Influence of early life adversity on nicotine-dependent behavior and circuit activity

Background

Smoking is the leading cause of preventable death in the United States, contributing to nearly 480,000 deaths per year[1]. While the overwhelming majority of adult smokers report wanting to quit, only 7% successfully quit in a given year[2]. These low rates of success are partially due to the highly addictive qualities of nicotine, the primary psychoactive ingredient found within tobacco products. Though cigarette use has waned in the United States[3], nicotine use has remained popular through e-cigarettes, which have become the 'new smoking'[4]. E-cigarettes—often used to describe any electronic device used to vape—encompass vape pens, vape boxes, JUULs, e-hookahs, and e-pipes. Many of these devices contain an e-liquid, a fluid with propylene glycol, vegetable glycerin, concentrated nicotine, and flavorings, that delivers more than 5 times the amount of nicotine than a cigarette[5]. Vape flavors, like cotton candy and blue raspberry, have directly contributed to the popularity of vaping amongst teenagers, with nearly a third of e-cig users citing flavors as their motivation for initiation[6]. Indeed, among 19-22 year-olds in the United States, vaping of nicotine has doubled in the past two years[7]. Nearly half of U.S. teens have vaped at least once in their lifetime[8]. Recent reports from the CDC identified a national outbreak of e-cigarette or vaping product use–associated lung injury (EVALI), and as of February 2020, there have been 2,807 hospitalized cases or deaths in the U.S. related to EVALI. As expected, vaping also increases risk of COVID-19 infection and is a predictor of worse outcomes in COVID-19 patients[9]. Left untreated, long-term nicotine exposure can lead to changes in brain circuitry involved in learning, reward, and memory, increasing responsiveness to nicotine, particularly in the case of adolescent nicotine exposure[10-11]. Therefore, it is imperative that we develop methods for aiding nicotine addiction.

A growing body of literature has indicated that certain populations demonstrate an elevated risk of addiction. In particular, individuals who have experienced adverse childhood events, such as abuse and household dysfunction, have a 4- to 12-fold increased risk for drug abuse and are 2- to 4-fold more likely to smoke tobacco[12]. Exposure to childhood trauma can "reshape" the brain, leading to changes in stress and emotional regulation and cognitive processes[13]. Studies have suggested that early adversity increases the likelihood of developing anxiety and depressive disorders[14-16]. These symptoms, in turn, have been linked to difficulties in smoking cessation and more severe withdrawal symptoms[17]. Furthermore, researchers have demonstrated that childhood trauma can affect impulsiveness in adulthood; impulsivity, characterized by urgency, sensation seeking, lack of premeditation, and lack of perseverance, increases vulnerability to substance-use disorders[18-19].

In order to mitigate nicotine addiction, it is critical to understand why individuals who have experienced early life adversity (ELA) are more susceptible to nicotine addiction than others. However, it remains unknown how early life adversity (ELA) influences neural circuitry that underlie nicotine addiction. In our lab, we model ELA in mice by using the limited bedding and

nesting model (LBN)[20]. Using assays for assessing addiction- and anxiety-like behaviors in mice, we can study the behavioral effects of ELA on nicotine addiction and withdrawal. To identify circuits activated by nicotine relapse, we can use whole-brain iDisco++ tissue clearing and light-sheet microscopy, using the intermediate early gene product cFos as a proxy for neuronal activity. By identifying circuits that play a role in nicotine relapse, we may be able to develop more targeted interventions to mitigate the cycle of nicotine abuse and withdrawal in victims of childhood trauma.

Objective and Approach

Our study aims to investigate how ELA influences neural circuitry that underlie nicotine addiction. To do so, we will model ELA in mice by limiting bedding and nesting (LBN). Using assays for assessing addiction- and anxiety-like behaviors in mice, we can study the effects of ELA on nicotine addiction-related behaviors and withdrawal. During conditioned place preference (CPP), mice will receive repetitive nicotine injections paired to a specific context over five days. Following conditioning, the locomotor activity assay will be used to determine if nicotine had a sensitization effect on locomotion. CPP will then be extinguished in order to test for reinstatement later on. After an additional five days of passive nicotine withdrawal, we will test for withdrawal-induced anxiety behaviors using the open field test (OFT), elevated plus maze (EPM), light/dark box (L/D), and marble burying task (MB). A final challenge dose of nicotine will be administered to test for reinstatement of nicotine CPP. One hour following reinstatement, mice will be perfused and their brains extracted. To determine any differences in cellular circuit activation between ELA and control mice in response to a "challenge" dose of nicotine, meant to simulate relapse, we will use the iDisco++ tissue-clearing protocol to allow for whole brain immunolabeling of the intermediate early gene cFos, as a proxy for neuronal activity. Brains will then be imaged for cFos staining using light-sheet microscopy. Neurons labeled by the cFos antibody will be quantified in ClearMap to allow for the identification of differentially activated brain regions between the LBN and control mice following a challenge dose of nicotine.

Overall, in order to further investigate the influence of ELA on the development of nicotine-dependent behavior, our goal is to (1) examine differences in anxiety-like behavior and preference for nicotine in LBN and control mice and (2) identify differences in activated brain regions of the LBN and control mice following a "challenge" dose of nicotine meant to simulate relapse. Our results will allow for the development of therapies that target brain regions identified in early life adversity models as a potential for treating the cycle of addiction.

Conditioned Place Preference
drug preference test

Elevated Plus Maze
anxiety-like behavior

Open Field Test
anxiety-like behavior

CPP Reinstatement in Nicotine Group

Nicotine conditioned place preference in LBN and control mice. LBN mice administered nicotine (0.5mg/kg; i.p.) trend towards increased time spent in the drug-paired chamber during CPP reinstatement. n=7 in both LBN and control groups.

Elevated Plus Maze

EPM performance in LBN and control mice. The data represent group mean (+SEM) time spent in open arms of the maze during a 5-min test after a saline or nicotine (0.5mg/kg; i.p.) injection. Nicotine-administered LBN mice trend towards decreased time spent in the open arms of the EPM. n=5-7 mice per group.

Open Field Test

OFT performance in LBN and control mice. The data represent group mean (+SEM) time spent in center of open field during a 5-min test after a saline or nicotine (0.5mg/kg; i.p.) injection. Nicotine-administered LBN mice trend towards decreased time spent in the center of the open field. n=5-7 mice per group.
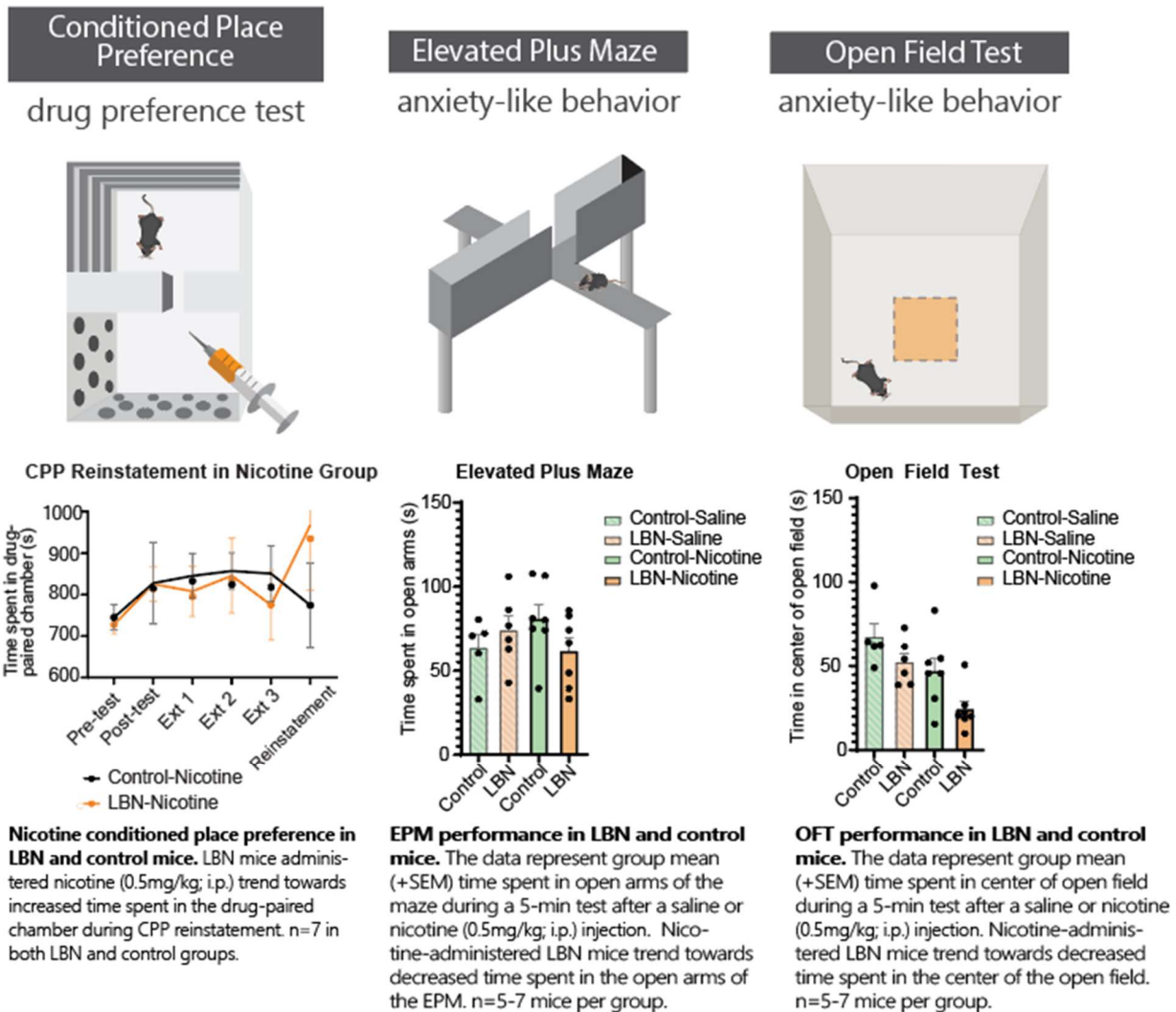
Figure 1. (left) LBN mice show increased preference for nicotine on the conditioned place preference test; LBN mice show a trend towards increased anxiety-like behavior on the elevated plus maze (center) and open field test (right).

Preliminary data from our lab has suggested that ELA mice show an increased susceptibility to the rewarding aspects of repetitive nicotine use. One cohort of LBN (n=7) and age-matched control mice (n=7) received nicotine and another cohort of age-matched control (n=5) and LBN (n=6) mice received saline as a control measure. Both cohorts of LBN and control mice were tested on the previously mentioned behavioral battery to assay reward and withdrawal behaviors. ELA mice trend towards increased time spent in the drug-paired chamber, indicating increased drug preference, as well as increased anxiety-like behavior on the EPM and OFT assays **(Fig. 1)**. Interestingly, on the L/D and MB assays, ELA mice trend towards decreased anxiety-like behavior **(Fig. 2)**. It is likely that the small cohort size of the initial study may have been a

contributing factor in the contradicting results observed; the LBN model and time sensitivity of the behavioral experiments made it difficult to allow for a large cohort size. Therefore, in order to capture a more accurate picture of the effect that ELA has on nicotine addiction, we aim to focus on exploring these promising preliminary findings by repeating these experiments with a greater number of mice.
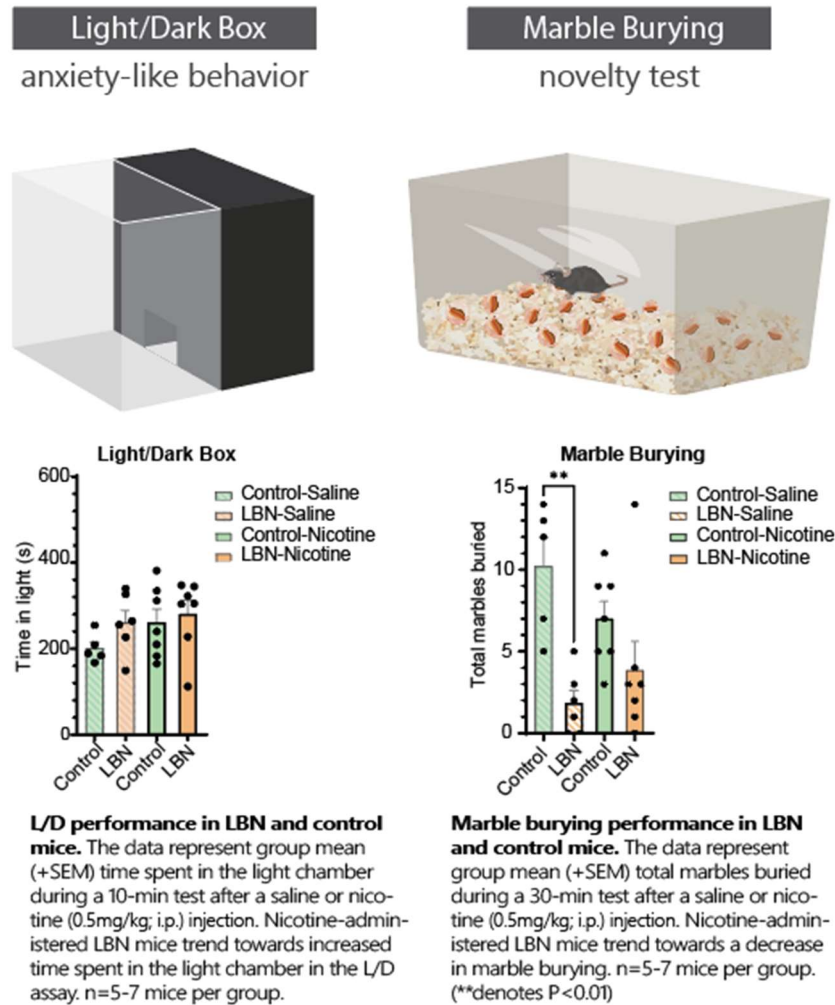

L/D performance in LBN and control mice. The data represent group mean (+SEM) time spent in the light chamber during a 10-min test after a saline or nicotine (0.5mg/kg; i.p.) injection. Nicotine-administered LBN mice trend towards increased time spent in the light chamber in the L/D assay. n=5-7 mice per group.

Marble burying performance in LBN and control mice. The data represent group mean (+SEM) total marbles buried during a 30-min test after a saline or nicotine (0.5mg/kg; i.p.) injection. Nicotine-administered LBN mice trend towards a decrease in marble burying. n=5-7 mice per group. (**denotes P<0.01)

Figure 2. (left) LBN mice trend towards decreased anxiety-like behavior on the L/D assay; (right) LBN mice trend towards decreased anxiety-like behavior on the MB assay.

To determine any differences in neural circuit activation between ELA and control mice in response to a "challenge" dose of nicotine, meant to simulate relapse, we will be using the iDisco++ tissue-clearing protocol to allow for whole brain immunolabeling of the intermediate early gene cFos, as a proxy for neuronal activity. Brains will then be imaged for cFos staining using light-sheet microscopy. Neurons labeled by the cFos antibody will be quantified in ClearMap to allow for the identification of differentially activated brain regions between the LBN and control mice following a challenge dose of nicotine.

Preliminary data from our ClearMap results highlight a cluster of brain regions that have significantly decreased activity in LBN animals relative to controls following the challenge nicotine dose. These brain regions, highlighted in grey **(Fig. 3)**, are all related to auditory and sensory processing: the primary, ventral, and posterior auditory cortex (AUDp, AUDv, AUDpo); temporal association area (TEa); and the nucleus of the brachium of the inferior colliculus (NB). Ongoing studies aim to investigate the role of the auditory cortex in the behavior battery described previously. These will be done by permanently silencing the auditory cortex through viral delivery of the inward rectifier potassium channel Kir2.1 prior to the start of repetitive nicotine administration. Additionally, we will be using the viral delivery of hM3D, an excitatory designer receptor activated by designer drugs (DREADD), to enhance neuronal activity in the midbrain. This will allow us to probe the normal function of the auditory cortex and midbrain in the behavior battery described previously.
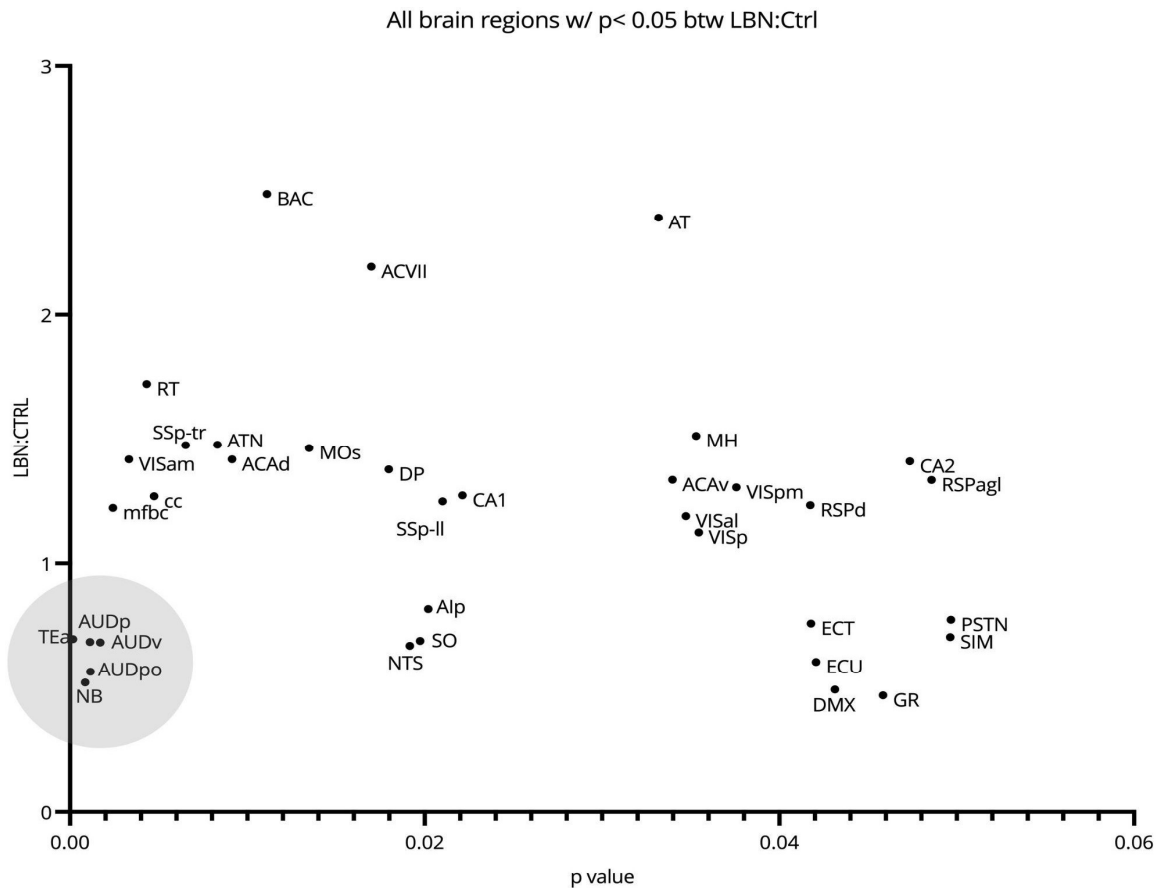

Figure 3. Plot of p-values for deviation in cell counts between LBN and control mice in major brain regions. The primary, ventral, and posterior auditory cortex (AUDp, AUDv, AUDpo); temporal association area (TEa); and the nucleus of the brachium of the inferior colliculus (NB), as highlighted in grey, show significantly decreased activity in LBN mice in comparison to controls after a challenge dose of nicotine (p<0.02, unpaired t-test).

Timeline

Fall+Winter

ELA protocol

- Mice are born on Day 0
- Maternal behavior is scored from Day 3-10
- Stereotaxic injections into auditory cortex/midbrain at Day 21
- Nicotine CPP pairings
- cFos results from previous quarter are analyzed to determine active brain regions following nicotine withdrawal

Behavior tests

- Locomotor Activity test
- CPP Extinction test
- OFT, EPM, L/D, MB behavioral tests, CPP reinstatement

Spring

Brain Imaging

- Brain perfusion and extraction
- Performing iDisco++ protocol to clear brains for cFos
- Image brains using light-sheet microscopy
- Count cells using ClearMap
- Data analysis and poster/manuscript presentation

Responsibilities

My responsibilities in this project include maintaining a cohort of 60+ LBN and control mice and carrying out the LBN protocol. In addition, I will perform all behavioral experiments and surgeries, analyze behavior, use iDisco++ to clear brains to examine cFos activity, and survey cFos expression data using the ClearMap software.

Budget

- 2 x 100ug vials of Synaptic Systems cFos monoclonal rat purified IgG (2 x $501 = $1002)
    - Used to label cFos+ cells during iDisco++ brain tissue immunostaining

Methods and Materials

ELA/LBN

The LBN model developed by the Baram Lab at UCI aims to simulate fragmented maternal care. Pups are paired with their dam and randomly assigned to either control or LBN conditions. Those assigned to control conditions are placed in cages with a standard amount of bedding (~0.33 cubic feet of shredded corn cob) without a platform, and with a cotton nestlet square. Those assigned to LBN conditions are placed in cages fitted with a plastic-coated mesh platform sitting ~2.5 cm above the cage floor with bedding sparsely covering the cage floor under the platform, and half of one cotton nestlet square[20].

TESTING

Conditioned Place Preference (CPP)

The conditioned place preference (CPP) procedure is used to assess the rewarding nature of an unconditioned stimulus with minimal distress to the animal. During this Pavlovian conditioning procedure, a previously neutral environment (a chamber of the CPP apparatus) is paired with a rewarding (e.g., drug) stimulus. The extent and nature of the rewarding properties of the unconditioned stimulus is reflected in the preference of the animal for the environment paired with the unconditioned stimulus compared with an unpaired, control environment on a drug-free test day. The drug injections were administered peripherally (intraperitoneal or subcutaneous). The procedure consisted of the following phases (two sessions per day): (1) pre-test (to determine inherent side preference in the chamber), 30 min; (2) conditioning (mice were placed in assigned chambers and injected with drug or saline in a counterbalanced design across sessions; 8 sessions, 30 min each); and (3) preference test (mice were free to move throughout all compartments of the apparatus and the time spent in each section was recorded; 1 session, 30 min). The advantage of this procedure relative to other tasks is that the training period is rapid, and the variable under investigation (conditioned environmental preference) is particularly sensitive to the effects of addictive drugs. As a result, the CPP test is a useful test for measuring the rewarding effects of addictive drugs in a learning paradigm. Thus, the data garnered from these studies may have implications for basic learning and memory processes involved in addiction. The testing chambers are cleaned in between each group with 70% ethanol.

Locomotor Activity Test

The locomotor activity test is a simple means of assessing activation and arousal in animals. Psychomotor stimulants increase spontaneous locomotor activity in humans, rats and mice. Hence, the locomotor activity test represents a simple procedure for detecting the stimulant actions of addictive drugs. Animals are placed in a chamber with no motivational constraints and are free to move about. Motor activity is quantified in locomotor activity boxes 43 x 43 cm^2. An overhead camera will be used to record 30-minute sessions; afterwards, a mouse tracking software will be used to calculate the total distance traveled in the locomotor activity chamber, and velocity of travel. Mice will be allowed to habituate to the chamber for 2 days (30 minutes each). Following habituation days, mice will be placed into the chamber following administration of drug and allowed to run freely for 30 minutes; these tests will occur once every day over five days. The testing chambers are cleaned in between each group with 70% ethanol.

Open Field Test

Mice are placed in a large square chamber and allowed to roam freely for five minutes. The time spent in the center square of the chamber, with decreased time in the center suggesting anxiety-like behavior, is determined using Biobserve, a software that records and tracks mouse movements[22].

Elevated Plus Maze Test

The elevated plus maze apparatus consists of a center region with two sets of opposing arms in a "plus" formation. One set of arms remains open while the other set of arms is enclosed. Mice are placed in the center of the apparatus and allowed to roam freely for five minutes. The time spent in the open arms, with decreased time suggesting anxiety-like behavior, is determined using Biobserve[22].

Light/Dark Box

The light/dark Box consists of a chamber divided into a light and dark compartment using a dark partition with an aperture. The "light" compartment has transparent walls and is uncovered while the "dark" compartment has black walls and is covered by a lid. Mice are placed in the center of the "light" side and allowed to roam freely between the two compartments for ten minutes. The time spent in the light box, with decreased time suggesting anxiety-like behavior, is determined using Biobserve[22].

Marble Burying Test

The marble burying apparatus consists of a plastic cage filled with 4 cm of corncob bedding. Twenty marbles are placed on top of the bedding in a 4 x 5 arrangement. Mice are placed in the center of the cage and allowed to roam freely for 30 min. The number of marbles buried is recorded at 10-min intervals[23].

Mechanical Allodynia (Von Frey Assay):

Von Frey filaments, pieces of nylon rod of varying diameters, are used to test mechanosensitivity in mice and rats. The filaments are applied to the plantar surface of the paw. Results are reported in several categories: (a) Frequency of paw withdrawal using 3 different filament forces (mild, moderate, and severe); (b) Maximum threshold, defined as the maximum filamentous force that does not cause paw withdrawal; (c) Frequency of vocalizations and other grooming behaviors (i.e. licking of the paw) associated with the filament application.

IMAGING

iDisco++

Mice are transcardially perfused using phosphate-buffered saline (PBS) and 4% paraformaldehyde. Brains are harvested and placed in 4% paraformaldehyde for 24hrs and washed 3 times in 1× PBS for 1h each, before undergoing the iDISCO++ protocol for whole-mount immunostaining. The brain tissue is incubated with rat anti-cFos (Synaptic Systems; 1:5000) and donkey anti-rat Cy3 (Jackson ImmunoResearch, 1:250) [24].

Light-Sheet Fluorescence Imaging

Brain samples treated with the iDisco++ protocol are imaged in the sagittal orientation using 3-d printed sample holders. Samples are imaged on the Z.1 LSFM (Zeiss) and excitation is done with 488nm, 561 nm, and 638nm laser lines coupled to BP 505–545, and LP 660 emission filters, respectively.

ClearMap Software

Images collected from light-sheet fluorescence imaging are automatically mapped to the Allen Brain Atlas. cFos-labeled cells are detected and counted in 3D. Distribution maps of immunolabeled cells are generated and available for statistical analysis. ClearMap detection parameters for each brain are close to each other and cell counts are manually inspected by a blinded experimenter to minimize bias[25].

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Timeline
ELA protocol
- Mice are born on Day 0
- Maternal behavior is scored from Day 3-10
- Mice are weaned at Day 21
- Stereotaxic injections into auditory cortex/midbrain at Day 21
- Nicotine CPP pairings Days 31-35

Behavior tests
- Locomotor Activity test Days 36-41
- CPP Extinction test Days 42-46
- CPP Withdrawal Days 47-51
- OFT, EPM, L/D, MB behavioral tests Days 52-53
- CPP reinstatement Day 55

Brain Imaging
- Brain perfusion and extraction Day 55
- Performing iDisco++ protocol to clear brains for cFos Days 55-76
- Image brains using light-sheet microscopy Days 77-80
- Count cells using ClearMap Days 80-87
- Data analysis and poster/manuscript presentation