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Supplementary Information for

Early Life Adversity Promotes Resilience to Opioid Addiction-Related Phenotypes in Male Rats and Sex-Specific Transcriptional Changes

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N/A

Supplementary Information Text

Supporting Information Methods

LBN details: On PND2, litters were culled to 10 pups with an even representation of the sexes when possible. Control animals were placed in standard housing conditions with access to bedding, 2 cotton nestlets, and 1 tunnel enrichment device. LBN animals were transferred to cages fitted with a custom stainless-steel metal platform to prevent access to bedding, only provided 1 paper towel for nesting material, and were denied enrichment as described(1). On PND10 all rats were transferred into standard housing conditions and rats were weaned at PND21 into same-sex pairs.

Housing and feeding: Rats were housed in standard polypropylene cages on a 12-hour reverse light-dark cycle (lights off at 1100h in breeding colony and 0830h after weaning). Once rats reached adulthood, they were maintained in same sex pairs and on *ad libitum* food (LabDiet 5001) and water with one exception. For delayed discounting, food access was restricted to reduce body weight to 85% of free feeding weight, with target weights increasing incrementally weekly to allow for continued growth.

Delay discounting: We used 8 operant conditioning chambers (30×24×30 cm, Med Associates Inc., USA), fitted with two retractable levers flanking a central food magazine that could deliver 45-mg sucrose pellets (Lot: 1811251, TestDiet, USA). Animals were kept on a reverse light/dark cycle and testing always occurred in the dark. Before training, rats were pre-exposed to 40 sucrose pellets in their home cage. Training began with teaching rats to lever press under a fixed-ratio 1 (FR1) schedule until they reached a criterion of 50 presses in 30min, first for the left then right lever. Next rats were trained on one session of the reward magnitude discrimination where they learned to associate one lever with a large reward and the other with a small reward (but there were no delays for the large reward). Then rats were trained on the delayed discounting task. For both the reward magnitude discrimination and delay discounting phases, there were four blocks of 12 trials. Each block was comprised of two forced-choice trials of which only one lever was presented (one trial for each lever, in randomized order) followed by 10 free-choice trials (both levers were out). If the lever was not pressed in 10s, an omission was recorded. One lever was assigned as the small reward lever, which delivered 1 pellet after a single lever press. The other lever was assigned as the large reward lever, which delivered 4 pellets after a single lever press. The position of the large reward lever was counterbalanced between rats and both levers immediately retracted following a lever press selection. The delay to the large reward increased across the four blocks of trials (0s Block 1, 15s Block 2, 30s Block 3, and 45s Block 4) and began immediately after animals selected the large/delayed lever. The primary dependent measure of interest was the proportion of choices of the large/delayed lever. For each block, the proportion of choices was calculated by dividing the number of choices of the large/delayed reward lever by the total number of successful trials. Once stability was reached, rats were tested for an additional 3d and these sessions were analyzed. We defined stable performance on delay discounting when a mixed factors ANOVA revealed that there was no effect of day or day × block interaction for three consecutive training days as detailed in(2). Three male control rats did not choose the larger/delayed option 7 out of 10 times during the 0s delay after the rest of their cohort reached stable performance, so they were dropped from the experiment for failing to understanding the task contingencies, leaving the final total 37 rats.

Morphine Self-Administration: A different cohort of adult rats underwent intravenous catheter surgery. Anesthesia was performed using a combination of ketamine hydrochloride (90 mg/kg, i.p.) and xylazine (2.1 mg/kg, i.p.). Silastic catheters (SAI Infusion Technologies, Lake Villa, IL) were attached to a back implant and inserted into the right jugular vein. Post-surgery, animals received carprophen (5mg/kg i..p) and were placed on heating pads until they awoke from anesthesia, then transferred to appropriate animal suites. Animals were given 7 days of surgical recovery during which they were flushed daily with 0.2mL of timentin dissolved in heparinized saline, a solution containing both antibiotic and blood thinner to prevent infection and blood clotting.

After 10 days of IVSA on an FR1 reinforcement schedule, animals were placed on an FR3 schedule, where three presses were required to earn a morphine infusion for two consecutive days. On Day 13, animals were switched to a PR reinforcement schedule. For progressive ratio responding, we used a schedule that has been shown to support morphine self-administration consistently over consecutive days(3, 4). The number of lever-pressing responses required to receive an infusion increased over the course of the session using the following equation:

Response requirement = Round (C1 x e[C2 x (Step Number C3)] – C1 + C4)
where results are rounded to the nearest integer value; step number is the number of ratios completed and C1, C2, C3 and C4 are constants with values of 10, 0.035, 1 and 0.5 respectively. The response requirement increases according to this sequence: 1, 1, 1, 2, 2, 2, 3, 3, 3, 4, 4, 5, 5, 6, 6, 7, 7, 8, 9, 9, 10, 11, 11, 12, 13, 14 etc. Two cohorts of rats were allowed to self-administer morphine at a lower dose (0.25mg/kg/infusion), while two separate cohorts self-administered morphine at a higher dose (0.75mg/kg/infusion). A subset of the animals that self-administered morphine at 0.75mg/kg/infusion were tested for drug seeking following 7 days of forced abstinence. During these 1-hour sessions, active lever responses were reinforced by presentation of the light cue in the absence of morphine.

Whole-cell recordings in the NAc: *Slice Preparation.* Naïve and morphine-experienced adult LBN rats were used to examine spontaneous excitatory transmission in the nucleus accumbens (NAc). Animals were euthanized using isoflurane, followed by decapitation. The brain was removed and 300μm coronal slices of the nucleus accumbens were cut with a Vibratome (VT1000S, Leica Microsystems) in an ice-cold artificial cerebrospinal fluid solution (ACSF), in which NaCl was replaced by an equiosmolar concentration of sucrose. ACSF consisted of 130 mM NaCl, 3 mM KCl, 1.25 mM NaH2PO4, 26 mM NaHCO3, 10 mM glucose, 1 mM MgCl2, and 2 mM CaCl2 (pH 7.2–7.4 when saturated with 95% O2/5% CO2). Slices were incubated in ACSF at 32–34 °C for 25 min and kept at 22–25 °C thereafter, until transfer to the recording chamber. The osmolarity of all extracellular solutions was 300–315 mOsm. Slices were viewed using infrared differential interference contrast optics under an upright microscope (Slice Scope Pro, Scientifica) with a 40 × water-immersion objective.

Recordings. The recording chamber was continuously perfused (1–2 ml/min) with oxygenated ACSF heated to 32±1 °C using an automatic temperature controller (Warner Instruments). Picrotoxin (100 μM) was added to all solutions to block the GABAA receptor-mediated currents. Recording pipettes were pulled from borosilicate glass capillaries (World Precision Instruments) to a resistance of 4–7 MΩ when filled with the intracellular solution (whole-cell recordings) or to a resistance of 3–6 MΩ when filled with extracellular solution (field recordings). All recordings were conducted with a MultiClamp700B amplifier (Molecular Devices). Intracellular solution contained (in mM): 100 CsCH3O3S, 50 CsCl, 3 KCl, 0.2 BAPTA, 10 HEPES, 1 MgCl2, 2.5 phosphocreatine-2Na, 2 Mg-ATP, 0.25 GTP-Tris, 1 QX-314 (pH 7.2–7.3 with CsOH, osmolarity 280–290 mOsm). All sEPSC recordings were conducted in whole-cell voltage-clamp mode (Vh = −70 mV). Currents were low-pass filtered at 1 kHz and digitized at 20 kHz using a Digidata 1550A acquisition board and pClamp10 software (both from Molecular Devices). Access resistance (10–30 MΩ) was monitored throughout the recordings by injection of 10 mV hyperpolarizing pulses and data were discarded if access resistance changed by >25% over the course of data acquisition. sEPSCs were detected using an automated sliding-template-based algorithm in pClamp 10. This method compares the shape of the detected current to that of a template and has been shown to detect events with amplitude of at least 3 times the square deviation of the noise(5). All detected events were verified by visual confirmation of a fast rise time and slower exponential decay to baseline. Mean sEPSC frequencies and amplitude were analyzed from 180-s long trace segments. Evoked responses were triggered by 100 μs constant-current pulses generated by an A310 Accupulser (World Precision Instruments) and delivered at 0.06 Hz via a bipolar tungsten stimulation electrode positioned within 100 μm of the recorded cell. The amplitude of the current pulses was controlled by a stimulus isolator (WPI Linear Stimulus Isolator A395) and was adjusted to elicit monosynaptic responses in the range of 100–300 pA (the required stimulus intensity ranged from 15 to 80 μA). AMPA/NMDA current ratios were computed

by dividing the mean peak eEPSC at -70mV (AMPA-mediated) by the mean amplitude at +40mV, 35 ms after the peak over a 2 ms window (NMDA-mediated). For all measures, cells from at least 3 animals, within each group, were used. Recordings were taken from cells within the accumbens core.

Behavior and physiology data analysis: Males and females were analyzed separately because we a priori predicted that LBN would have a bigger effect on males based on previous studies(1, 6), and we wanted the behavior and physiology comparisons to mirror the RNAseq data analysis that typically compares two groups (so here we separated males and females). As we do not compare the sexes for the behavior and physiology, we cannot conclude that there are sex differences and so limit our discussion of these findings to effects in males or females. Mixed factors ANOVAs were used for delay discounting with condition (control vs. LBN) as a between factor and delay (0s, 15s, 30s and 45s) as within factor. Mixed factors ANOVAs were also used for the FR1 self-administration data with (control vs. LBN) as a between factor and day as a within factor. Independent samples t-tests were used for PR. For electrophysiology studies, a 2×2 ANOVAs with condition (control vs. LBN) and drug (drug naïve vs. morphine SA) were conducted.

RNAseq and data analysis: RNA quality was assessed via Nanodrop (260/280nm and 260/230nm). RNA quantity was measured using the Qubit™ RNA HS Assay kit (Invitrogen, USA) following the manufacturer's protocol. RNA quality was further assessed via RNA integrity number by Beijing Genomics Institute (BGI) Americas Corporation using an Agilent 2100 Bioanalyzer RNA 6000 nano kit (Agilent, USA). All RNA samples used for sequencing had an RNA integrity number (RIN) > 8. Library preparation was performed by BGI Americas Corporation and sequenced on a Hiseq 4000.

To evaluate the quality of reads, the Fastqc software (version 0.11.8) was used (7) and, based on its results, adaptors and non-paired reads were removed using Trimmomatic (version 0.39)(8). RNA-seq analysis was done using a suggested pipeline by Sahraeian et al.(9). Briefly, to identify differences in gene expression, sequenced reads were aligned to the rn6 genome assembly using the Hisat2 software (10) and were quantified using StringTie(11). To determine differentially expressed genes (DEGs), the Deseq2 library in R was used(12). An adjusted p-value < 0.1 and 50% change in the expression (|log 2 Fold change| > 0.58) were used as cutoffs to determine significant DEGs. R statistical software (ver 4.0) was used for downstream analysis and visualization of the RNA-seq output including, but not limited, to hierarchical clustering analysis, drawing heatmaps, and volcano plots(13). HOMER motif analysis was used to find putative transcription factors of the DEGs(14). The parameters were set to − 2000 to + 1000 bp of the transcriptional start site and a length of 8–12 bp for TFs. DEGs in males (LBN vs. control) and females (LBN vs. control) were first analyzed separately. DEGs represented in the control males vs. control females analysis were also used to predict transcription factors orchestrating baseline sex differences in the NAc.

Gene set enrichment analysis (GSE analysis) is a method to detect part of genes that are over-represented in a large group of genes and may have an association with analyzed phenotypes (e.g. LBN treatment). GSE analysis was done using g:Profiler website service as previously described(15), using DEGs from the Male and Female groups, separately. KEGG pathways with adjusted p-value < 0.1 were selected for further analysis. The WebGestalt online toolkit (16) was used for Panther enrichment analysis. The rank-rank hypergeometric overlap version 2 (RRHO2) (17) test was used to evaluate the degree of overlap in gene signatures between sexes. The full table of comparisons between LBN samples and their control in each sex was extracted. The adjusted-p-values were multiplied by the sign of the effect to show up or down-regulated genes. These tables were used to make the RRHO heatmap.

Supporting Information Results

Delayed Discounting Results: Before rats were trained on delay discounting, they were all required to learn to lever press both left and right levers 50 times or more. Lever press training takes 2-3 days. LBN exposure did not affect males ability to learn to lever press (no effect of day [F1,16=2.364, p=.144]; no effect of LBN [F1,16<1]; no effect of interaction [F1,16<1]. Similarly, LBN exposure in females did not affect their ability to learn delayed discounting at the same rate as control females (no effect of day [F1,17=.028, p=.868]; no effect of LBN [F1,17=.226, p=.641]; no effect interaction [F1,17=.197, p=.663]). LBN exposure did not affect the time it took males to reach stable baseline criteria (no effects of day [F1.084, 17.343= 2.858, p=.107]; no day × block interactions [F6, 96= 1.012, p=.422]), indicating they learned the task as quickly as controls. LBN exposure also had no effect on female's ability to reach stable baseline criteria at the same time as controls (no effect of day [F1.213, 20.619= 1.839, p=.190]; no effect of day × block interactions [F6, 102=1.555, p=.168]). For all statistics where sphericity was violated, we used Greenhouse Geisser corrections.

Response latencies were not significantly different between LBN and control males (no effect of delay [F1.802, 28.836= 2.168, p=.137], no effect of LBN [F1, 16=.016, p=.902; interaction [F1.802, 28.836=.364, p=.676]. Response latencies were also not significantly different between LBN and control females [no effect of delay [F1.441, 24.502= .823, p=.415]; no effect of LBN [F1, 17=.217, p=.647]; nor interaction [F1.441, 24.502= 1.294, p=.282].

Lastly, there were no differences in the number of omissions made between LBN and control males (no effect of day [F1.054, 16.857=2.184, p=.158], no effect of LBN [F1, 16<1]; no interaction [F1.054, 16.857<1]. There were no differences in the number of omissions made between LBN and control females as well (no effect of day [F1.473, 25.033<1]; no effect of LBN [F1, 17=2.825, p=.111]; no interaction [F1.473, 25.033= .062, p=.890].

LBN had no significant effects on self-administration of the high dose of morphine: LBN had no effect on drug-taking behavior using the high dose of morphine (0.75mg/kg/infusion) in males or females. Specifically, we found no differences in drug-taking between control (n=7) and LBN (n=11) males on an FR1 schedule (effect of LBN: [F1,16=1.13, p=.304]; days of morphine IVSA: [F2.64,42.22=2.901, p=.0522]; interaction: [F9,144<1]). There was no effect of LBN on PR for the high dose (Fig.2F, effect of LBN: [t16=0.641, p=.53)]. Similarly, there were no differences in drug-taking for the high morphine dose between control (n=12) and LBN females (n=11) on an FR1 schedule (effect of LBN: [F1, 21<1]; days of morphine IVSA: [F2.51,52.77=4.321, p=.012]; interaction: [F9,189=3.756, p=.0002]). Breakpoint scores on PR for the high dose of morphine were not altered by LBN in females (effect of LBN: [t21=1.8, p=.086]. Animals were tested for drug seeking behavior after 7 days of forced abstinence. During this one-hour test, morphine was not available and active lever presses were reinforced by presentation of the previously drug-paired cue light above the active lever. Females [t21=0.3847, p=.7043] and males [Mann-Whitney test, p=.5494] from both groups showed similar responding during the cue tests, indicating that LBN does not impact drug-seeking behavior at this time point.

LBN does not alter glutamate transmission in the NAcC of females: Females did not show any group differences in sEPSC frequency (7-13 cells/group from 3 rats/group) [effect of LBN, F1,29=1.31, p=.26; effect of morphine, F1,29=1.11, p=.30; interaction, F1,29<1]. No effect of early life adversity on sEPSC amplitude was detected in females [effect of LBN, F1,29<1; effect of morphine, F1,29=1.07, p=.31; interaction, F1,29<1].

LBN causes sex-specific changes in gene expression in the nucleus accumbens: LBN produced unique patterns of gene expression in the nucleus accumbens of males and females. When comparing up and downregulated genes between the sexes, only 2 genes were identified as downregulated in both males and females and 3 genes were identified as upregulated in both sexes. Additionally, only 7 genes were oppositely regulated in males and females. HOMER motif analyses were conducted to identify the potential master regulators orchestrating LBN-induced

changes in gene expression in the NAc. The first analysis compared motifs in the DEGs of males and females, respectively (SI Fig 3A). DEGs accounting for baseline sex differences (control males vs. control females) were also analyzed using HOMER and yielded a distinct set of predicted transcription factors (SI Fig 3B).

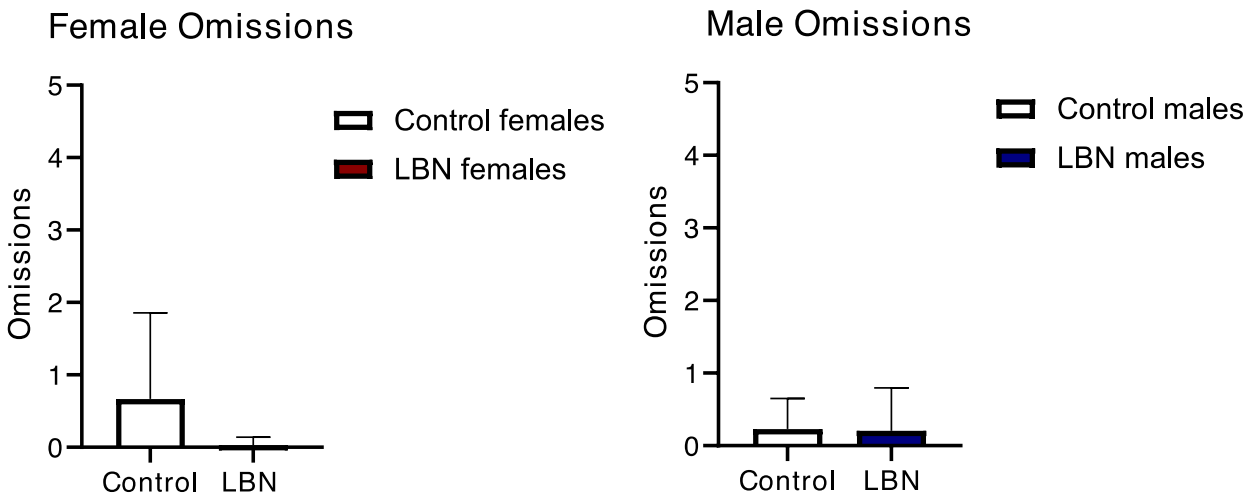

Fig. S1. LBN had no significant effect on omissions in either males or females. Omissions were very low in both sexes. Mean ± S.E.M.

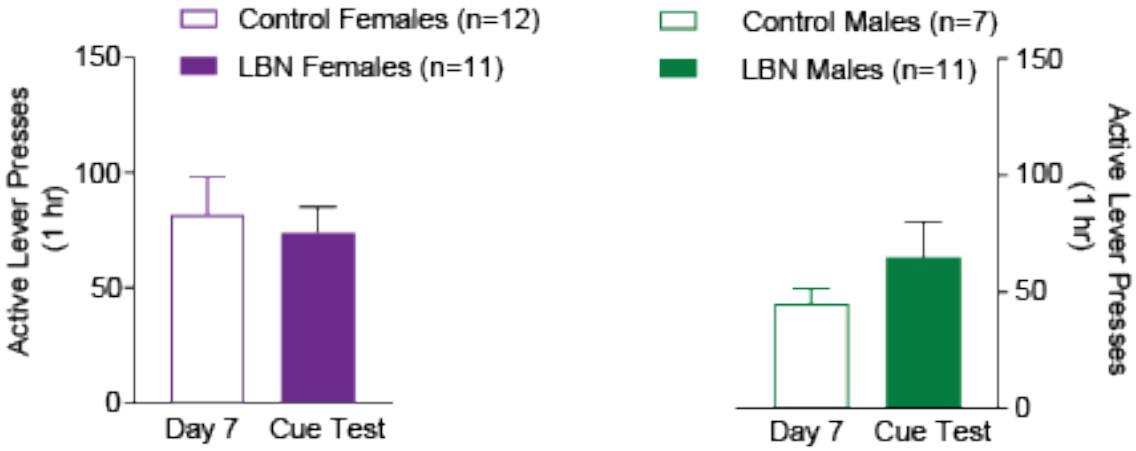

Fig. S2. LBN did not impact cue-induced drug seeking behavior. Animals were tested for drug-seeking behavior after 7 days of abstinence from morphine IVSA at 0.75mg/kg/infusion. During the one-hour test, active lever presses were reinforced by presentation of previously drug-paired cue light above the lever. Mean ± S.E.M.

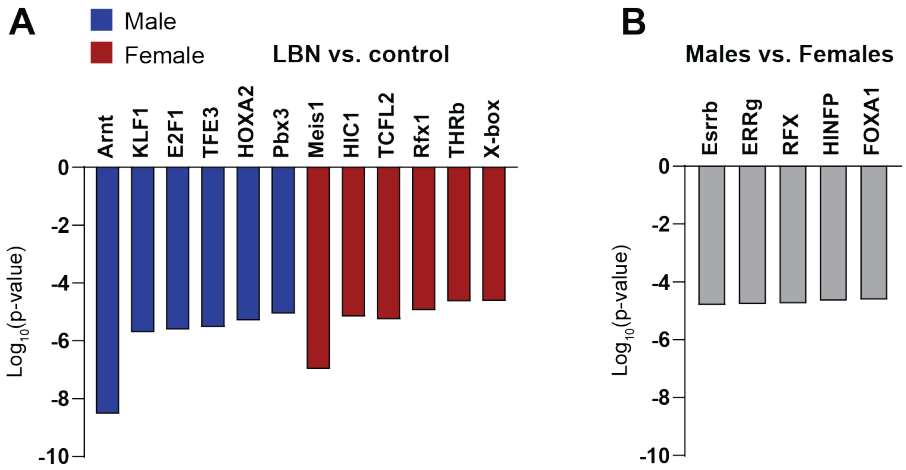

Fig S3. The predicted transcription factors regulating differentially expressed genes following LBN are sex-specific. (A) Transcription factors predicted by HOMER motif analysis to regulate the DEGs in both males and females (LBN vs. control). (B) Transcription factors predicted by HOMER motif analysis to regulate DEGs comparing control males to control females.

Table S1. Physiology Infusions. Total number of infusions earned at 0.75mg/kg/infusion prior to electrophysiological recordings.

Group	total infusions (avg +/- s.e.m)	p-value
Control Males	224.43 ± 52.62	
LBN Males	285.64 ± 31.77	.304
Control Females	300.92 ± 28.30	
LBN Females	272.91 ± 43.66	.5899

Table S2 (separate file). DEGs. List of differentially expressed genes (DEGs) in the NAc of males and females in response to LBN. DEGs overlapping in males and females are in bold font.

Table S3 (separate file). Pathways. List of GO terms enriched in males and females DEGs associated with LBN.

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