, even a transient reduction in ejaculation latency relative to controls could meaningfully increase the reproductive success of LBN males.

Not all models of early life adversity enhance sex behavior. Some find that maternal separation stress increases latencies to behave[35] and decreases frequencies of sex

behaviors[36] compared to control males. Although, others have reported decreased latencies for reproductive behavior in maternal separated male rats[37]. Discrepancies in the impact of early life stress between laboratories are not uncommon. The impact of early life stress is dependent on the timing, severity, type of stress, and test species[38]. More severe stressors tend to induce negative outcomes whereas more mild stressors can often confer resilience and adaptability[39]. Davis and colleagues used a version of LBN in which the metal grate is absent, and rats are given a lower volume of bedding compared to controls[40]. This study reported no impact of LBN on male sex behavior, but an enhancement of sexual motivation as measured in the partner preference test, with LBN males spending more time with a stimulus female compared to controls[40]. We did not conduct a specific sexual motivation test in the present study, but the reduced latency to mount and intromit in early sessions is consistent with enhanced motivation due to LBN. Collectively, these studies suggest that early life adversity does impact male reproductive behavior, with milder models enhancing motivation.

The observed enhancements in sex behavior are consistent with our previous finding that adult LBN males have elevated levels of plasma estradiol[4]. Estradiol is involved in both motivational and consummatory aspects of male sexual behavior[31,32]. One target of the estrogenic effects on male reproductive behavior is the mPOA. Administration of estradiol into the mPOA restores sexual behavior in castrated male rats[41-44]. Estrogens in the mPOA can further impact motivated behavior via projections to the mesocorticolimbic dopaminergic system [45]. There is an afferent from the mPOA to the ventral tegmental area (VTA)[17,46] that is both organized by estrogens in early life[47] and regulated by estrogens in adulthood[46]. Thus, the increased levels of estradiol in LBN males may contribute to the enhanced sex behavior observed in LBN males via regulation of the mPOA. Interestingly, the enhancement of sexual motivation

in the present study is in contrast with the reduced motivation for opioid self-administration in LBN males previously observed in our laboratory[48]. The reason for this apparent disconnect is not immediately clear but is likely due to a difference in underlying circuitry. While both natural rewards and drug rewards act on the mesocorticolimbic system, the mPOA is selectively engaged during sex behavior. Thus, LBN effects on the mPOA may drive enhanced male reproductive behavior. Future studies should investigate how LBN differentially regulates the mPOA and mesocorticolimbic system and alters connections between these regions.

LBN Does Not Affect Masculinization of the SDN/POA or Pubertal Onset in Males

Our prior report found that, in adults, LBN males had higher levels of plasma estradiol than controls[4]. The perinatal sensitive period for brain masculinization overlaps with the LBN model[21]. Thus, if the LBN-induced increase in estradiol is present during the perinatal sensitive period, there could be a hypermasculinization of the SDN/POA. One way to assess the masculinization of the SDN/POA is with calbindin, because estradiol (converted from the testosterone released during the perinatal surge in males), increases the number of calbindin neurons in this region[21]. Thus, here we quantified the number of cb-ir cells in the SDN/POA. We replicated a significant sex difference in the number of cb-ir cells in the SDN/POA, with males displaying much higher numbers of cb-ir cells than females[21,49]. However, there was no effect of LBN on the number of cb-ir cells in males or females. Thus, the LBN manipulation does not appear to increase estradiol in the perinatal period, and the LBN facilitation of male sex behavior is not attributable to a hypermasculinization of the size of the SDN/POA.

Another developmental measure that is dependent on gonadal hormones is the timing of puberty onset. We have previously shown that LBN does not affect puberty onset in females as

measured by the timing of vaginal opening[4]. Here, we took the analogous measure in males, preputial separation. Preputial separation is dependent on androgenic signaling in male rats, as testosterone and dihydrotestosterone but not estradiol replacement in castrated rats restores the normal onset of preputial separation[50]. We found that LBN had no effect of the timing of preputial separation. These results along with the finding that LBN does not alter the size of the SDN/POA, suggest LBN does not alter gonadal hormone-mediated endpoints until adulthood. Future studies should track the onset of the previously observed elevation of estradiol in LBN males.

LBN Has Sex-Specific Effects on mPOA Gene Expression

We investigated gene expression in the mPOA of male and female adult rats raised in LBN versus control housing conditions. RNAseq analysis revealed that LBN-induced gene expression changes in the mPOA were highly sex-specific, as can be seen in the generated RRHO, Venn diagram, and heatmaps. The sex specificity of these findings is not unexpected. The mPOA is a highly sexually dimorphic region of the brain and is involved in many sexually dimorphic behaviors including male sexual behavior, as highlighted here, as well as female parental behavior[51]. LBN may thus alter these sexually dimorphic endpoints in females as well, which will be tested in future studies.

One gene that was downregulated in LBN males compared to control males was *Slc6a9*, which encodes glycine transporter 1. A decrease in the amount of available glycine transporter would allow for glycine to remain in the synapse longer, protracting glycine's effects. Glycine in the mPOA is linked to male sex behaviors. Microinjections of glycine into the mPOA decreased the latency to first ejaculation[52], while blocking mPOA glycine receptors leads to a deficit in both appetitive and consummatory aspects of male sex behavior in rats[53]. Furthermore, *Slc6a9*

expression is regulated by stress. Following social defeat, male mice exhibit increased expression of *Slc6a9* in the hypothalamus[54]. We found the opposite effect, with male rats exhibiting decreased *Slc6a9* expression following early life stress as induced by the LBN model. However, we also observed that LBN males tend to show an adaptive behavioral response to this stress. It is possible that an LBN-induced decrease in glycine transporter availability enhances glycine signaling in the mPOA to reduce ejaculation latency.

KEGG and Wikipathways analyses revealed that LBN altered multiple pathways involved in cellular signaling and plasticity in males, including phosphatidylinositol signaling, inositol phosphate metabolism, VEGF signaling, and Wnt signaling. Phosphatidylinositol, inositol, and VEGF signaling in the mPOA have been linked to estrogens. For example, estrogens regulate the hypothalamic expression of genes involved in phosphatidylinositol 3-kinases, which can be metabolized into inositols[55]. VEGF triggers the release of nitric oxide from endothelial cells, a critical molecule for the acquisition of male sex behavior in rats. Thus LBN-derived changes in the VEGF pathway may impact the acquisition of male sex behavior via alterations to nitric oxide signaling. Sexually experienced male rats show greater expression of nitric oxide synthase in the mPOA than sexually naïve males[56], and blocking nitric oxide synthase prevents sexually inexperienced male rats from showing enhanced sexual performance following sexual experience[57]. The relationship between VEGF signaling and nitric oxide within the mPOA is one target pathway for future investigations into the mechanism by which LBN alters sex behavior.

While the current study did not investigate any behavioral endpoints in females, we did examine the impact of LBN on mPOA gene transcription in females. There were more pathways significantly regulated by LBN in females than in males, including pathways involved in

signaling (including phospholipase D signaling, HIF-1 signaling, and mTOR signaling) and the immune system (including IL-1, IL-2, IL-3 IL-5, IL-6, IL-9). How these altered pathways may impact the functioning of the mPOA and the behaviors it regulates, such as maternal behavior, remain to be investigated.

Conclusions

The present study describes an enhancement in the acquisition of male sex behaviors following early life adversity in the form of the LBN model of resource scarcity. LBN males exhibited significantly shorter latencies to engage in reproductive behaviors compared to controls at earlier timepoints in the sex behavior assay. This may represent an evolutionarily adaptive response to the experience of early life adversity. Although we did not observe any effect of LBN on the structure of subregions in the mPOA, LBN does induce sex-specific transcription in this region. LBN-induced downregulation of the glycine transporter 1 in males or alterations in cell signaling may drive the changes in reproductive behavior, and this will be explored in future studies. Importantly, studying the mechanisms by which early life adversity affects reproductive behavior has implications for understanding sexual dysfunction disorders and motivated behavior more broadly.

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Author Contributions

S.R.E. contributed to study design, all data collection and analysis, interpretation of data, and manuscript drafting. J.P. contributed to study design, behavioral data collection and analysis, interpretation of data, and manuscript drafting. C.C.B. contributed to RNA-seq data collection, analysis, and interpretation. R.K. contributed to RNA-seq data collection and analysis. E.O.S. contributed to behavioral data collection. J.F. contributed to behavioral data collection. A.H. contributed to study design and behavioral data interpretation. M.E.W. contributed to study

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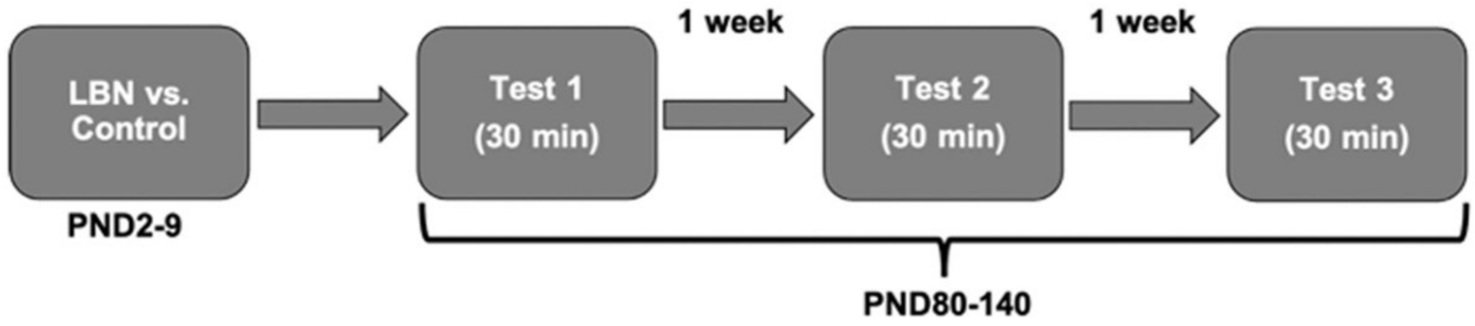
Figure Captions

Figure 1. Male sex behavior across three consecutive weeks in control and LBN rats. Experimental timeline (A). Mount latency (B), total number per week (C), and number per block during week 2 (D). LBN males had a shorter latency to mount compared to control males during week 2. Intromission latency (E), total number per week (F), and number per block during week 2 (G). LBN males had a shorter latency to intromit compared to control males during week 1. There was a significant housing condition by block interaction for the number of intromissions during week 2, with LBN males exhibiting more intromissions than controls during the first 10 minutes of the test. Box plots displaying median latency to ejaculate per session (H). LBN males had a shorter latency to ejaculate compared to control males during week 2. Mean number of ejaculations per week (I) and per block during week 2 (J). Asterisks indicate $p<.05$

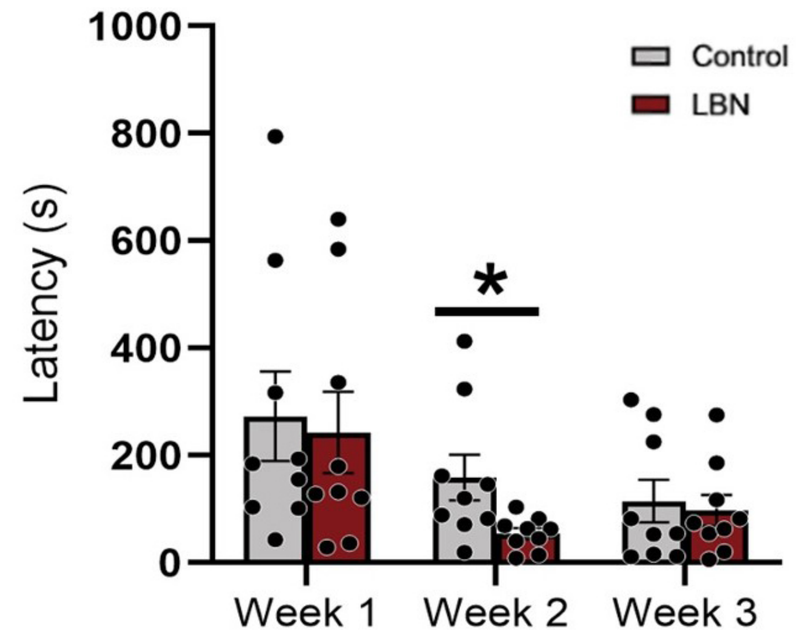
Figure 2. LBN does not alter calbindin cell count or preputial separation. Experimental design for calbindin immunohistochemistry and preputial separation (A). Note that females and a portion of males used for calbindin immunohistochemical staining did not undergo sex behavior testing prior to tissue collection. LBN did not change number of cb-ir cells or timing of preputial separation. Representative images showing cb-ir cells in the SDN/POA of adult control and LBN males and females. (B) Males showed significantly more cb-ir cells in the SDN/POA than females, as has been previously reported [20]. (C) LBN did not affect the timing of preputial separation in adolescent males measured in a separate cohort of rats. (D) Survival curves show that the percentage of rats displaying preputial separation over development did not differ between LBN and control males (E). Asterisks indicates $p<.001$

Figure 3. Transcriptomic regulation in the mPOA by LBN. (A) Bilateral mPOA punches were collected from naïve males and females. The insert depicts sex differences in the SDN subregion of the POA, which was included in the punch collected. (B) Threshold-free rank-rank hypergeometric overlap. Color-coded pixels show overlap of gene transcription changes in LBN males and females with warmer colors indicating more overlap. Upper left and lower right quadrants include genes with expression that changed in opposite directions in males and females. Upper right and lower left quadrants represent co-upregulated and co-downregulated genes, respectively. (C) Venn diagram of DEGs in males and females. RNA-sequencing analysis identified 176 DEGs in LBN males and 212 DEGs in LBN females, with 15 DEGs overlapping between the sexes. (D) Heatmaps sorted by fold change of DEGs in males compared to the expression of those genes in females (top) and of DEGs in females compared to the expression of those genes in males (bottom). (E) Heatmap of the 15 DEGs overlapping in both sexes, sorted by fold change of DEGs in males (top) compared to the expression of those genes in females (bottom). (F) Venn Diagram of DEGs for 4 comparisons: male LBN vs. male control (blue), female LBN vs. female control (red), male control vs. female control (grey) and male LBN vs. female LBN (white). (G) Number of DEGs for which sex difference were maintained, reversed or for which LBN produced patterns comparable to baseline sex differences. LBN eliminated baseline sex differences for some genes. If the loss of sex resulted in a control male-like pattern of gene expression, it was termed "masculinized" and if it resulted in a control female-like pattern of expression it was termed "feminized". (H&I) Selected top KEGG and Wikipathways enrichment terms identified in males (H) and females (I) following LBN. The number of DEGs within each term is listed in parentheses to the right of the term. All selected enrichment terms have an adjusted p-value < .05.

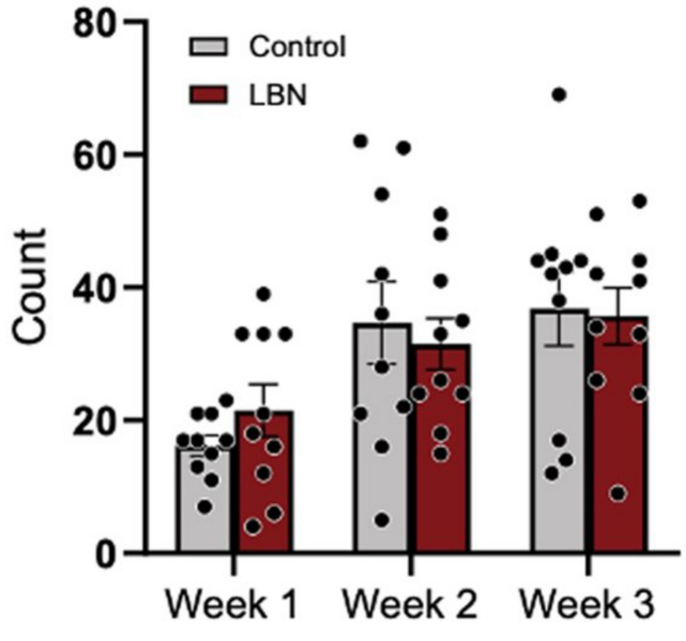
A


LBN vs. Control
PND2-9
1 week
Test 1 (30 min)
1 week
Test 2 (30 min)
Test 3 (30 min)
PND80-140

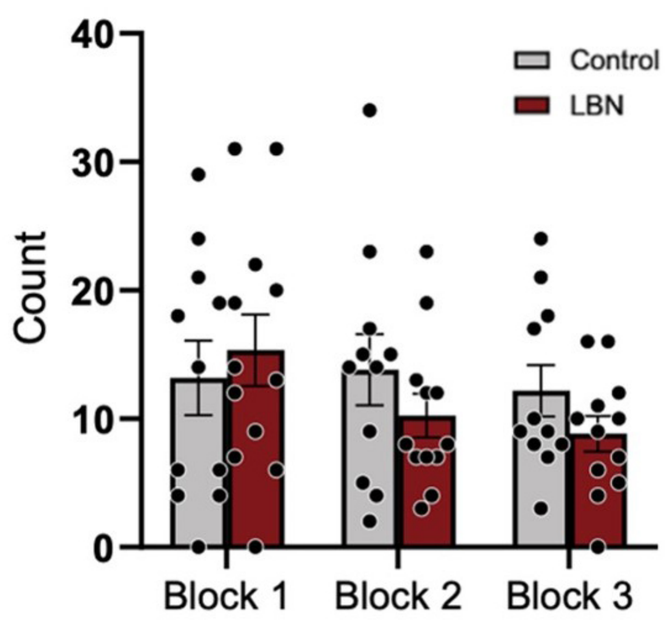
B


Mounts
Latency (s)
1000
800
600
400
200
0
*
Control
LBN
Week 1
Week 2
Week 3

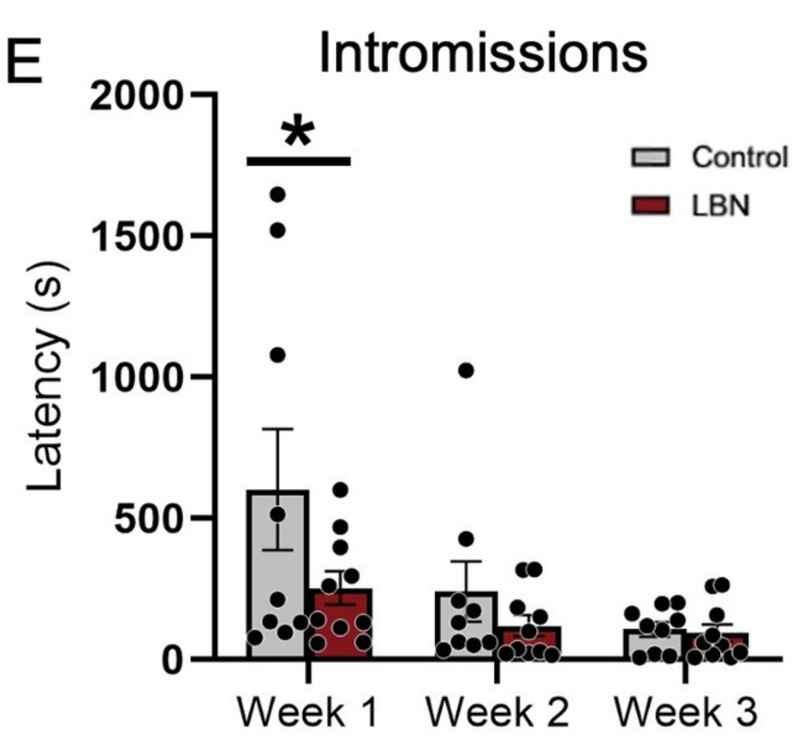
C


Mounts
Count
80
60
40
20
0
Control
LBN
Week 1
Week 2
Week 3

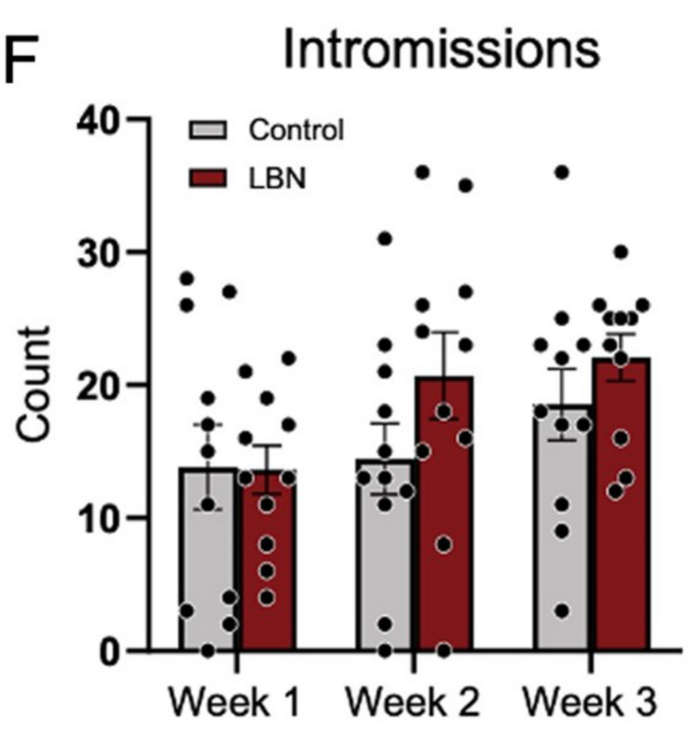
D


Week 2 Mounts by Block
Count
40
30
20
10
0
Control
LBN
Block 1
Block 2
Block 3

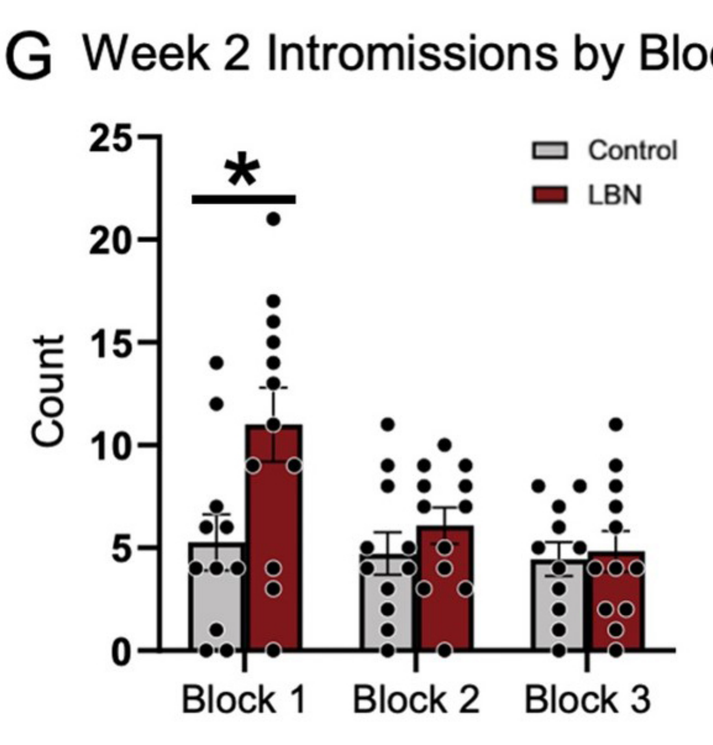
E


Intromissions
Latency (s)
2000
1500
1000
500
0
*
Control
LBN
Week 1
Week 2
Week 3

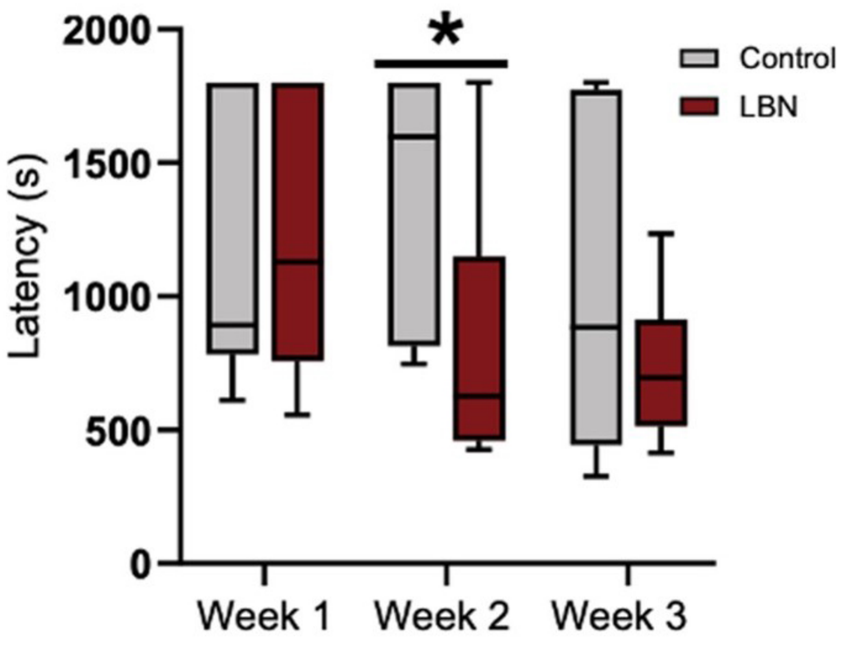
F


Intromissions
Count
40
30
20
10
0
Control
LBN
Week 1
Week 2
Week 3

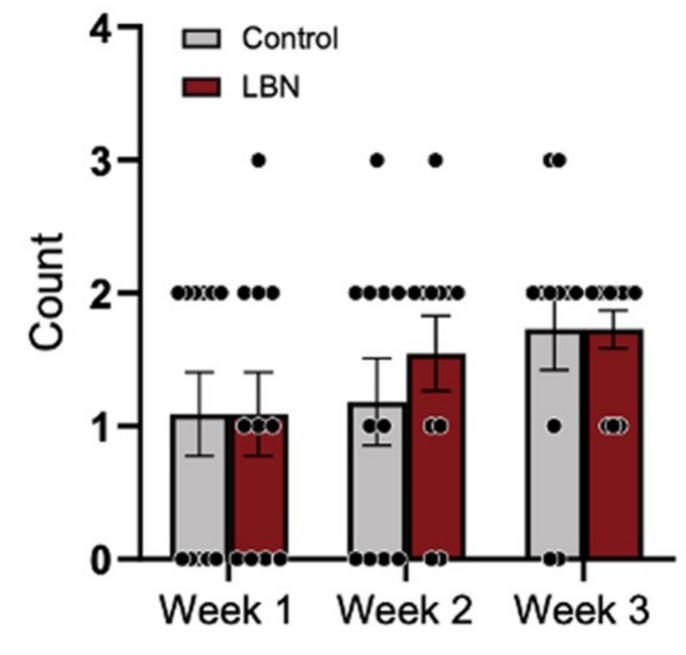
G


Week 2 Intromissions by Block
Count
25
20
15
10
5
0
*
Control
LBN
Block 1
Block 2
Block 3

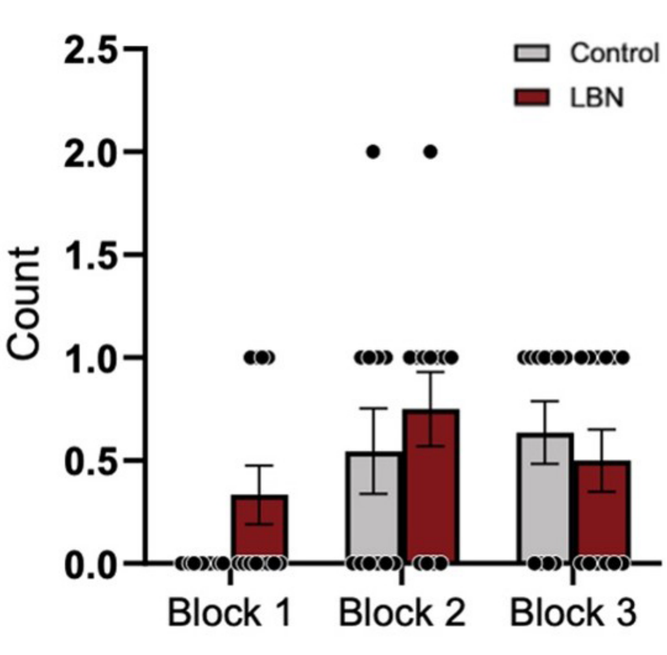
H


Ejaculation
Latency (s)
2000
1500
1000
500
0
*
Control
LBN
Week 1
Week 2
Week 3

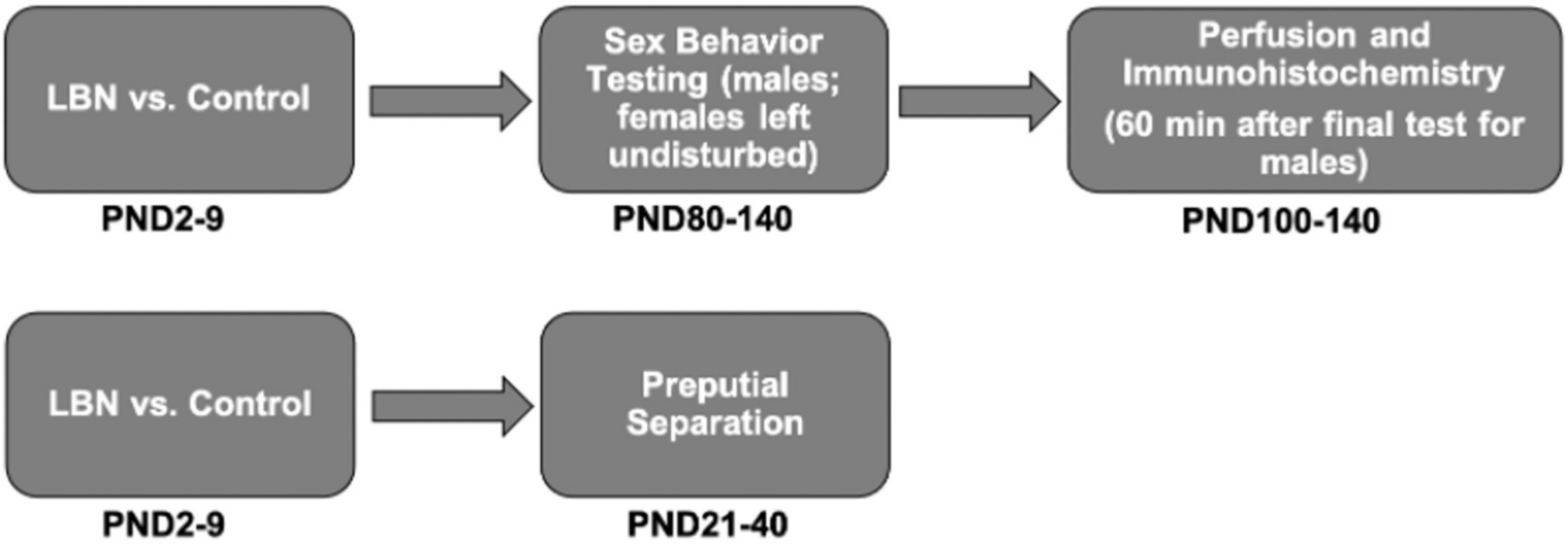
I


Ejaculations
Count
4
3
2
1
0
Control
LBN
Week 1
Week 2
Week 3

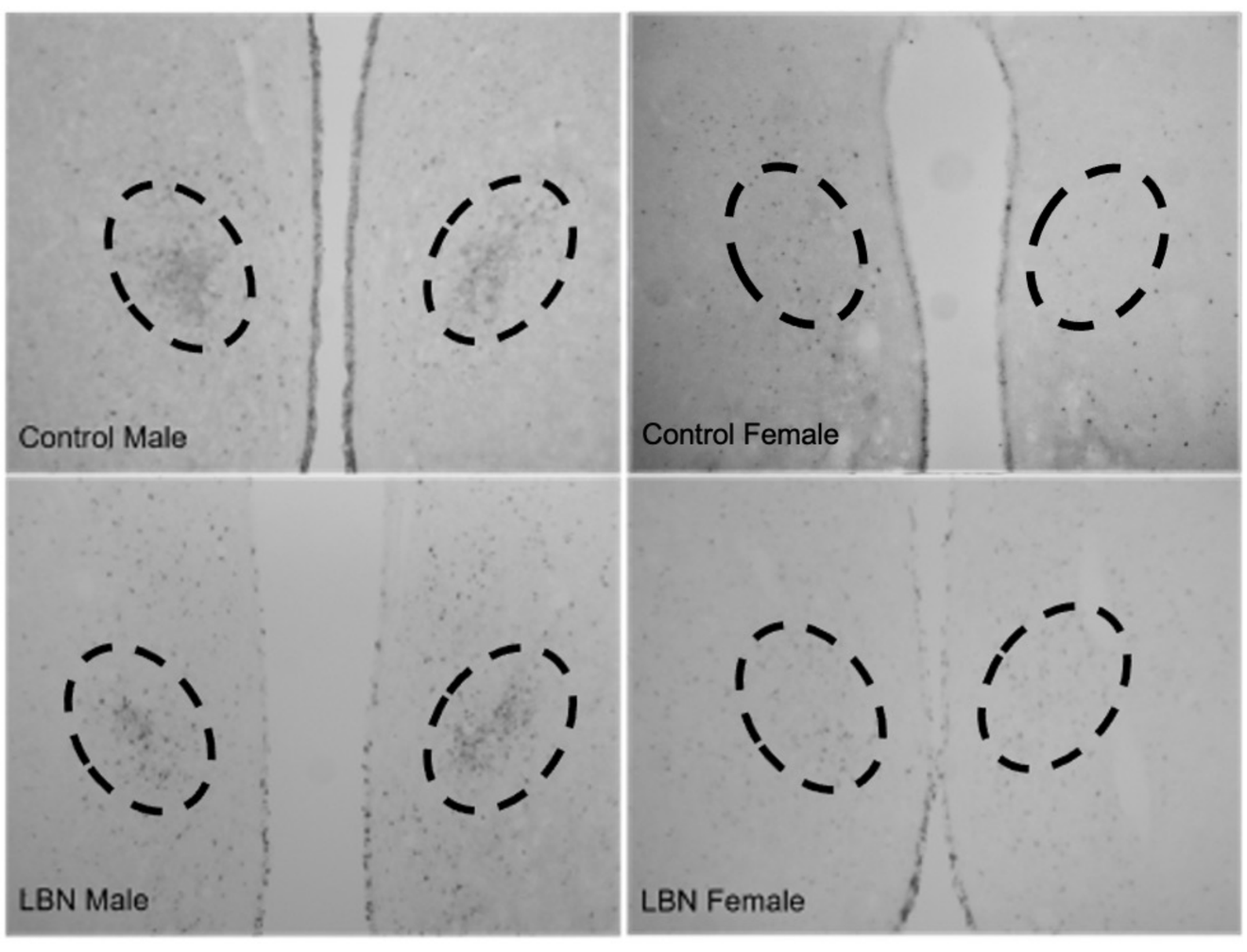
J


Week 2 Ejaculations by Block
Count
2.5
2.0
1.5
1.0
0.5
0.0
Control
LBN
Block 1
Block 2
Block 3

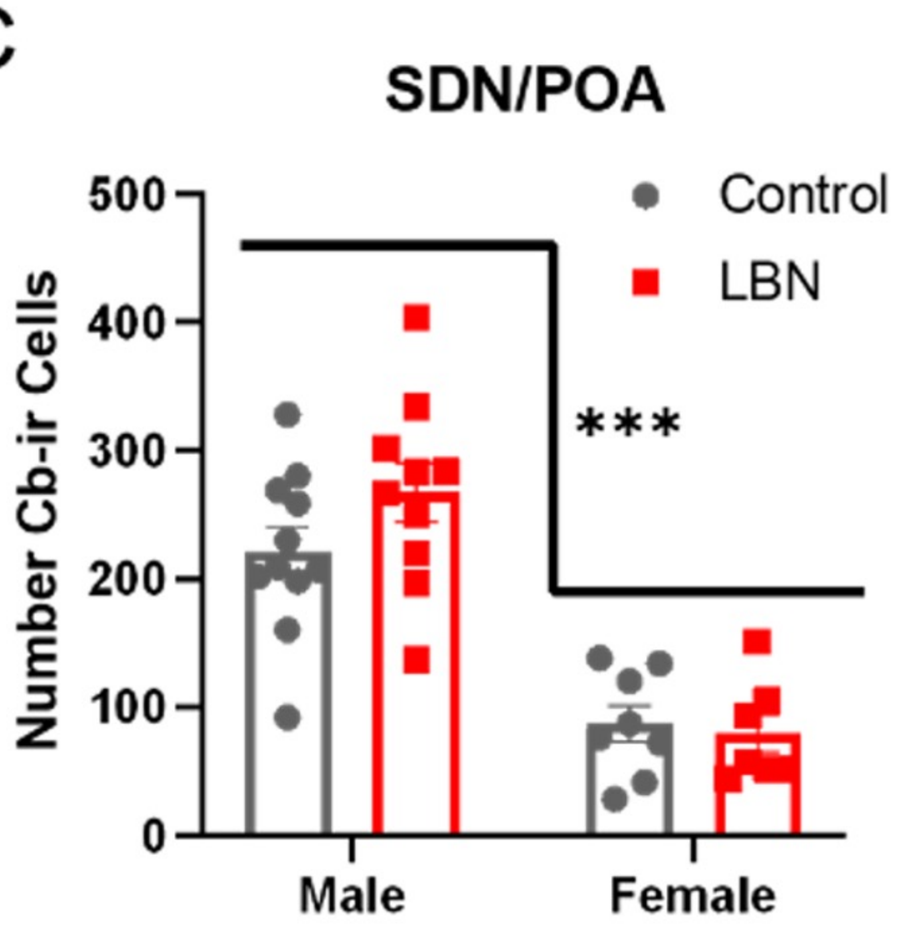
A


LBN vs. Control
PND2-9
Sex Behavior Testing (males; females left undisturbed)
PND80-140
Perfusion and Immunohistochemistry
(60 min after final test for males)
PND100-140
LBN vs. Control
PND2-9
Preputial Separation
PND21-40

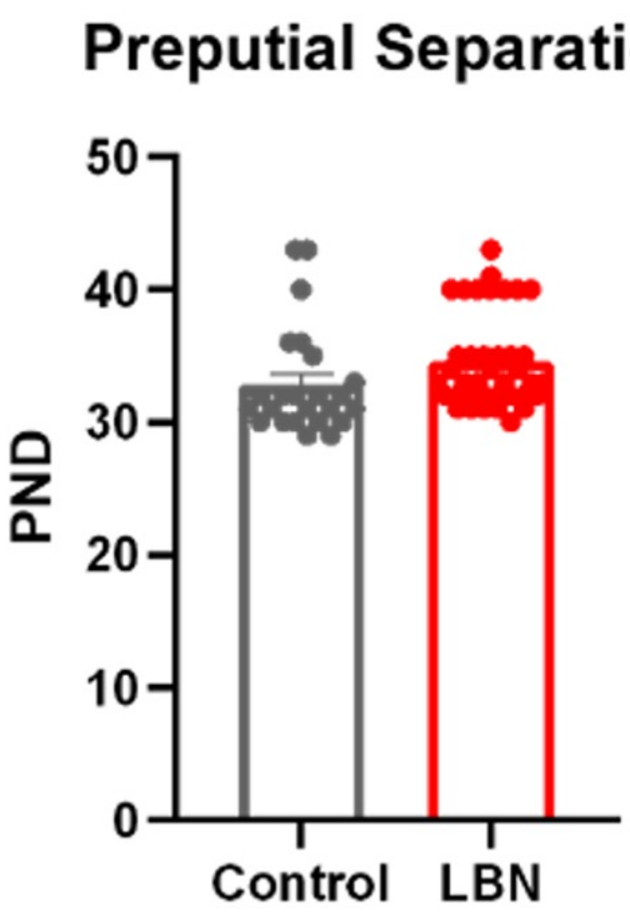
B


Control Male
Control Female
LBN Male
LBN Female

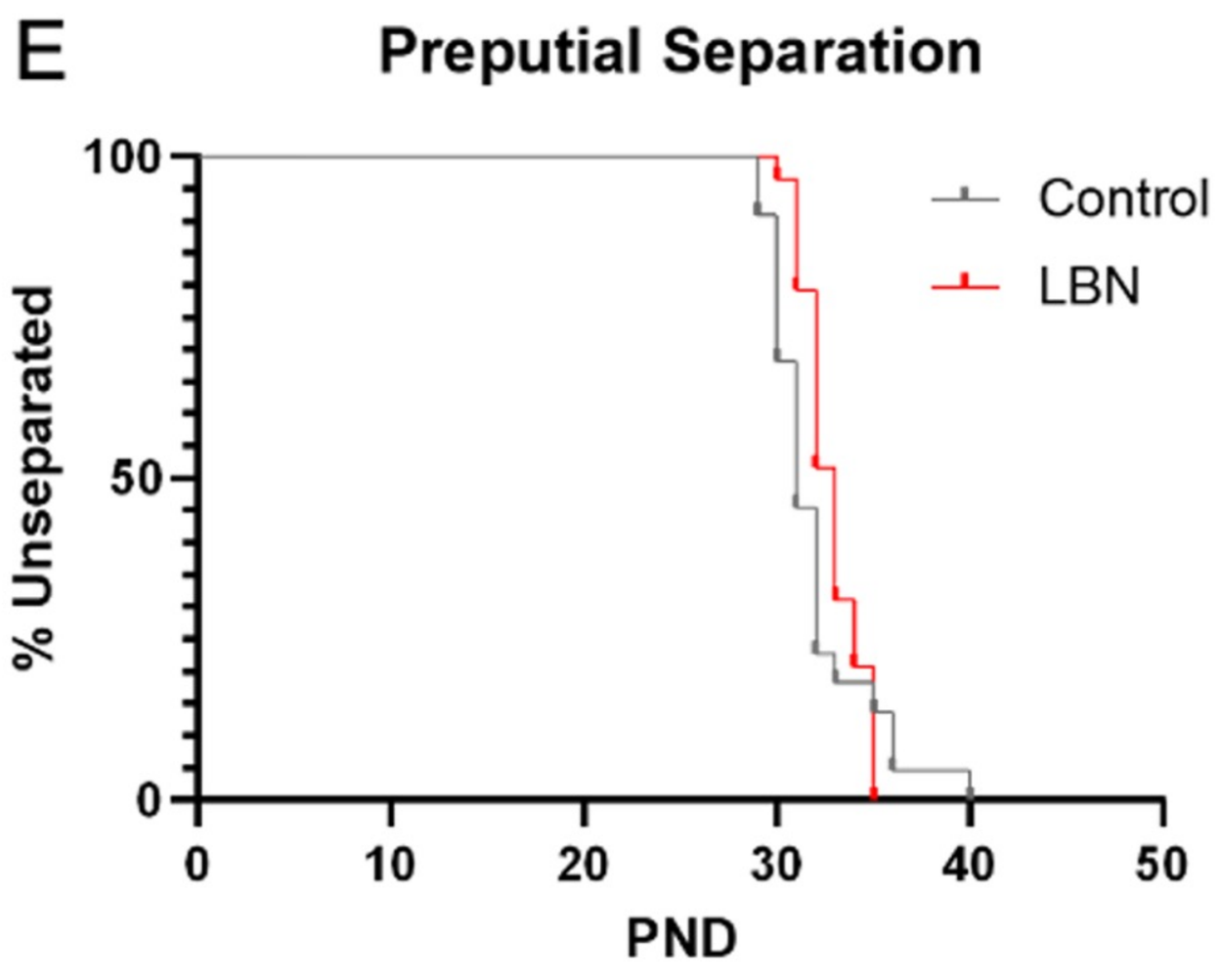
C


SDN/POA
Control
LBN
Number Cb-ir Cells
500
400
300
200
100
0
***
Male
Female

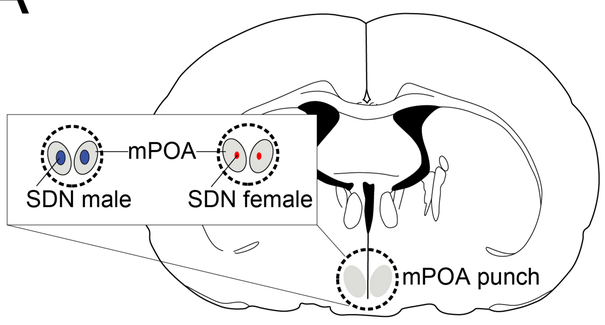
D


Preputial Separation
PND
50
40
30
20
10
0
Control
LBN

E


Preputial Separation
Control
LBN
% Unseparated
100
50
0
0
10
20
30
40
50
PND

A



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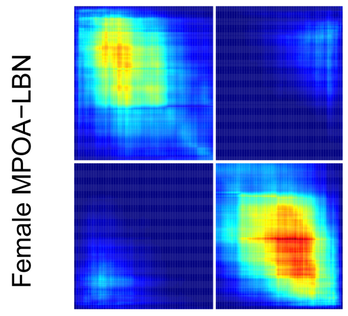
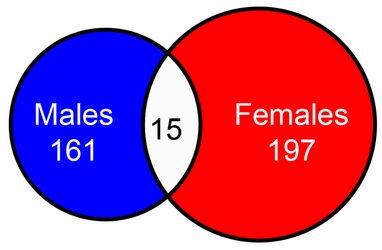
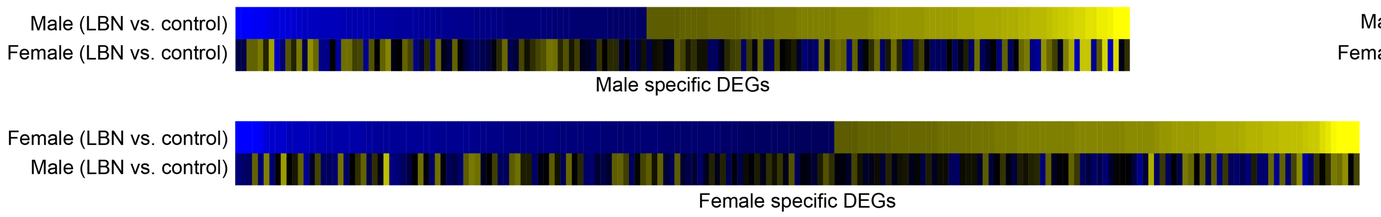


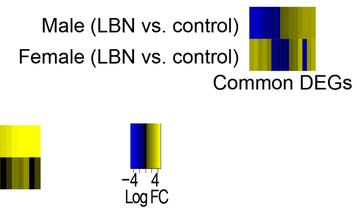
C



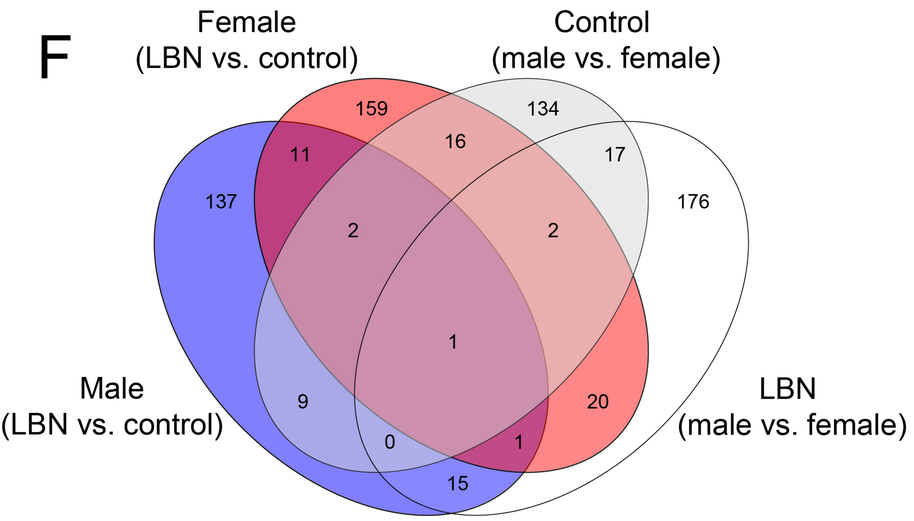
D



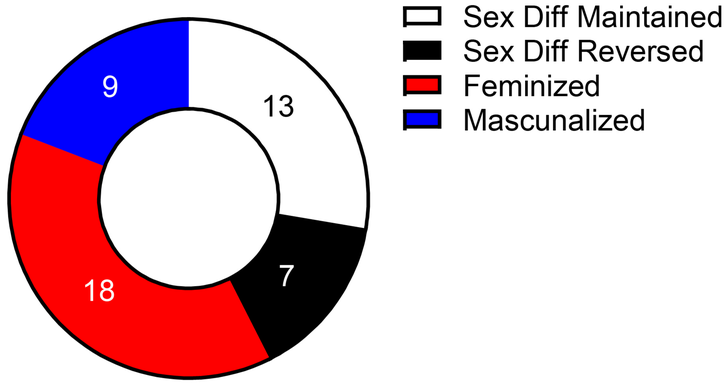
E



F



G



Effect of LBN on baseline sex differences

H

Male Selected Enrichment Terms
KEGG (3/6)
Phosphatidylinositol signaling system (5)
VEFG signaling pathway (4)
Inositol phosphate metabolism (4)

Wikipathways (1/2)
Ionotropic glutamate receptor pathway (4)

I

Female Selected Enrichment Terms
KEGG (6/19)
Endocytocis (10)
EGFR tyrosine kinase inhibitor resistance (6)
Fc gamma R-mediated phagocytosis (3)
Phospholipase D signaling pathway (5)
HIF-1 signaling pathway (5)
Aldosterone synthesis and secretion (5)

Female Selected Enrichment Terms
Wikipathways (6/10)
IL-1 Signaling Pathway (3)
EGFR1 Signaling Pathway (8)
IL-5 Signaling Patway (5)
IL-6 Signaling Pathway (5)
IL-3 Signaling Pathway (5)
IL-9 Signaling Pathway (3)

Table 1. LBN alters expression of astrocytic and endothelial cell markers.

Gene	Males		Females		Category
	Log Fold Change	FDR	Log Fold Change	FDR	
Gfap	0.5557	0.1347	-0.6443	0.0688	Astrocyte marker
Hspb1	0.8610	0.0629			VEGF pathway
Plcg1	-1.2557	0.0996	-1.8047	0.0103	VEGF pathway
Prkcb	-1.8188	0.0365	2.4061	0.0753	VEGF pathway
Rac1	-1.0334	0.0478			VEGF pathway
Akt1			2.5045	0.0003	Endothelial Cell Marker
Hsd17b4	2.8006	0.0703			Endothelial Cell Marker
Lamb2	1.4854	0.0837			Endothelial Cell Marker
Nr1d1			-1.5424	0.0844	Endothelial Cell Marker
Nr1h2	2.3009	0.0569	-2.1668	0.0403	Endothelial Cell Marker
Ptk2b			1.5637	0.0510	Endothelial Cell Marker