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Combined early life stressors: prenatal nicotine and maternal deprivation interact to influence behavioral phenotypes in rats

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Abstract

Early life stress (ELS) increases the risk for later cognitive and emotional dysfunction, and has been implicated in the etiology of multiple psychiatric disorders. We hypothesize that combined insults during gestation and infancy, critical periods of neural development, could exacerbate neuropsychiatric outcomes in later life. Thus, we investigated the effects of maternal deprivation (MD) stress alone or combined with prenatal nicotine exposure (PNE) on negative affective states, ethanol drinking, and development of mesolimbic loci that regulate depression and drug dependence. On the elevated plus maze (EPM), MD rats exhibited ~50% increase in risk-taking behavior/decreased anxiety when compared to control, but the combined MD+PNE did not affect this specific behavior. In the open field test, however, both MD and MD+PNE groups showed 2-fold greater locomotor activity. Furthermore, whereas MD showed greater latency to fall at 40 RPM on the rotarod compared to CTL, the MD+PNE animals' latency to fall was significantly greater at all RPMs tested, with an approximate ~15% enhancement in motor coordination overall compared to control and MD. Analyses of depressive symptomatology with the forced swim test (FST) yielded 2- and 3-fold higher immobility times in MD and MD+PNE respectively. When tested in an operant drinking paradigm to quantify the effect of treatment on 10% v/v ethanol drinking, the MD and MD+PNE groups showed heightened ethanol consumption by ~3- and 2-fold respectively, with the experience of PNE reducing ethanol consumption in adults. To test the stressors' impact on neurons in the amygdala and ventral tegmental area (VTA), mesolimbic anatomical regions associated with mood and reward, unbiased stereological measurements were performed and revealed ~15% increase in number and density of neurons in the amygdala for both MD and MD+PNE, and ~13% reduction in dopaminergic-like neurons in the VTA compared to control. We report here that multiple early stressors including prenatal nicotine and MD can

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Declarations of Interest:

None

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modulate the neuroanatomy of the amygdala and VTA. These early life stressors can interact to influence the development of depressive-like and addictive behaviors.

Keywords

Early life Stress; Maternal deprivation; Maternal separation; Nicotine; Depression; Anxiety; Addiction

1. INTRODUCTION

Upwards of 46 percent of children in the United States have experienced at least one form of early life stress (ELS) [1]. Numerous studies have shown that the experience of childhood stress and early life adversity through neglect, maltreatment, and abuse exerts prominent, persistent effects on the brain's emotional circuits [2–4] and reward circuitry [5–7] and contribute significantly to the neurobiology of depression [8]. One of the most salient forms of ELS in humans and animals is maternal deprivation (MD) [9, 10]. MD has been associated with an earlier-onset and more chronic depression, a finding supported by concordant twin studies [11–13]. ELS increases the risk for anxiety and depression in adolescents and adults by facilitating exaggerated responses to stressful events later in life [14] and for developing alcohol dependence/substance use disorder [15, 16]. Stress-related psychiatric disorders are estimated to affect 20% of people in the United States within their lifetime, and remain a major cause of disability leading to significant social and economic costs [17, 18].

Whereas a single chronic stressor during childhood increases the lifetime risk for anxiety or depressive disorders by approximately 30%, two or more adverse events early in life, more than doubles the risk to develop these disorders [19]. The neural mechanisms of heightened vulnerability to affective disorders due to multiple ELS exposures, compared to a single stressor is not known. Earlier studies have primarily focused on the impact of individual stressors. For instance, prenatal drug exposure - a form of ELS - has been associated with an increased prevalence of hyperactivity, anxiety- and depression-like behaviors in adolescent and adult rats, increasing susceptibility to addictive and Attention Deficit/Hyperactivity Disorders (ADHD) [20, 21]. Behavioral studies on another form of ELS, maternal deprivation (MD), showed increased ambulatory time and vertical count [22], increased immobility time during a forced swim test [23], and increased ethanol self-administration during adolescence and adulthood [24–27].

Although the mechanisms for these behaviors in humans and animal models are not well understood, recent findings suggest that ELS in the form of maternal separation stress (MS) impacts the structural plasticity of neurons, leading to stoichiometric reductions in granule neuron numbers and dendritic rearrangement of cells in ventral (anterior) dentate gyrus of hippocampus [28–30] with extensive reciprocal afferents to the amygdala. The cognitive and behavioral effects of nicotine differ greatly dependent on whether the exposure is experienced developmentally during the gestational/early postnatal period or during adulthood [31]. When exposed in utero or in early postnatal period, the effects are deleterious, with modulation of limbic circuits and cortical circuits respectively or in

concert, impacting emotion[32], and cognitive function [21, 33]. Thus, prenatal nicotine exposure (PNE) is postulated to induce long-lasting changes in the brain structure and function of the individual [34, 35].

Stress reactivity is regulated in the central nervous system through the coordinated circuitry coupling of limbic, midbrain, and prefrontal brain regions, including the basolateral amygdala and ventral tegmental area (VTA) [36]. The amygdala controls negative emotions such as fear, anxiety, and depression, and closely interacts with the hippocampus and prefrontal cortex to process and store emotional memory. As a key locus in the limbic-prefrontal circuit, the amygdala is central to emotional and social information processing, and damage to this area results in problems with evaluation of social stimuli [37, 38]. The VTA to ventral striatum circuit mediates dopamine-dependent reward mechanisms implicated in mood and depression [39]. Excitatory and inhibitory synapses on VTA dopaminergic neurons express long-term potentiation (LTP) that is altered by exposure to acute stress [40–42]. Thus, these two regions are relevant structural sites to evaluate mechanisms of affective behaviors and substance use disorders.

High comorbidity exists between major depression and substance use disorders [43], which may be attributable to common etiological conditions, such as stress [44]. Specifically, exposure to stressful life events is believed to precipitate depressive episodes [45] and to increase craving for drugs of abuse [46]. Furthermore, dysregulation of reward function and altered reward sensitivity comprises a pathophysiological state that is common to both disorders. For example, one of the key features of major depressive disorder (MDD) and of addiction is anhedonia, or decreased interest or pleasure in rewarding events [47].

There is but one previous study in the literature investigating the combine effects of PNE and MD. In a “2-hit” stress model, the combination of developmental drug exposure (prenatal nicotine (PNE) and stressful experiences (postnatal maternal deprivation (MD)), first described by Wang *et al.* [30] synergistically exacerbated perturbations in hippocampal development. To date, strategies to determine how perinatal environmental stressors alone or combined interact to manifest themselves in neuroanatomical and neuro-adaptational deficits have not been thoroughly investigated. To fill this gap, the current study uses rat models of combined MD and nicotine exposure to mimic maternal neglect, combined with early drug exposure to study their effects on negative affective states and neuroanatomical loci reported to regulate depression and drug dependence [44]. We find that MD has a strong impact on behaviors associated with locomotor activity, anxiogenic/ risk-taking measures, alcohol consumption, and decidedly impacts neurons in VTA and amygdala, respectively involved in brain reward and affect. We report for the first time that the combinatorial exposure to prenatal nicotine prior to postnatal MD specifically exacerbates depressive-like behaviors, and may enhance amygdala function in adolescents and young adults.

2. MATERIAL AND METHODS

2.1 Animals

In this study, we used a total of 75 rat offspring, and 38 dams. Timed-pregnant Sprague-Dawley rats weighing between 250 g and 300 g were obtained from Harlan Laboratories

(Frederick, MD, USA) and housed under 12 h light/dark cycles, with access to food and water *ad libitum*. Rats were implanted with osmotic delivery pumps for saline or nicotine during gestation. The pups used for this study were born at the Howard University veterinary facility, where a subset was thereafter subjected to the MD paradigm, also described below. To control for litter effects, approximately 1 male and 1 female was used from each litter such that the smallest experimental group of $n=10$, represents at least 5 dams. Ex-vivo studies of $n=6$ animals were from at least 3 litters per group. Upon weaning, the animals were housed in groups of 2 or 3 in plastic cages. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of Howard University.

2.2 Prenatal nicotine exposure

This procedure was designed to emulate human gestational development and the human infancy period where nicotine may be passed to the infant through mother's milk, as previously described [48]. The nicotine used was freshly prepared in 0.9% saline on the pump implantation day, and the pH was adjusted to 7.4–7.6. This dose is usually used to achieve circulating blood nicotine levels of a smoker, about 1–2 packs/day [49]. On gestational day 7 (G7), the dams were surgically implanted with a mini-osmotic infusion pump (model #2004) (Alzet, Cupertino, CA, USA) containing either 0.9% saline or (–)-nicotine hydrogen tartrate (Sigma-Aldrich, St. Louis, MO, USA), which was delivered at a rate of 4 mg/kg/day until parturition at G21/P0 [48]. The pumps were thereafter removed by aseptic surgery on G21. The surgical procedures were performed under isoflurane anesthesia, and all efforts were made to minimize suffering.

2.3 Maternal deprivation paradigm

The MD paradigm was carried out using established protocols [30]. This paradigm has been shown to mimic early adverse stressful experiences. Beginning at postnatal day (P) 2, pups were removed from their mothers' home cage, moved to a different room altogether and isolated from other pups for 3 hours daily, from 11 am to 2 pm. The temperature of the MD room was monitored and maintained at 29°C with a heater to simulate the warmth of the dam's body. After the 3 hours, the pups were returned to the nursing dams. This was carried out until they were weaned at P21.

2.4 Experimental design

Whole litters were assigned to 1 of 3 groups: Controls were from dams who received Alzet pumps filled with saline and were kept with their mothers during the entire postnatal period; maternally deprived groups (MD) received saline during the prenatal period and were subjected to the MD paradigm from P2 to P21; maternal deprivation plus nicotine groups (MD+PNE) were first exposed to prenatal nicotine followed by the MD paradigm. Although sex differences were not analyzed in this study, both sexes were represented in each group.

2.5 Elevated Plus maze (EPM)

EPM is an established test to measure anxiety in laboratory animals [50]. A total of 34 SD rats were used for this experiment — Controls (n=14), MD (n=14), MD+PNE (n=10). The EPM consisted of 2 open, unprotected arms (45 cm (l) × 5 cm (w)) and 2 closed, protected arms (45cm length × 5 cm width × 45 cm height) that extended from a central platform (5 cm × 5 cm), and elevated 50 cm above the floor. Each rat was placed in the central platform facing an open arm. This experiment was performed in a dark room, and so a camera with an infrared filter, fitted over the EPM, and a computer with pre-installed EthoVision XT video tracking software was used to track the activities of the rats. The number of entries into the open and closed arms, and time spent in open and closed arms were calculated and considered an index of anxiety response and overall exploratory behavior, respectively.

2.6 Open field Test

Following EPM, 30 rats were used to determine the effects of double-hit exposure to prenatal nicotine and maternal deprivation on locomotor activity [51], with 10 rats per group. At P28, they were placed in individual Opto-varimex Plexiglas cages (30 cm × 42 cm × 42 cm) with a homogenous black plastic floor (Columbus Instruments, USA) designed to measure photo beam interruptions was used to assess locomotor activity of the animals 30 minutes daily for a duration of 4 days. These experiments were carried out in the dark. The walls of the cages housed infrared emitters and detectors, which automatically registered the locomotor activity using the Opto-varimex software (Columbus Instruments, USA). The mean horizontal activity, vertical activity, ambulatory time, and stereotypic time for the 4 days were thereafter analyzed.

2.7 Forced Swim Test (FST)

FST measures learned helplessness, quantified by the immobility of animals in an inescapable cylinder of water. A total of 42 SD rats were used for this experiment. Swimming sessions were conducted by placing rats (N = 12–15/treatment group) in individual Pyrex cylinders (46 cm tall, 20 cm in diameter) containing 28 ± 0.5 °C water at a depth of 30 cm, so that rats could not support themselves by touching the bottom with their hind paws or tails. 24 hours prior to testing, the SD rats underwent 15 minutes FST training to induce helplessness, and 5 minutes of forced swimming on test day [52]. The experiment was recorded on the Noldus EthoVisionXT video tracking software, and thereafter behavior sampling of the frequency of immobility in each 5-s period of the 300-s test was analyzed.

2.8 Sucrose Home Cage Drinking

The rats were separated into single cages and a 10-mL sipper tube containing a 3% (w/v) sucrose solution in tap water was placed in each cage (n = 10/treatment group). Animals were given access to the solution with simultaneous free access to food for 2 hours. Total sucrose intake was calculated based on bottle volumes before and after the 2 h period [53].

2.9 Rotarod

The Columbus instrument's rotarod system, used to quantify motor function, consists of 4 horizontal plastic cylinders 7 cm in diameter and 9.5 cm in length, which rotate around their

axis. The rotational speed of the rotarod spindle is controlled by the system software, and infrared beams are used to detect when a subject has fallen from the Rotarod. A total of 30 SD rats were used in this experiment, with 10 rats per group. Each experiment was set for a maximum of 3 minutes, and took place for four consecutive days, each day consisting of different speeds ranging from 13 to 40 rounds per minute (RPM). Four animals were placed simultaneously on the apparatus, 1 animal per cylinder. Motor coordination was estimated by the average time each animal group managed to remain on the cylinder [54].

2.10 Ethanol drinking protocol

The animals were trained to lever press for ethanol beginning at P60-P70. This experiment was carried out in a total of 30 SD rats, with 10 rats per group. The training to lever press for a reward (10% sucrose) first on the FR1 schedule, and then on FR4, took place for approximately 4 weeks, was followed by the sucrose fading technique, and stabilization on 10% EtOH. Once responding was animals were stable on FR4 (within 20% from day-to-day), 21 days of testing commenced. Standard operant chambers (Coulbourn Instruments, Inc., PA) were enclosed in an isolated box. The operant apparatus contained two levers, two dipper manipulanda, triple cue lights over each lever, and a house light. The dipper cup size was 0.1 ml. The Coulbourn Graphic State “3” operant software was used to capture lever presses for the alcohol. Rats were allowed access to the chamber for 2×30 mins with a 30-minute rest period between sessions, over a 3-week period using a 5-day access and 2-day resting modifications of well-established procedures [53].

2.11 Tissue processing and histology

Immunocytochemical analyses of the amygdala and VTA was performed according to previously published protocol [27, 30]. Brains ($n=6$ per group) were post-fixed overnight in the 4% PFA/4% sucrose/0.1 M phosphate buffer, and then transferred to and stored in phosphate buffered saline (PBS), pH 7.4, at 4°C until ready for processing. Brains were sunk in 30% sucrose/PBS over 3 days, mounted onto a platform with OCT, and freeze-sectioned on a sliding microtome to generate free-floating 50 μm coronal sections, and every 9th section from Bregma (A-P= -4.5 to -6.9 mm for VTA, and A-P= -1.3 to -4.2mm for amygdala) was mounted onto gelatin-coated slides, and dried overnight for staining with Nissl, dehydrated in serial ethanol concentrations, and coverslipped with Permount.

2.12 Stereological analysis

Unbiased stereology was used to estimate mean total number and density of neurons in the amygdala and VTA, via the optical fractionator method [30, 55]. Data collection was carried out using both the Stereo Investigator program (MicroBright Field, Inc.). The system uses an X-Y-Z motorized stage. The Stereo investigator was attached to a Nikon E800 microscope and Optromics high-resolution video camera. The region of interest was outlined at low power (4 $\times$) over the rostral-caudal length of the region of interest in the right hemisphere. Cells were counted on a thin focal plane scanning with a 63 $\times$ objective in the z-axis. The appropriate grid size and counting frames were determined based on results of pilot studies for each region (VTA and amygdala) [56]. Sampling grid sizes (200 $\mu\text{m} \times 200 \mu\text{m}$) and counting frames (30 $\mu\text{m} \times 30 \mu\text{m}$) were optimized to achieve a mean Gundersen coefficient of error (CE) of <0.1 [56]. A guard volume 2.0 μm deep on both sides of the section was

used to avoid introduction of errors due to sectioning artifacts, including uneven section surfaces. Neurons within the VTA or amygdala were identified as Nissl positive cell bodies containing a nucleolus clearly in focus within the counting frame, with lightly stained surrounding cytoplasm.

2.13 Data analysis

The data, presented as the mean $\pm$ standard error of the mean, were calculated with the Microsoft Excel program, employing student's *t*-test for the behavioral tests and ANOVA for ex vivo calculations of neuronal number and density; to determine whether the differences between two experimental groups were significant.

RESULTS

3.1 Increased Risk-taking/reduced anxiety behavior is correlated with MD, but not when combined with prenatal nicotine

Some early life stressors have been associated with development of anxiety disorders or risky behaviors [57, 58], therefore we measured if MD alone or in combination with PNE induces anxiety-like behaviors on the elevated plus maze, where the number of open and closed arm entries, and the time spent in the open and closed arms were evaluated as measures of anxiety. The MD group showed a statistically significant 100% increase in open arm entries (4.2 ± 0.7 vs. 7.8 ± 0.8 , $p = 0.01$; Figure 1A) and time spent in the open arms (24.6 ± 5.6 vs. 46.8 ± 6.3 sec, $p = 0.01$; Figure 1B) relative to control, but pre-exposure to nicotine reduced these open arm entries (7.8 ± 0.8 vs. 3.8 ± 0.6 , $p = 0.01$) and time spent (46.8 ± 6.3 vs. 19.8 ± 3.7 , $p = 0.01$) when compared to MD alone and did not produce any significant effect relative to control. Finally, MD+PNE rats did result in a reduction in the number of closed arm entries (16.2 ± 3.4 vs. 12.4 ± 0.7 , $p = 0.01$) (Figure 1C) but not the time spent in closed arms (Figure 1D).

3.2 Combined Prenatal nicotine and postnatal MD exposure is associated with an increase in locomotor activity

It was previously reported that MD induces heightened locomotor activity [22], we repeated this behavioral measure, but evaluated the mean locomotor activity for 4 consecutive days during early adolescence (P28-P35) in MD +/- prenatal nicotine exposure. When compared to control, exposure to MD resulted in a statistically significant, ~100% increase in the averaged horizontal (1011.0 ± 94.7 vs. 1971.1 ± 122.1 , $p = 0.001$; Fig 2A) and vertical beam breaks (885.05 ± 85.65 vs. 1749.1 ± 111.9 , $p = 0.001$; Fig 2B), a 67% increase in time spent on stereotypic grooming (170.3 ± 14.2 vs. 284.6 ± 15.2 , $p = 0.001$; Fig 2C) and a 72% increase in ambulatory time (184.4 ± 15.8 vs. 316.1 ± 17.9 , $p = 0.001$; Fig 2D). Combined exposure to prenatal nicotine and MD stress (MD+PNE) resulted in a statistically significant increase in horizontal (1011.0 ± 94.7 vs. 1805.7 ± 191.8 , $p = 0.001$) and vertical beam breaks (885.05 ± 85.65 vs. 1646.83 ± 175.57 , $p = 0.001$), stereotypic time (170.3 ± 14.2 vs. 245.1 ± 21.2 , $p = 0.001$) as well as ambulatory time (184.4 ± 15.8 vs. 293.5 ± 27.2 , $p = 0.001$) (Figure 2).

3.3 Combined Prenatal nicotine and postnatal MD exposure causes an increase in motor activity on the Rotarod test

Next, performance of adolescents on the rotarod was evaluated. This test is frequently used as a measure of motor coordination mediated by the cerebellum, but is also useful to assess neural deficits associated with hyper-excitability/activity, sedation, and stamina [59]. Overall, the MD group did not significantly differ from control at lower speeds, but had a greater latency to fall at 40 rpm (48 ± 12.9 vs 80.2 ± 14.5 $p < 0.05$; Fig 3A). However, with gestational PNE exposure, MD animals exhibited a sustained increase in latency to fall at each time point from 20 RPM to 40RPMs, that was statistically significant from control and MD alone (Figure 3A). At 20 RPM, the MD+PNE group had a 55% increase in time spent on the rod, when compared to control (98.4 ± 23.16 vs. 152.7 ± 15.39 , $p = 0.05$) and 46% increase when compared to MD group (103.9 ± 14.54 vs. 152.7 ± 15.39 , $p = 0.01$). At 30 RPM, the MD +PNE group also spent 97% more time on the rod, when compared to control (78.1 ± 24.24 vs. 154.6 ± 16.95 , $p = 0.05$). At 40 RPM, when compared to control, there was a 67% increase in time spent on the rod by the MD group (48.0 ± 12.86 vs. 80.22 , $p = 0.05$), and 48% increase by the MD+PNE group (48.0 ± 12.86 vs. 119.0 ± 18.94 , $p = 0.01$) (Figure 3A). When the average latency to fall for the different groups was analyzed, MD+PNE group was observed to have a 41% higher latency to fall from the rod when compared to control (106.97 ± 9.50 vs. 150.22 ± 7.0 , $p = 0.001$), and 31% when compared to the MD group (114.41 ± 9.22 vs. 150.22 ± 7.0 , $p = 0.001$) (Figure 3B).

3.4 Depressive-like behavior and anhedonia is enhanced with combined exposure to prenatal nicotine and postnatal MD

We have previously published the finding that the experience of maternal deprivation stress enhances anhedonia and depressive-like behavior in rodents [27]. Here, we examine how prenatal nicotine exposure influences the MD effect. Whereas the MD group spent greater than 2-fold more time immobile than controls (16.7 ± 3.5 vs. 35.2 ± 4.6 , $p = 0.01$), the MD +PNE 3-fold more time immobile than controls (16.7 ± 3.5 vs. 52.1 ± 4.3 , $p = 0.001$) and ~50% greater immobility than the MD group (35.2 ± 4.6 vs. 52.1 ± 4.3 , $p = 0.01$) (Figure 4A). The sucrose intake of the MD and MD+PNE groups was not lower than control (Figure 4B).

3.5 Prior exposure to prenatal nicotine does not enhance MD-induced increased ethanol self-administration.

MD has been previously correlated with increased impulsivity and increased ethanol consumption on operant self-administration tasks [16, 27]. We tested dual exposure to life adversity. Figure 4c shows the mean number of lever presses for each of 3 weeks during 1 hour in the operant chamber. The MD group consistently exhibited 2- to 3-fold greater ethanol intake relative to control (week 1: 362.5 ± 36.4 vs 131.22 ± 15.0 , week 2: 258.9 ± 30.2 vs 81.5 ± 9.2 , week 3: 485.0 ± 31.3 vs 196.1 ± 43.9). The MD+PNE group also consistently exhibited higher ethanol intake relative to control (week 1: 224.5 ± 35.1 vs 131.22 ± 15.0 , week 2: 169.3 ± 28.1 vs 81.5 ± 9.2 , week 3: 332.6 ± 45.4 vs 196.1 ± 43.9). However, the ethanol intake for MD+PNE group was significantly lower than MD (week 1: 362.5 ± 36.4 vs 224.3 ± 35.2 , week 2: 258.9 ± 30.2 vs 169.3 ± 28.1 , week 3: 485.0 ± 31.3 vs 332.6 ± 45.4).

Therefore, MD+PNE show reduced alcohol-drinking phenotype than MD animals, and increases intake compared to control. (Figure 4C).

3.6 Combined exposure to prenatal nicotine and MD is correlated with increased in neurons in amygdala and decrease in neurons in VTA

Since stress can alter transcriptional regulation in the amygdala and VTA, which may increase risk for addictive disorders, we tested if the combined PNE and MD altered cell dynamics in the amygdala and VTA. The number of neurons in the amygdala of MD animals increased by 24% over control ($8.4 \times 10^5 \pm 2.4 \times 10^4$ vs. $6.8 \times 10^5 \pm 2.9 \times 10^4$, $p = 0.01$). MD +PNE enhanced even more robustly, to 35%, the number of neurons in the amygdala ($1.0 \times 10^6 \pm 2.5 \times 10^4$ vs. $6.8 \times 10^5 \pm 2.8 \times 10^4$, $p = 0.001$), and 16% greater than the MD group ($1.0 \times 10^6 \pm 2.5 \times 10^4$ vs. $8.4 \times 10^5 \pm 2.4 \times 10^4$, $p = 0.01$) (Figure 5A). These data were mirrored in neuron density values, which increased in the amygdala with MD (16%) and with PNE+MD (32%) (Figure 5B). By contrast, there was a 27% reduction of neurons in the VTA of both MD and MD+PNE groups ($6.1 \times 10^4 \pm 3.7 \times 10^3$ (CTL) vs. $4.8 \times 10^4 \pm 1.7 \times 10^3$ (MD) vs. $4.8 \times 10^4 \pm 1.7 \times 10^3$ (MD+PNE), $p = 0.01$, (Figure 5C), and a reduction of neuron density estimated at 34% decrease in the MD group when compared to control ($3.1 \times 10^4 \pm 2.4 \times 10^3$ vs. $2.4 \times 10^4 \pm 1.6 \times 10^3$, $p = 0.01$) (Figure 5D). Therefore, MD and PNE additively impacted the amygdala, whereas the MD effect and the MD+PNE effect registered as one in the VTA.

4. DISCUSSION

In the current study, we investigated the effect of a “two-hit” early life adversity model of prenatal nicotine exposure followed by MD on behavioral correlates of neuropsychiatric and affective disorders. ELS is known to alter several neural circuits involved in the stress response as well as developing dopaminergic, serotonergic, and glutamatergic systems, all implicated in binge drinking/alcohol addiction vulnerabilities, and mental health [60–62]. In this study, the experience of MD alone caused increased locomotor activity in the open field, increased motor activity on the rotarod, and increased number of entries and time spent in open arms of the EPM. LCA activity was heightened with equivalence for MD and MD +PNE, and on the rotarod, prior PNE further enhanced MD-mediated performance. However, in the EPM test of anxiety, PNE interacted with MD to reduce MD-generated anxiolytic behavior to control levels. In the FST test of learned helplessness and depression, MD-induced depressive-like symptomatology was exacerbated in the presence of PNE, whereas by contrast, an MD-induced increase in ethanol intake was ‘attenuated’, i.e., fell between MD and control. These behavioral findings are mirrored with anatomical findings that the amygdala, which mediates learned helplessness and depression had many more neurons, and the VTA had fewer. This study provides compelling evidence expanding our earlier findings that combined exposure to prenatal nicotine and postnatal MD influences neural architecture of stress-induced reward systems that may functionally converge or diverge, as exhibited in the many behavioral phenotypes tested here.

Locomotor activity was significantly increased to the same extent whether exposed to MD alone or MD+PNE. This suggests that the two stressors worked to elicit an MD-informed

behavioral response (Figure 1). Increased locomotor activities observed in the MD group may be due to hyperactivity of the mesolimbic dopaminergic system, as MD is known to alter the dopaminergic pathway [63, 64]. Prenatal nicotine exposure alone has also been reported to provoke similar hyperlocomotor [65–68] and anxiogenic behaviors in rats [68, 69]. Clinical and epidemiological studies have consistently reported increased prevalence of Attention-Deficit-Hyperactivity-Disorder (ADHD) in offspring prenatally exposed to nicotine, and so these results indicate that PNE by itself is also a risk factor for emergence of hyperlocomotive behaviors. It has been suggested that behaviors reported with PNE are due to pathological activation of acetylcholine nicotinic receptors (AChNR) during neonatal stages of neurodevelopment [66]. PNE aberrantly activates, or most likely desensitizes nAChRs in the developing catecholaminergic circuitry, which may not manifest until childhood and adolescence [70]. Furthermore, prenatal and postnatal stress results in HPA reactivity and therefore an increase in anxiety-like behaviors, which is mediated by increased glucocorticoid receptor (GR) gene expression, and is accounted for partly, by demethylation of the rat GR gene (*Nr3c1*) in the hippocampus of the offspring [71].

The MD rats exhibited reduced anxiety and increased risk-taking behavior in the elevated plus maze, evidenced by the number of entries and time spent in the open arms. However, PNE prior to the secondary stressor of MD, negated the effects of MD alone, as the number of entries and time spent in the open arms was not significantly different from that of control, perhaps due to anxiogenic effects of PNE [69, 70], and recruitment of divergent mechanisms from PNE and MD-activated pathways. The number of closed arm entries by the MD+PNE group was significantly lower than control and MD, indicating that combined MD+PNE exposure may have rendered animals in an intermittent, less active state, as observed in the reduced number of entries in both open and closed arms (Figure 2). The prolonged development of the cerebellum also leaves it susceptible to long-term stress exposure, which may alter cerebellar cytoarchitecture and therefore interfere with motor coordination and motor cognition [72]. However, MD did not negatively impact and in fact, enhanced motor coordination at 40 rpm. MD with PNE enhanced motor coordination at all rpms tested (Figure 3). In rodent models, anxiety is typically measured by a lack of exploration of the open arms (i.e., more time spent in the closed arms) of the EPM, and preference for the periphery of the open field box in locomotor activity tests. It is possible that hyperlocomotion and risky activity may also represent a display of anxiety. This example has parallels with human behavior, where a person with anxiety may shun social interaction, or they may overcompensate with nervous energy and risk-taking behavior [73].

Chronic stress is known to result in amygdaloid hypertrophy [74], and MD is known to increase neuron number and density in the amygdala [74, 75], linked to increased fear circuitry, and depressive symptoms. FST, a behavioral parameter that measures learned helplessness and depression, showed a greater immobility time in MD alone compared to control, and an increase in immobility time with MD+PNE compared to control and MD. This suggests that PNE and MD likely acted independently to potentially impact amygdala and VTA circuits (Figure 4). Hyperactivity of the HPA axis in depressed patients results in increased levels of corticosterone [76] similar to ELS which is also known to cause an elevation in corticosterone and adult. GR dysfunction may be the cause of HPA axis hyperactivity in depression, as glucocorticoids usually regulate the HPA axis through

negative feedback inhibition and thereby reduce the production of glucocorticoids themselves.

The FST data was mirrored in the results of stereological analyses, where the pattern of neuron increases in the amygdala show enhanced numbers compared to MD alone. Figure 5 shows that the experience of PNE greatly enhances MD-induced increases in amygdala neurons in a pattern similar to the effect MD and PNE on depression symptomatology (Figure 4). This shows a somewhat exacerbating effect of the combined stressors, with MD stress possibly building up on the effects of prenatal nicotine exposure. Although the amygdala is functionally dormant in the rat neonatal period, significantly stressful events as used in this study, can precociously activate the amygdala [77], and such amygdala effects can last until adulthood [78, 79], resulting in increased anxiety- and depressive-like behaviors in adulthood [80]. Previously, we showed that PNE and MD impact neurons in subfields of the hippocampus differently. This regimen resulted in additive (CA1) or synergistic (CA3) increases in pyramidal neuron but a reduction of granule neuron number in the DG, similar to MD alone, and unaffected by PNE [30]. Moreover, analysis of dorsal or ventral hippocampus revealed that MD+PNE induced synergistic effects in dorsal CA1 and CA3 but the effects on DG were primarily by MD on caudal/ventral DG [21, 81, 82]. The caudal hippocampus is believed to be extensively connected to the amygdala and work together to process affective memories, specifically associated with negative emotions [83, 84]; to determine potential correlation with neural structures in the reward pathway, anxiety and depressive behaviors, and alcohol drinking.

CRF signaling in the VTA is particularly compelling since drugs of abuse and stressful/aversive stimuli activate the VTA similarly. Because both prior exposures to drugs of abuse, and the experience of stress enhances drug-seeking behavior [85, 86], we tested the two on an operant self-administration assay. The increased ethanol intake observed with MD (Figure 4C) has been reported in previous work [16, 27], and confirmed in other studies [24, 25, 87] indicating that MD facilitates alcohol preference in adulthood. PNE prior to MD seemed, however, to reduce the ethanol intake observed in MD alone (Figure 4C). Although the mechanism for stress-induced binge drinking is unknown, studies show that MD permanently alters expression of various GABA_A receptor subunits in the stress circuits [16, 88]. GABAA $\alpha 1$ and $\alpha 2$ receptors are upregulated whereas GABA neurotransmitters are downregulated in humans with alcohol use disorder, major depressive disorder [89], and in preclinical models of stress and alcohol preference [16] even as the levels of GABA neurotransmitter is reduced. Furthermore, dopaminergic neurons of the VTA are downregulated in both stress and alcohol-preferring models whereas projection neurons of the amygdala are upregulated [27]. Consequently, the reduction in neuron number and density in the VTA may have resulted in low dopaminergic tone and thus increased consumption of ethanol, whereas PNE may act to influence the reward pathway differently [90]. These data suggest specific, targeted effects of stress on reward and emotional centers.

In the future, it will be important to study more directly the contribution of the HPA axis, and the relative glucocorticoid receptor activation from each stressor on the output behaviors studied here. These findings highlight how little is known about the physiology and biochemistry of the convergence/divergence of the reward and affective pathways.

5. Conclusion

In summary, our study indicates that combined exposure to prenatal nicotine and MD elicits increased hyperactivity, risk-taking behaviors, and depressive-like symptomatology, which indicates a converging mechanism in the pathogenesis associated with some mood disorders. In other neuropsychiatric diseases such as excessive alcohol consumption they interact in a seemingly subtractive manner. The mechanism by which the combined exposure to prenatal nicotine and MD conferred resilience to impulsive and addictive behavior should however be further investigated.

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Figure 1: Anxiety behavior on Elevated Plus Maze following combined prenatal nicotine exposure and/or maternal deprivation.

Compared to the control SD rats (blue), the MD group (red) made a significantly greater number of (A) open arm entries and (B) spent more time in the open arms, while the MD +PNE group (green) made significantly fewer (A) open arm entries and (B) spent less time in the open arms, when compared to MD. (C) The MD+PNE group had significantly fewer closed arm entries when compared to control. Control (n=14), MD (n=14), MD+PNE (n=10). Data are expressed as mean ± SEM. * When compared to control, # When compared to MD. **/## $p < 0.01$ by T-test.

Figure 2: Effects of prenatal nicotine exposure and/or maternal separation on locomotor activity. (A) mean horizontal beam break count, (B) mean stereotypic time, (C) mean ambulatory time, (D) mean vertical beam break count were higher in the MD and MD+PNE groups when compared to control. $n=10$ per group. Data are expressed as mean $\pm$ SEM. *** $p < 0.001$ by T-test.

Figure 3: Analyses of motor coordination following prenatal nicotine exposure and/ or maternal deprivation.

(A) Motor coordination was tested in SD rats at different rotations per minute (RPM). At 20, 30, and 40 RPM, the MD+PNE group (green) spent significantly more time on the rod compared to control (blue), and compared to MD (red), whereas MD showed greater latency to fall at 40RPM. $n=10$ per group. Data are expressed as mean $\pm$ SEM. *When compared to control, #When compared to MD. */# p 0.05, **/# p 0.01 by T-test.

Figure 4: Immobility time, anhedonia and One-hour Analyses of Ethanol intake in nondeprived Sprague-Dawley rats following combined prenatal nicotine exposure and maternal deprivation. (A) Immobility time was 2-fold greater in the MD group compared to control. Immobility time was 3-fold greater in the MD+PNE group compared to control, and 50% greater than MD; (B) Anhedonia, measured via 3% (w/v) sucrose drinking indicated that sucrose intake of the MD and MD+PNE groups was lower than control, though it did not reach significance. (C) 10% ethanol intake was significantly higher in MD and MD+PNE groups. However, MD+PNE exhibited significantly lesser intake when compared to MD. Data are expressed as mean $\pm$ SEM. $n=10$ per group. $n=12$ per group for A and B, $n=10$ per group for C. Data are expressed as mean $\pm$ SEM. * When compared to control, # When compared to MD. */# p 0.05, **/## p 0.01, ***/### p 0.001 by T-test.

Figure 5: Analysis of neuron number and neuron density in VTA and amygdala following prenatal nicotine exposure and/or maternal deprivation.

(A) amygdala neuron number, and (B) amygdala neuron density were greater in both the MD group (red) and the MD+PNE group (green) compared to control, and in the MD+PNE group compared to MD. (C) VTA neuron number and (D) VTA neuron density were significantly reduced with MD and MD+PNE. $n=10$ per group. Data are expressed as mean $\pm$ SEM. *When compared to control, #When compared to MD. */# $p < 0.05$.