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High-Salt Exposure During Perinatal Development Enhances Stress Sensitivity

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Abstract

Excess consumption of dietary sodium during pregnancy has been shown to impair offspring cardiovascular function and enhance salt preference in adulthood, but little is known regarding the long-term impact of this nutritional surplus on offspring brain morphology and behavior. Using a combination of cellular and behavioral approaches, we examined the impact of maternal salt intake during the perinatal period on structural plasticity in the prefrontal cortex (PFC) and nucleus accumbens (NAc) in weanling and adult offspring as well as reward- and stress-driven behaviors in adult offspring. We found that weanling rats born to 4% NaCl fed dams exhibited an increase and decrease in thin spine density in the infralimbic PFC (IL-PFC) and prelimbic PFC (PL-PFC), respectively, as well as an increase in mushroom spine density in the NAc shell, compared to 1% NaCl fed controls. Structural changes in the IL-PFC and NAc shell persisted into adulthood, the latter of which is a phenotype that has been observed in rats exposed to early life stress. There was no effect of maternal salt intake on reward-driven behaviors, including sucrose preference, conditioned place preference (CPP) for cocaine, and forced swim stress (FSS)-induced reinstatement of cocaine-induced CPP. However, rats born to high-salt fed dams spent less time swimming in the FSS and displayed heightened plasma CORT levels in response to the FSS compared to controls, suggesting that early salt exposure increases stress sensitivity. Overall, our results suggest that perinatal salt exposure evokes lasting impacts on offspring physiology and behavior.

Keywords

Dietary Salt; Perinatal Development; Stress; Dendritic Spines; Prefrontal Cortex; Nucleus Accumbens

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INTRODUCTION

Research indicates that for a variety of reasons not all pregnant women meet perinatal nutritional recommendations (Bodnar and Siega-Riz, 2002, Siega-Riz et al., 2002, Laraia et al., 2004). In fact, roughly one third of pregnant women report consuming fast foods, which are high in fat, sugar, and salt, weekly during their pregnancies (Santiago et al., 2013). Excess fat consumption during pregnancy increases the risk that offspring will develop obesity, metabolic syndrome, cardiovascular disease, diabetes, and fatty liver disease in adulthood (Vickers et al., 2000, King, 2006, Aimukhametova et al., 2012). Relative to macronutrients like fats and carbohydrates, little is known regarding the long-term consequences of excess salt consumption during this sensitive period.

Using a model of perinatal salt exposure, in which rat dams are fed either a 1% or 4% NaCl diet in the 21 days prior to mating and throughout gestation and lactation, (Gray et al., 2015) demonstrated that offspring born to high-salt fed dams exhibit an increase in systolic blood pressure and a hypertensive phenotype as late as postnatal day 135, despite being fed standard laboratory chow since weaning. Additionally, early salt exposure has been linked with aberrant stress responsiveness in adulthood. Specifically, adult offspring from high-salt fed dams, using the model described above, display increased basal corticosterone (CORT) levels compared to controls (Gray et al., 2013). When maternal salt consumption is limited to the prenatal period only, offspring no longer exhibit heightened *basal* CORT levels but do demonstrate a relatively elevated CORT and adrenocorticotrophic response to acute stress (McBride et al., 2008) as well as an increase in corticotrophin-releasing hormone mRNA levels in the paraventricular nucleus under both basal and stress-induced conditions (Porter et al., 2007). In addition to physiological parameters, early excess salt exposure has been shown to augment behavioral responsiveness to both natural and drug rewards. Specifically, adult offspring born to high-salt fed dams display an enhanced preference for salt (Contreras, 1989, McBride et al., 2008) and glucose solutions (Contreras, 1989) as well as a heightened locomotor response to amphetamine (McBride et al., 2008). These observations suggest that perinatal salt exposure may serve as an early life stressor, predisposing offspring to diseases of reward dysregulation, much like other forms of stress increase vulnerability to drug use and addiction (Sinha, 2008). Yet, the effect of early salt exposure on behavior and neuronal morphology in the prefrontal cortex (PFC) and nucleus accumbens (NAc), areas highly involved in both stress (Radley et al., 2006, Jones et al., 2011, Ronzoni et al., 2016) and reward processing (Russo and Nestler, 2013), remains to be elucidated.

To evaluate the changes in dendritic morphology after perinatal salt exposure dams were fed either a standard (1% NaCl) or high-salt (4% NaCl) diet during both gestation and lactation. After which, dendritic spine type and density were assessed in weanling (postnatal day 21, PND21) and adult (PND60) male offspring. Results from this analysis indicated that high-salt exposed offspring, at PND21 and PND60, exhibited changes in spine density within the PFC and NAc resembling spine plasticity present in offspring exposed to early life stress (Muhammad et al., 2012). We further assessed cocaine-induced conditioned place preference (CPP) and forced swim stress (FSS)-induced reinstatement for cocaine CPP as well as sucrose preference, in order to examine stress- and reward-driven behaviors, but observed no dietary effect. Interestingly though, rats born to high-salt fed dams exhibited a

significant reduction in mobility time in the FSS as well as heightened plasma CORT levels in response to the FSS compared to controls, both indicative of alterations in stress sensitivity. Our conclusion is therefore that early salt exposure serves as an early life stressor which increases the behavioral and physiological response to future stress.

MATERIALS AND METHODS

Animal Ethics

All procedures in this study were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and with approval from the Institutional Animal Care and Use Committee (IACUC) committee at the University of Wyoming. With the exception of single housing of pregnant and lactating dams and of offspring during fluid consumption for sucrose preference testing (SPT), all animals were group housed in clear plastic cages in a temperature-controlled (25°C) vivarium with *ad libitum* access to food and water under a standard 12-hour light/dark cycle (0700–1900).

Breeding and Dietary Manipulation

Adult female Wistar rats were pair housed with a single male Wistar rat for approximately 8 days during which time estrous cycling was monitored with vaginal smearing as previously described (Westwood, 2008). Onset of pregnancy was determined by the lack of estrous as well as vaginal plug, when visible. Once pregnant, dams were singly housed and fed either a 1% or 4% NaCl diet (Tab. 1) during gestation and subsequent lactation. Food consumption of the dams was measured during this time. At birth, litters were culled to 8 pups, 4 males and 4 females to ensure equal access to milk, but only male offspring were used for experimentation. Once 21 days of age, offspring were either immediately euthanized for structural analysis or were weaned, placed in group housing, and fed standard laboratory chow until experimentation took place at PND60 (Fig. 1A).

Quantification of Dendritic Spine Type and Density

Quantification of dendritic spine type and density was conducted at PND21 and PND60 (Figs. 2–4). Methods for staining and quantification closely followed those previously described by our laboratory and others (Ferrario et al., 2005, Bloss et al., 2011, Dingess et al., 2016). Briefly, offspring were anesthetized with isoflurane and euthanized via decapitation, immediately following cardiac perfusion of 200 mL 0.9% saline followed by 300 mL 1.5% paraformaldehyde (PFA; in 0.1M phosphate buffered saline (PBS)). A Leica VT 1200S vibratome (Leica, Buffalo Grove, IL) was used to acquire 200 μ m coronal slices of the PFC and NAc, which were then incubated in 4% PFA at room temperature (RT), DiI labeling solution (1:200, Invitrogen, Carlsbad, CA) at RT, and PBS at 4°C for 20min, 1 hr, and 48 hrs, respectively. After incubation, slices were mounted on glass slides with Vectashield (Vector, Burlingame, CA). A Zeiss 710 confocal microscope and Zen imaging software were used for image acquisition. All representative images were acquired using a 40 $\times$ oil immersion objective (NA 0.55) with the same zoom ($\sim$ 10 $\times$) under identical acquisition settings. Representative images are Z-stacks comprising of 5–10 optical sections each 1 μ m in thickness. Thickness of the stack was determined by the dendrite itself, such that the lower and upper limits of the dendrite were included in the image. Acquired stacks

were reconstructed as max projections using ImageJ software, with no manipulation to brightness or contrast.

Cortical pyramidal cells in the PFC (Yang et al., 1996) and medium spiny neurons in the NAc (Wilson and Groves, 1980) were visually identified by their morphology. Spines were then quantified on the final 10 μm of terminal dendrites. In the PFC, both basal and apical dendrites of layer II/III were analyzed. Layer II/III was selected for analysis as it has been repeatedly demonstrated that this layer, receiving direct input from the thalamus and basolateral amygdala (Viola et al., 2016), exhibits structural deficits in the presence of stress (Seib and Wellman, 2003, Liston et al., 2006, McEwen and Morrison, 2013). Spines were classified based on parameters previously described (Bloss et al., 2011, Dingess et al., 2016). Importantly, spines were classified as mushroom type if the ratio of head to neck diameter was greater than 1.1 and the maximum head diameter was greater than 0.4 μm . In total, 24 dendrites were analyzed per dietary group (8 dendrites per animal, 4 from each hemisphere) for each brain region.

Cocaine-induced Conditioned Place Preference & Stress-Induced Reinstatement

Cocaine-induced CPP and stress-induced reinstatement for cocaine was assessed following methods previously described (Brown et al., 2007). Briefly, a three-chambered CPP apparatus with white (metal grid flooring) and black (wire mesh flooring) compartments separated by a central gray compartment was utilized for this study (Med Associates, Inc., Fairfax, VT). Rats were habituated to the apparatus for 2 days in which they were able to freely explore all three chambers for 15 minutes. The time spent in each compartment on the second day was used to determine initial preference (IP). This study followed an unbiased design, such that half the rats received cocaine administration on their initially preferred side and half on the initially non-preferred side. The conditioning sessions (training) were conducted once per day for 8 consecutive days. On alternating days, rats were given either an intraperitoneal injection of cocaine (12 mg/kg) and confined to one compartment or saline (1mL/kg) and confined to the other for 30 minutes. Testing occurred 24 hours after the last conditioning session and was conducted in the same drug-free manner as the preconditioning sessions, with 15 minutes of free access to all three chambers (Test 1).

Extinction followed identical procedures to IP and Test 1 and occurred once daily until rats spent no more than 10% more time in the cocaine-paired side than they did on IP for two consecutive days. Once this extinction criterion was met, rats were subjected to a modified Porsolt FSS (3-min, 4°C) as previously described (Porsolt et al., 1977, Niehaus et al., 2010). During the FSS, locomotor behavior was video recorded and manually quantified as mobile or immobile. 24 hrs following FSS, animals were retested (Test 2) for CPP in the same manner as Test 1.

Open Field Test

Adult male offspring from 1% and 4% NaCl fed dams were subjected to a 30-minute open field test in order to assess both basal locomotion (Fig. 5B) and anxiety (data not shown), as measured by the time spent in the center of the locomotor chamber compared to the time spent against the chamber walls (Seibenhener and Wooten, 2015). On the day of testing, rats

were moved from the vivarium to the testing room and allowed to acclimate to the new environment in their home cage for 30 minutes. Following acclimation, rats were placed into Omnitech locomotor chambers (Columbus Instruments, Columbus, OH) for 30 minutes, during which time photobeam breaks were quantified using VersaDat software. These sessions were video recorded and locomotor behavior manually quantified.

CORT Analysis

Plasma Corticosterone (CORT) levels were measured using The DetectX Corticosterone Immunoassay Kit (Arbor Assays, Ann Arbor, MI, Cat # K014) following the manufacturer's instructions. Blood was collected from adult male rats born to 1% or 4% NaCl fed dams under basal conditions as well as following 3-min and 5-min cold (4°C) FSS. Plasma was extracted by centrifugation at $2000 \times g$ for 15 min in EDTA treated tubes. Resulting supernatant was transferred into clean tubes and analyzed for CORT levels. Samples and Standards were prepared and 50 μ l each were pipetted into plate wells. Assay buffer, DetectX, Corticosterone Conjugate and DetectX Corticosterone Antibody were pipetted into each well and after adequate mixing the plate was incubated for 1 hr at room temperature on a shaker. Following incubation, wells were rinsed with wash buffer and dried. Then TMB substrate was added to each well and plate was incubated for 30 min at room temperature. After the incubation ended Stop Solution was added to each well and the optical density (OD) was read at 450 nm using Multiscan FC microplate reader (Thermo Scientific, USA). Standard curve was created after averaging the duplicate OD readings and sample concentrations were obtained from the standard curve.

Sucrose Preference Test

Sucrose preference testing was modified from methods described previously (Willner et al., 1987, Strekalova et al., 2004). Animals were singly housed for 3 days during which time they were given *ad libitum* access to food and two bottles. On the first day, the bottles were filled with tap water only, to acclimate the rats to the housing conditions of this experiment. During the subsequent two days, one bottle contained tap water and the other 2% sucrose dissolved in water (Fig. 6A). Bottle placement was switched after 24 hr such that each rat received the sucrose solution on both the left and right side, eliminating effects of strong side preferences. Fluid consumption was measured to the nearest mL daily at 0900.

Statistical analysis

All statistical tests were conducted using Prism 6 (GraphPad Software). Importantly, dendritic spines were analyzed using two-way ANOVA with Sidak's multiple comparisons test in which experimental groups at PND21 and PND60 were compared for each spine type. Behavioral analyses utilized unpaired t-test to compare differences between experimental groups. Behavioral data collected from offspring born to the same dam were averaged together. All data are shown as mean $\pm$ standard error of the mean (S.E.M.)

RESULTS

Dietary Manipulation

Dams fed the 1% and 4% NaCl diet both exhibited the anticipated increase in food consumption during lactation required to provide nutrients to developing offspring (Fig. 1B). Offspring weight gain was also monitored but two-way ANOVA with Sidak's multiple comparisons test revealed no significant dietary effect (Fig. 1C).

Dendritic Spine Density

Stress (Popoli et al., 2011) and reward processing (McFarland et al., 2003, Williams and Steketee, 2004) rely heavily on glutamatergic transmission in the PFC and NAc. We therefore assessed dendritic spines, structures which receive glutamatergic input (Hering and Sheng, 2001), in these stress and reward circuit regions. Our analysis of dendritic spines in weanling rats revealed significant group differences. In the PL-PFC, two-way ANOVA with Sidak's multiple comparisons revealed that offspring born to 4% NaCl fed dams exhibited a reduction in thin spines compared to standard-fed controls at PND21 on both basal (spines/10 μ m: 1%; 7.92 ± 0.04 , 4%; 7.38 ± 0.22 , $p < 0.05$, Fig. 2D, Tab. 2) and apical (spines/10 μ m: 1%; 7.88 ± 0.19 , 4%; 7.17 ± 0.11 , $p < 0.01$, Fig. 2E, Tab. 2) dendrites. In the IL-PFC, two-way ANOVA with Sidak's multiple comparisons revealed a potentiation of thin spines in high-salt exposed offspring on both basal (spines/10 μ m: 1%; 7.69 ± 0.11 , 4%; 8.63 ± 0.19 , $p < 0.01$, Fig. 3D, Tab. 2) and apical (spines/10 μ m: 1%; 7.63 ± 0.26 , 4%; 8.67 ± 0.23 , $p < 0.001$, Fig. 3E, Tab. 2) dendrites. We also observed a significant increase in mushroom spines on medium spiny neurons of the NAc-Sh (spines/10 μ m: 1%; 2.46 ± 0.08 , 4%; 3.21 ± 0.15 , $p < 0.01$, Fig. 4D, Tab. 2). There was no dietary effect on spine density in the NAc-C (Fig. 4E).

Dendritic spines were also quantified at PND60. Two-way ANOVA with Sidak's multiple comparisons revealed some of these adaptations were still present, including the increase of thin spines in the IL-PFC (basal, spines/10 μ m: 1%; 7.45 ± 0.33 , 4%; 8.40 ± 0.23 , $p < 0.05$; apical, spines/10 μ m: 1%; 7.46 ± 0.10 , 4%; 8.53 ± 0.27 , $p < 0.05$, Fig. 3D-E, Tab. 3) and mushroom spines in the NAc-Sh (spines/10 μ m: 1%; 2.56 ± 0.04 , 4%; 3.14 ± 0.07 , $p < 0.05$, Fig. 4D, Tab. 3).

Cocaine-Induced Conditioned Place Preference & Stress-Induced Reinstatement

To investigate the effect that perinatal salt exposure has on pathological reward-related behaviors, we measured cocaine-induced CPP, which assesses the preference for drug-associated environments. Cocaine was selected because of its direct mechanism of action in the regions of interest. Prior to CPP testing, we measured basal and FSS-induced (3 min and 5-min) plasma CORT levels in offspring born to 1% and 4% NaCl fed dams. Unpaired t-test comparing plasma CORT between groups under basal conditions revealed no group differences (Fig. 5A). Similarly, both groups exhibited an increase in plasma CORT following 5-min FSS (Fig. 5A). However, following 3-min FSS plasma CORT levels were significantly elevated in rats born to 4% NaCl fed dams compared to controls (plasma CORT: 1%; 812.4 ± 46.13 , 4%; 1267 ± 146.0 , $p < 0.05$, Fig. 5A). We further assessed basal locomotion in an open field test and found no dietary effect (Fig. 5B). Time spent in the

open field was also assessed as a crude measure of anxiety, but no group differences were detected (data not shown).

Unpaired t-test comparing the increase in time spent on the cocaine-paired side during Test 1 compared to IP revealed that both groups exhibited cocaine-induced CPP (Fig. 5D). No group differences in FSS-induced reinstatement of cocaine-induced CPP, measured as time spent on the cocaine-paired side during the last extinction session subtracted from Test 2, were detected (data not shown). However, unpaired t-test comparing time spent mobile and immobile between groups in the FSS revealed that offspring born to high-salt fed dams displayed a significant decrease in mobility (mobility time: 1%; 141.5 ± 3.42 sec, 4%: 108.5 ± 7.07 sec, $p < 0.01$, Fig. 5E) and increase in immobility time (immobility time: 1%; 38.48 ± 3.42 sec, 4%: 71.50 ± 7.07 sec, $p < 0.01$, Fig. 5E) compared to standard-fed controls suggesting a salt-induced depressive affect.

Sucrose Preference Test

Given that attenuated mobility in the FSS is considered a sign of depression (Yankelevitch-Yahav et al., 2015), we quantified depression-induced anhedonia using a sucrose preference test. Unpaired t-test comparing sucrose consumption between groups on day 1 and day 2 revealed that offspring born to high-salt fed dams did not display a decrease in sucrose consumption compared to controls (Day 1 sucrose consumption: 1%; 168.25 ± 29.27 mL, 4%: 196.25 ± 15.29 mL, $p > 0.05$, Fig. 6).

DISCUSSION

The primary aim of our study was to expand upon previous work by our colleagues that showed perinatal high-salt exposure facilitated physiological adaptations to stress (McBride et al., 2008). In our structural analysis of weanling and adult rats, we observed an increase in spine density in the NAc-Sh (Fig. 4). This observation is reminiscent of a 2012 study which reported an increase in spine density in the NAc-Sh of adult rats exposed to prenatal stress in which dams were placed on an elevated Plexiglas platform during gestation (Muhammad et al., 2012). The present study goes a step further to characterize changes in individual spine types. Our results demonstrated that structural plasticity in the NAc was attributed to changes in mushroom spines, which is reflective of activity-dependent insertion of ionotropic glutamate receptors, such as AMPA and NMDA, to the postsynaptic membrane (Matsuzaki et al., 2004). Interestingly, stress has been shown to increase the AMPA/NMDA ratio in the ventral tegmental area (Saal et al., 2003) and importantly in the NAc-Sh (Campioni et al., 2009).

It should be noted that the technique used for spine analysis allowed us to observe changes in spine morphology but does not afford the ability to discern the origin of synaptic contacts. Given that the NAc-Sh receives excitatory input from several regions, such as the PFC, hippocampus, and amygdala (Russo and Nestler, 2013), it is impossible to determine which synapses are affected by early salt exposure. Moreover, despite knowledge that the development of mushroom spines correlate with functional changes at the synapse (Matsuzaki et al., 2004), changes in spine density do not necessarily correlate with changes

in cellular activation and therefore future studies should aim to elucidate salt-induced changes to neuronal activity.

We further observed a decrease and increase in thin spine density in the PL-PFC and IL-PFC, respectively (Figs. 2–3), the latter of which persisted into adulthood. The opposing shifts in spine density in these regions is unsurprising given the distinct roles these regions play in stress regulation. Stimulation of the PL-PFC, for example, has been demonstrated to inhibit HPA activation to stress (Radley et al., 2006, Jones et al., 2011) and inhibition of the PL-PFC is sufficient to increase heart rate in response to stress-provoking psychological stimuli (Akana et al., 2001). Meanwhile, activation of the IL-PFC has been shown to increase basal plasma levels of CORT (Ronzoni et al., 2016) as well as blood pressure (Fryszak and Neafsey, 1994). The observed dendritic spine plasticity in the PL-PFC and IL-PFC is therefore intriguing in that it may predispose salt-exposed offspring to heightened stress sensitivity in a two-prong fashion, by attenuating and augmenting activity in stress-repressing and stress-inducing brain regions, respectively.

In addition to regulating stress, the PFC and NAc have long been examined as critical structures in brain reward circuitry (Russo and Nestler, 2013). It has been demonstrated that exposure to rewarding stimuli, such as cocaine and sucrose, induces structural plasticity in these regions (Robinson and Kolb, 2004, Klenowski et al., 2016). Given the observed effect of perinatal high-salt exposure on spine morphology in the PFC and NAc, we became curious about whether perinatal high-salt exposure might influence reward-driven behaviors. Our results showed that adult offspring born to both 1% and 4% NaCl fed dams exhibited equivalent cocaine-induced CPP, which could not be attributed to changes in general locomotion as basal locomotion was not different between groups in an open field locomotor test (Fig. 5B). This was curious given the previous reports that early high-salt exposure increases the locomotor response to amphetamine (McBride et al., 2008) and that sodium appetite and cocaine-induced locomotor behavior cross sensitize (Acerbo and Johnson, 2011). The CPP model however assesses a *preference* for drug-associated environments rather than the mere physiological response to a drug. Early salt exposure may therefore influence the locomotor response to psychostimulants without affecting an animal's preference for them. Given the role of the PL-PFC in cocaine-induced CPP (Zavala et al., 2003) and our observation of spine changes in the PL-PFC at PND21, but not PND60, it is possible that salt exposure alters adolescent, but not adult, drug preference. It is also plausible that we would have observed different results using amphetamine. Future studies should aim to elucidate these possibilities.

To follow-up on our histological data, which suggests that 4% NaCl exposed rats have structural adaptations reminiscent of a chronically stressed brain, we assessed whether a mild stressor (3 min FSS) would facilitate an increase in CORT levels. Specifically, we found that basal plasma CORT levels of adult offspring born to 1% fed dams were not different from those born to 4% NaCl fed dams were not different from each other. Additionally, the CORT levels were not different between groups following 5-min FSS (Fig. 5A). However, rats born to 4% NaCl fed dams exhibited an increase in plasma CORT following 3-min FSS compared to 1% NaCl controls (Fig. 5A). We therefore chose to examine whether acute 3-min FSS was sufficient to cause stress-induced reinstatement for

cocaine-induced CPP following extinction. Curiously, neither group exhibited FSS-induced reinstatement to cocaine CPP. However, offspring born to 4% NaCl fed dams exhibited reduced mobility/swimming behavior in the 3-min FSS. Given that attenuated mobility in the FSS is often considered a sign of depressive-like affect (Yankelevitch-Yahav et al., 2015) we implemented a sucrose preference test, used to quantify depression-induced anhedonia (Forbes et al., 1996, Krishnan and Nestler, 2011) to further investigate this behavior. However, 4% NaCl exposed offspring exhibited no significant differences in sucrose preference compared to their 1% NaCl fed counterparts.

Taken together, the results of our CORT and dendritic spine analyses coupled with the behavioral results of the FSS, lead us to favor the hypothesis that early salt exposure alters neuronal morphology in a way that mimics early life stress and increases offspring sensitivity to future stressors. Although stress sensitivity can often be correlated with aberrant reward seeking behavior, offspring born to high-salt fed dams failed to exhibit differences in cocaine and sucrose preference compared to controls. However, future studies will look to utilize an operant self-administration task, which may be more sensitive to detecting stress-induced changes in reinstatement behavior (Bardo and Bevins, 2000).

It should be noted that our findings are correlative and suggest that perinatal exposure to high-salt impacts the brain in a manner consistent with stress and that these animals appear more sensitive to typical readouts of physiological stress (CORT) but the impetus behind the observed changes to physiology and behavior were not explored and should be addressed in future studies. Salt exposure, for example, may alter maternal behaviors towards the pups. Indeed, dams with heightened CORT levels exhibit impairments in maternal care which have lasting impacts on the offspring (Patin et al., 2002). Moreover, given the relationship between salt consumption and vasopressin (Kjeldsen et al., 1985), it remains possible that maternal vasopressin levels could play a role in mediating the observed plasticity. Another remaining question would be whether the observed changes can be reversed with glucocorticoid receptor antagonism.

Nevertheless, our findings add to a growing literature suggesting that maternal salt consumption during perinatal development alters offspring physiology and behavior. While there are no current guidelines on salt consumption during pregnancy, aside from adhering to the recommended daily intake (RDI) of 2,300 mg, which many fail to follow, these results highlight the need for more research on the effects of early salt exposure.

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Figure 1. Model of Dietary Manipulation.

A. Experimental timeline. Sexually mature male and female Wistar rats were housed together and female estrous cycles were monitored until pregnancy was confirmed. Females were then separated and fed either a 1% or 4% NaCl diet throughout gestation and lactation (denoted in gray). At weaning, offspring were either immediately euthanized for structural analysis or put into group housing until experimentation at PND60. Experimentation included cocaine conditioned place preference (CPP), sucrose preference testing (SPT), corticosterone (CORT) analysis or open field test. **B.** Maternal food consumption of 1% (white circles, $n=8$) and 4% (black squares, $n=8$) fed rats during dietary manipulation. **C.** Weight of offspring during postnatal development born to 1% (white circles, $n=23/6$, where first and second numbers indicate the number of animals and dams, respectively) and 4% (black squares, $n=42/9$) fed dams.

Figure 2. Spine Analysis of the Prelimbic Prefrontal Cortex (PL-PFC).

A. Schematic of region analyzed and example dendrite with depiction of individual spine types. **B.** Representative images of terminal basal dendrites from DiI stained pyramidal cells of 1% (left) and 4% (right) NaCl fed offspring at postnatal day 21 (PND21, top) and PND60 (bottom). **C.** Representative images of terminal apical dendrites from DiI stained pyramidal cells of 1% (left) and 4% (right) NaCl fed offspring at PND21 (top) and PND60 (bottom). **D.** Quantification of spine types analyzed from terminal basal dendrites in 1% (white, n=24/3 for each time point, where first, second, and third numbers indicate the number of total

dendrites analyzed, number of animals, and number of dams respectively) and 4% (black, n=24/3/3 for each time point) NaCl fed offspring at PND 21 and PND60. **E.** Quantification of spine types analyzed from terminal apical dendrites in 1% and 4% NaCl fed offspring at PND 21 and PND60. Values represent the mean $\pm$ SEM (*p<0.05).

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Figure 3. Spine Analysis of the Infralimbic Prefrontal Cortex (IL-PFC).

A. Schematic of region analyzed. **B.** Representative images of terminal basal dendrites from DiI stained pyramidal cells of 1% (left) and 4% (right) NaCl fed offspring at postnatal day 21 (PND21, top) and PND60 (bottom). **C.** Representative images of terminal apical dendrites from DiI stained pyramidal cells of 1% (left) and 4% (right) NaCl fed offspring at PND21 (top) and PND60 (bottom). **D.** Quantification of spine types analyzed from terminal basal dendrites in 1% (white, n=24/3/3 for each time point, where first, second, and third numbers indicate the number of total dendrites analyzed, number of animals, and number of dams respectively) and 4% (black, n=24/3/3 for each time point) NaCl fed offspring at PND

21 and PND60. **E.** Quantification of spine types analyzed from terminal apical dendrites in 1% and 4% NaCl fed offspring at PND 21 and PND60. Values represent the mean $\pm$ SEM (* $p < 0.05$).

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respectively) and 4% (black, n=24/3/3 for each time point) NaCl fed offspring at PND 21 and PND60. **E.** Quantification of spine types analyzed from terminal dendrites in the NAc-C in 1% and 4% NaCl fed offspring at PND 21 and PND60. Values represent the mean $\pm$ SEM (*p<0.05).

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Figure 5. Conditioned Place Preference (CPP) for Cocaine.

A. Plasma corticosterone levels basally and following 3-min and 5-min FFS in offspring born to 1% (white, $n=3/3$, $4/4$, and $3/3$, for basal, 3-min FFS, 5-min FFS, respectively, where first and second numbers indicate the number of animals and dams, respectively) and 4% (black, $n=3/3$, $6/6$, and $5/5$ for basal, 3-min FFS, 5-min FFS) NaCl fed dams. **B.** Basal locomotion measured as photobeam breaks in a 30-min open field test of offspring born to 1% (white, $n=6/6$) and 4% NaCl (black, $n=6/6$) fed dams. **C.** Experimental timeline for cocaine CPP. Animals were habituated to the 3-chambered CPP apparatus for 15 minutes on day 1 (H). The following day initial preference (IP) was determined by how much time the

animal spent in each chamber. Test 1 (T1) occurred 24 hr after the last training session and was followed by an extinction period. Test 2 (T2) occurred 24 hr after a 3-min forced swim stress (FSS). **D.** Time spent on cocaine-paired side during IP, T1, Last extinction, and T2 in 1% (white, n=9/4) and 4% (black, n=11/5) NaCl fed adult offspring. **E.** Locomotor behavior during the 3-min FSS for 1% (white) and 4% (black) fed offspring expressed as time mobile and immobile. Values represent the mean $\pm$ SEM. *p<0.05 indicates group difference. #p<0.05 indicates that both groups displayed CPP for cocaine, as measured by a significant increase in time spent in the cocaine-paired chamber from IP to T1.

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Figure 6. Sucrose Preference Test.

A. Experimental Timeline. Animals were acclimated to having 2 bottles in their cage for 24 hr. During the next two days, they were given *ad libitum* access to two bottles, one containing 2% sucrose dissolved in H₂O, one containing tap water. Consumption of both solutions was measured. **B.** Consumption of 2% sucrose solution (left) and water (right) for 1% (white, n=4/4, where first and second numbers indicate the number of animals and dams, respectively) and 4% (black, n=4/4) NaCl fed offspring.

Table 1.

Macronutrient composition of the 1% and 4% NaCl diet fed to the dams.

	1% NaCl	4% NaCl
Protein (kcal%)	29	29
Carbohydrate (kcal%)	58	58
Fat (kcal%)	14	14
Sodium Chloride (%)	1.02	4
kcal/gm	3.89	3.78

Table 2.

Spine quantification in the PL-PFC, IL-PFC and NAc at PND21. Bold numbers indicate difference between 1% and 4% salt fed offspring.

A Prelimbic PFC					
	Basal			Apical	
	1% NaCl	4% NaCl		1% NaCl	4% NaCl
Filopodium	0.29 ± 0.04	0.29 ± 0.04	Filopodium	0.29 ± 0.08	0.33 ± 0.04
Thin	7.92 ± 0.04	7.38 ± 0.22	Thin	7.88 ± 0.19	7.17 ± 0.11
Mushroom	2.38 ± 0.14	2.25 ± 0.14	Mushroom	2.25 ± 0.14	2.46 ± 0.18
Stubby	1.29 ± 0.17	1.25 ± 0.07	Stubby	1.33 ± 0.04	1.25 ± 0.07
Branched	0.21 ± 0.04	0.17 ± 0.04	Branched	0.17 ± 0.04	0.21 ± 0.04
Total	12.08 ± 0.11	11.33 ± 0.34	Total	11.92 ± 0.15	11.42 ± 0.15

B Infralimbic PFC					
	Basal			Apical	
	1% NaCl	4% NaCl		1% NaCl	4% NaCl
Filopodium	0.29 ± 0.04	0.29 ± 0.04	Filopodium	0.25 ± 0.07	0.29 ± 0.04
Thin	7.69 ± 0.11	8.63 ± 0.19	Thin	7.63 ± 0.26	8.67 ± 0.23
Mushroom	2.25 ± 0.07	2.38 ± 0.22	Mushroom	2.33 ± 0.11	2.50 ± 0.13
Stubby	1.38 ± 0.19	1.25 ± 0.14	Stubby	1.38 ± 0.13	1.29 ± 0.11
Branched	0.17 ± 0.04	0.17 ± 0.04	Branched	0.21 ± 0.04	0.17 ± 0.04
Total	11.79 ± 0.30	12.71 ± 0.18	Total	11.79 ± 0.36	12.92 ± 0.25

C Nucleus Accumbens					
	Shell			Core	
	1% NaCl	4% NaCl		1% NaCl	4% NaCl
Filopodium	0.29 ± 0.04	0.33 ± 0.04	Filopodium	0.29 ± 0.04	0.29 ± 0.04
Thin	9.75 ± 0.33	9.79 ± 0.11	Thin	9.83 ± 0.25	9.96 ± 0.42
Mushroom	2.46 ± 0.08	3.21 ± 0.15	Mushroom	2.33 ± 0.11	2.25 ± 0.07
Stubby	1.63 ± 0.07	1.67 ± 0.11	Stubby	1.46 ± 0.04	1.54 ± 0.11
Branched	0.29 ± 0.04	0.17 ± 0.04	Branched	0.21 ± 0.08	0.17 ± 0.04
Total	14.42 ± 0.27	15.17 ± 0.17	Total	14.13 ± 0.19	14.21 ± 0.55

Table 3.

Spine quantification in the PL-PFC, IL-PFC and NAc at PND60. Bold numbers indicate difference between 1% and 4% salt fed offspring.

A Prelimbic PFC					
	Basal			Apical	
	1% NaCl	4% NaCl		1% NaCl	4% NaCl
Filopodium	0.21 ± 0.08	0.24 ± 0.01	Filopodium	0.15 ± 0.03	0.19 ± 0.04
Thin	7.54 ± 0.18	7.40 ± 0.14	Thin	7.58 ± 0.23	7.50 ± 0.13
Mushroom	2.67 ± 0.11	2.76 ± 0.08	Mushroom	2.61 ± 0.07	2.83 ± 0.23
Stubby	1.67 ± 0.22	1.69 ± 0.18	Stubby	1.75 ± 0.07	1.65 ± 0.07
Branched	0.14 ± 0.01	0.19 ± 0.04	Branched	0.25 ± 0.07	0.18 ± 0.04
Total	12.21 ± 0.08	12.58 ± 0.54	Total	12.33 ± 0.14	12.29 ± 0.22

B Infralimbic PFC					
	Basal			Apical	
	1% NaCl	4% NaCl		1% NaCl	4% NaCl
Filopodium	0.25 ± 0.02	0.25 ± 0.02	Filopodium	0.25 ± 0.02	0.22 ± 0.05
Thin	7.45 ± 0.33	8.40 ± 0.23	Thin	7.46 ± 0.10	8.53 ± 0.27
Mushroom	2.59 ± 0.14	2.57 ± 0.04	Mushroom	2.73 ± 0.07	2.60 ± 0.09
Stubby	1.71 ± 0.07	1.68 ± 0.10	Stubby	1.79 ± 0.11	1.75 ± 0.20
Branched	0.19 ± 0.03	0.15 ± 0.01	Branched	0.15 ± 0.01	0.25 ± 0.02
Total	12.04 ± 0.25	13.11 ± 0.32	Total	12.32 ± 0.13	13.51 ± 0.24

C Nucleus Accumbens					
	Shell			Core	
	1% NaCl	4% NaCl		1% NaCl	4% NaCl
Filopodium	0.18 ± 0.04	0.17 ± 0.04	Filopodium	0.13 ± 0.01	0.14 ± 0.07
Thin	9.46 ± 0.44	9.60 ± 0.36	Thin	9.49 ± 0.07	9.54 ± 0.36
Mushroom	2.56 ± 0.04	3.14 ± 0.07	Mushroom	2.68 ± 0.06	2.63 ± 0.07
Stubby	1.56 ± 0.04	1.67 ± 0.22	Stubby	1.75 ± 0.14	1.79 ± 0.04
Branched	0.25 ± 0.14	0.18 ± 0.04	Branched	0.24 ± 0.01	0.19 ± 0.04
Total	13.92 ± 0.29	15.13 ± 0.83	Total	14.38 ± 0.14	14.46 ± 0.48