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Research article

After a period of forced abstinence, rats treated with the norepinephrine neurotoxin DSP-4 still exhibit preserved food-seeking behavior and prefrontal cortex fos-expressing neurons

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ABSTRACT

Aims: Relapse is a common characteristic of compulsive behaviors like addiction, where individuals tend to return to drug use or overeating after a period of abstinence. PFC (prefrontal cortex) neuronal ensembles are required for drug and food-seeking behaviors and are partially regulated by Norepinephrine (NE). However, the contributions of neuromodulators, such as the adrenergic system, in food-seeking behavior are not fully understood.
Main methods: To investigate this, we trained male and female rats to press a lever in an operant chamber to obtain banana-flavored food pellets for ten days. We then administered DSP-4 (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride), a neurotoxin that diminishes norepinephrine levels in the brain. The rats were kept in their home cages for ten more days before being returned to the operant chambers to measure food-seeking behavior.
Key findings: Despite receiving DSP-4, the PFC neuronal ensembles measured by Fos and food-seeking behavior did not differ between groups, but rather sex.
Significance: Although both NE and Fos expressing neurons are implicated in food-seeking, they do not seem to be involved in a cue-contextual induced re-exposure response.

1. Introduction

The recurrence of drug-seeking is a common characteristic of addiction, which can occur following a period of voluntary or forced abstinence [1,2]. This phenomenon has been observed in animal models of addiction, where stimuli present during initial substance use (e.g., cues) can trigger drug-seeking behavior even after a period of abstinence [3], for review see [4]. Furthermore, responding to palatable food such as sugar follows a similar behavioral trajectory where responses are initially low but begin to increase over days. After forced abstinence, responses to obtain palatable food can be reinstated by reintroducing the stimuli available during food conditioning [5–7].

Cell assemblies [8], now commonly referred to as 'neuronal ensembles,' are groups of coactivated neurons sparsely distributed

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throughout the mammalian brain that are believed to be critical for cues predicting drug or food taking and seeking [9–11]. One way to identify these neuronal ensembles is by their expression of Fos, a protein product of the immediate-early gene Fos; Fos is found in highly active neurons [12] and has been studied by several researchers within the medial prefrontal cortex (mPFC) in relation to different phases of food-seeking behavior [9,13–15]. For example, exposure to cues predicting food delivery induces Fos mRNA in multiple cortical and subcortical regions [16]. Finally, chemogenic disruption of Fos-expressing neurons in the mPFC that predicted food delivery attenuated previously established food-seeking behavior [13].

The locus coeruleus (LC) is the primary source of Norepinephrine (NE) innervating the Prefrontal Cortex (PFC). Several studies have established a strong connection between the LC and PFC [17–20]. When the LC is electrically stimulated, the firing rate of PFC neurons increases [20], and lesions of the LC lead to an augmented firing rate of PFC neurons [19]. Tracing studies have also demonstrated direct afferent inputs from the LC to the PFC, and feedback projections from the PFC to the LC [18].

Manipulation of the NE system has resulted in changes to drug and food-seeking behavior. For instance, the non-selective β-antagonist propranolol retards the escalation of cocaine intake across days [21], and the selective depletion of mPFC NE results in diminished conditioned place preference (CPP) for amphetamine [22]. Additionally, norepinephrine-uptake inhibitors desmethyl imipramine (DMI) and thionisoxetine (TNIX) can suppress the intake of palatable food [23]. Even a human Positron Emission Tomography (PET) of the insular cortex has revealed a negative correlation between the NE transporter and hunger levels [24]. Combined evidence from preclinical and human studies suggests a critical involvement of NE in reward-seeking behaviors. However, the role of NE in Fos ensembles and food reward seeking has not been fully explored.

We *hypothesized* that disruption of the LC-NE system would *decrease* Fos expressing neurons in the PFC and food-seeking behavior after forced abstinence. To do this, we pharmacologically ablated LC-NE using the selective neurotoxin for the LC (N-(2-chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride) or DSP-4 and measured, Fos and DβH (dopamine-β-hydroxylase) protein levels and food-seeking behavior after forced abstinence. When injected systemically, the compound DSP-4 undergoes a chemical transformation, forming a reactive aziridinium derivative. It accumulates in the NE terminals and inhibits the noradrenaline transporter [25], resulting in reduced NE levels in multiple brain regions [26]. This reduction can manifest itself in a decrease in dopamine-β-hydroxylase [DβH] as measured by immunofluorescence [27–29]. Our results showed that despite DSP-4 injections, food-seeking and Fos-expressing neurons in the PFC were preserved.

2. Materials & methods

2.1. Subjects

Male and female Wistar rats [weighing ~250 g at the beginning of the study] (8 saline, 8 DSP-4, N = 16) were bred in-house and maintained on a 12/12 light on-off cycle. All experiments were conducted during the light phase. Food and water were available ad libitum. The Mercer University Animal Care and Use Committee approved all experiments.

2.2. Procedure

Behavioral procedures: For ten days, all rats were trained to lever press on a fixed ratio schedule of reinforcement (FR1) for banana-flavored sugar pellets (see Fig. 1)*;* each response resulted in the delivery of a single banana-flavored food pellet, lever pressing during the ITI (which was 20s) did not have any programmable consequences. The chambers *(Med Associates, VT)* maintained a house light and a discrete light cue above the active lever, *indicating* reward availability. Sessions lasted for 1 h each day for 10 days. On day 10, after food training, all rats received a single injection of either saline (0.9 %) or DSP-4 (50 mg/kg/ml/i.p.) [Sigma (CAS No 40416-75-9)]. Rats were counterbalanced and divided into the DSP-4 and saline groups based on their last day of lever pressing. Rats that exhibited high and low levels of lever-pressing were evenly allocated to saline and DSP-4 groups. Rats were returned to their home cages for 10 days to allow for DSP-4 incubation and NE depletion. On test day, the rats were placed in the operant chambers, with access to both levers (light cue still present). No reward or consequence was implemented for either lever. Ninety minutes following the start of the re-exposure session, the rats were transcardially perfused with 4 % paraformaldehyde, and their brains were frozen to −20

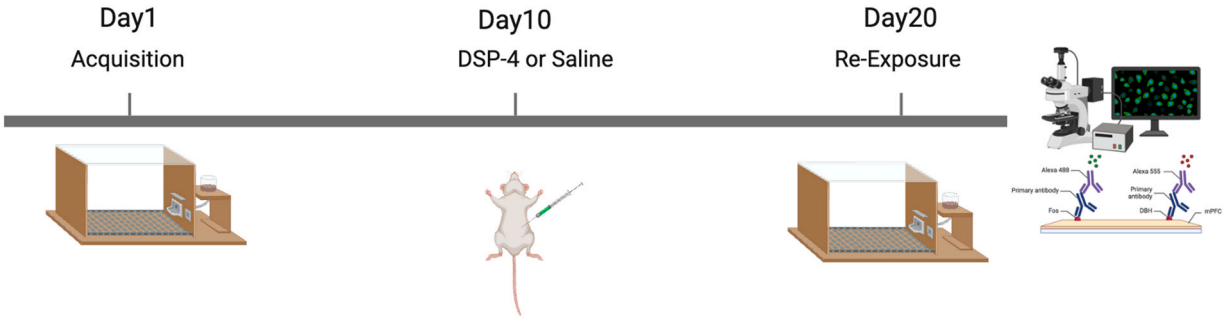

Fig. 1. Experimental timeline.

°C for cryosectioning and immunofluorescence analysis.

Lavage: Cytological assessment of the female rat estrus cycle was performed as described previously [30]. Female rats were lavaged during the last five days of acquisition. A small amount of sterile saline (~200 μl) was inserted at the entrance of the vaginal canal and collected again in the glass dropper. Immediately after, the smear was placed on a microscope slide, and was imaged with a light microscope for cytological and staging profiles. The males received sham lavage.

Cryosectioning and Immunofluorescence: 30 μm coronal sectioning of the PFC at stereotaxic coordinate 3.20 from bregma [31] was used for free-floating immunofluorescence of Fos and Dopamine β Hydroxylase DβH [Sigma (CAS No MAB308)]. The sections were washed with 1X PBS (Phosphate-buffered saline), blocked with 0.2 % Triton X [Sigma (CAS No 9002-93-1)], 5 % BSA (Bovine serum albumin) in 1X PBS, and then incubated with primary antibodies overnight at 4 °C [Fos: Cell Signaling, DβH: Sigma]. The next day, after washing the sections with 1X PBS, secondary antibodies Alexa 488 for Fos [Abcam (CAS No Ab150077) and Alexa 555 for DβH [Abcam (CAS No Ab150170) were applied at room temperature for 3 h. The sections were then washed with PBS +0.3 % tween and placed on slides for microscopy. The cells were imaged with a Zeiss Epifluorescence microscope aided by an Apotome at 20X magnification; each image's exposure time was constant. The right and left hemispheres of the cingulate (Cg), prelimbic (PrL), and infralimbic (IL) cortex were imaged, and the hemispheres for each area were averaged.

2.3. Data analysis

Behavioral and Immunofluorescent data were analyzed separately. Acquisition data from sessions 1–10 was analyzed using a mixed model ANOVA, and lever press-type (Active vs. Inactive) as a factor. Since sphericity was violated in this data set, the Geiser-Greenhouse correction was used. Re-exposure data was analyzed using a two-way ANOVA and independent sample T-test. DβH and Fos data were analyzed with a 3-factor-MANOVA with brain regions (Cg, IL, PrL), Group (Saline vs. DSP-4) and Sex (M, F) serving as factors. Notable interactions were followed up with Bonferroni post hoc.

Fos and DβH cells were quantified by two experimenters blind to the conditions (see representative images). The number of Fos-positive cells was counted manually, while DβH was quantified by selecting 'total red' areas using FIJI ImageJ. Pearson's correlations were used to compare total session lever pressing with total Fos per PFC subregion, delineating the relationship between lever presses during reinstatement and Fos activation. Simple linear regression was used to correlate Fos counts with the number of lever presses on re-exposure day. Due to procedural issues leading to poor image quality or missing data points, results from two males and one female were omitted. We used SPSS v29 and Prism 10 to analyze and graph all data; the significance threshold was set at $p \leqq 0.05$.

3. Results

Behavioral Results: Acquisition session results varied [main effect of session: $F(2.10, 54.59) = 12.64$, $p < 0.05$]. During the sessions, rats preferred the active lever [main effect of lever: $(1, 26) = 33.4$, $p < 0.05$], and the number of active lever presses increased [lever × session interaction $F(9, 234) = 12.46$, $p < 0.05$]. Post-hoc comparisons showed that active lever pressing had increased as early as day five ($p < 0.05$, Fig. 2).

On the re-exposure day (Fig. 2), After ten days of forced abstinence in the home cage, lever pressing was still greater for the active than the inactive lever [main effect of Lever Type $F(1,16) = 19.87$, $p < 0.001$]. On the re-exposure day, both the Saline and DSP-4

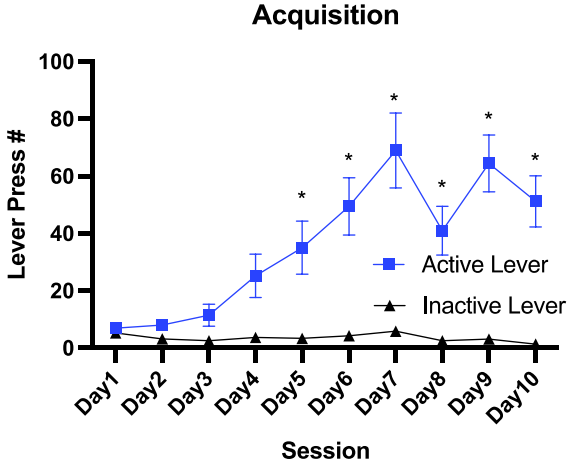

Fig. 2. Banana-flavored sugar pellets are reinforcing. Before receiving DSP-4 or saline, all rats were provided with an opportunity to lever press for banana-flavored sugar pellets for 10 days. There was a main effect of Lever-type $F(1, 26) = 33.4$, $p < 0.05$, session $F(2.1, 54.59) = 12.6$, $p < 0.05$ and Lever-type X Session interaction $F(9,234) = 12.6$, $p < 0.05$. Post-hocs showed that by day 5, lever pressing for the active lever was greater than the inactive one. * = $p < 0.05$ *vs. inactive lever*.

groups were pressing the active lever more than the inactive ($p < 0.01$ for both groups) (Fig. 3). Females also had higher total lever presses [main effect of Sex F(1,16) = 7.19, $p < 0.05$], and active lever pressed more than males [Lever Type × Sex interaction, F(1, 16) = 6.56, $p < 0.05$]. Lever pressing during the first 15 min also did not differ between DSP-4 and saline-treated rats (Fig. 4). Lavage Data: By the end of food-seeking training, most female rats were in the non-estrus phase (data not shown).

Immunofluorescent results: DβH immunofluorescence showed that rats injected with DSP-4 had lower DβH levels in the prefrontal cortex (IL, Prl and Cg) [main effect of Group F(1,27) = 9.7, $p < 0.05$.] and females tended to have lower DβH [main effect of Sex F(1,27 = 13.99, $p < 0.01$]. The males in the saline group had higher DβH as well [GroupXSex Interaction: F(1, 27) = 7.550, $p < 0.05$] (Fig. 5 Left and Right). Representative images of Immunofluorescence for Fos and DβH is shown in Fig. 8.

The number of Fos-positive cells in the PFC also did not differ between the saline and DSP-4 groups (Fig. 6 left). However, when collapsed across groups, females had higher Fos-positive counts in the PFC [Main effect of Sex, F(1,27) = 8.77, $p < 0.01$] [Fig. 6 right] overall and in particular, males in the saline group had higher Fos numbers [SexXGroup Interaction: F(1,27) = 7.69, $p < 0.05$]. When we assessed the correlation between the number of Fos-positive cells and the number of lever-pressing during the entire reinstatement, we observed a positive correlation in the number of lever presses and Fos number for the females, and a negative correlation in males. However, both of these effects only approached significance ($p = 0.057$ in males, and $p = 0.06$ in females) [Fig. 7].

4. Discussion

4.1. NE and lever pressing

Here, we tested the hypothesis that NE in the PFC regulates Fos-expressing neurons associated with palatable food-seeking behavior following forced abstinence. Our hypothesis, however, was not confirmed. We found that rats injected with DSP-4 or saline exhibited similar lever-pressing responses after forced abstinence in the home cage. This was observed for the first 15 min and throughout the re-exposure session. Similarly to the behavioral data, the number of Fos-positive cells in the PFC did not differ between the DSP-4 and the saline groups.

It is worth mentioning that lever pressing increased over consecutive days of palatable food seeking, consistent with previous work [13,15,32] and the rats preferred the active lever even after ten days of forced abstinence, which is consistent with previous research [13,15,32]. Reports on the role of NE in food reinstatement to palatable food-seeking behavior are infrequent [33,34]. However, several findings indicate the involvement of NE in multiple drug relapse paradigms [35–37]. For example, the α-2 receptor agonists clonidine and lofexidine block stress induced reinstatement to cocaine [35], while yohimbine [an α-2 receptor antagonist] enhances priming induced reinstatement [36]. The selective β hydroxylase inhibitor nepicastat can also block cue-induced reinstatement [37]. The potential for these pharmacological interventions to influence the reinstatement of food-seeking behavior following a period of abstinence remains an open question that needs to be explored.

Our assessment was confined to only rats tested on cue/contextual re-exposure. NE could modulate stress and/or priming-induced reinstatement to palatable food seeking. It is important to note that despite the different uses of the reinforcer (sugar in our study vs. cocaine in other studies), the above studies all employed an extinction period before reinstatement testing. The absence of an extinction phase in our study may have diminished the role of norepinephrine (NE) in the resurgence of food (or drug) seeking behaviors. This could explain the lack of observable effects in our investigation. However, there are advantages and disadvantages to

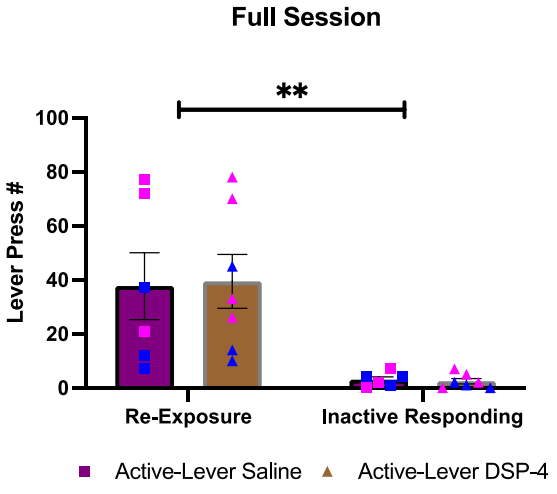

Fig. 3. Rats remember the active lever after 7 days in the home cage. When exposed to the self-administration chamber after forced abstinence, rats pressed the active lever more than the inactive one [main effect of Lever Type F(1,16) = 19.87, $p < 0.001$], active lever responses did not differ between the Saline and DSP-4 groups however. Additionally, we observed a higher number of lever presses in females [main effect of Sex F(1,16) = 7.19, $p < 0.05$], and females pressed active lever more than males [Lever Type × Sex interaction, F(1, 16) = 6.56, $p < 0.05$].

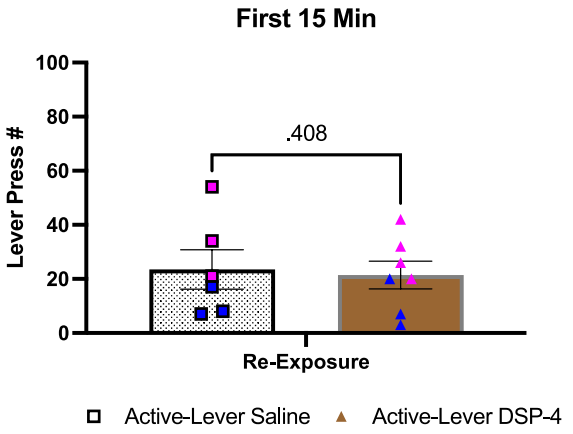

Fig. 4. Comparable levels of lever pressing in all rats re-exposed to the self-administration chamber (First 15 min).

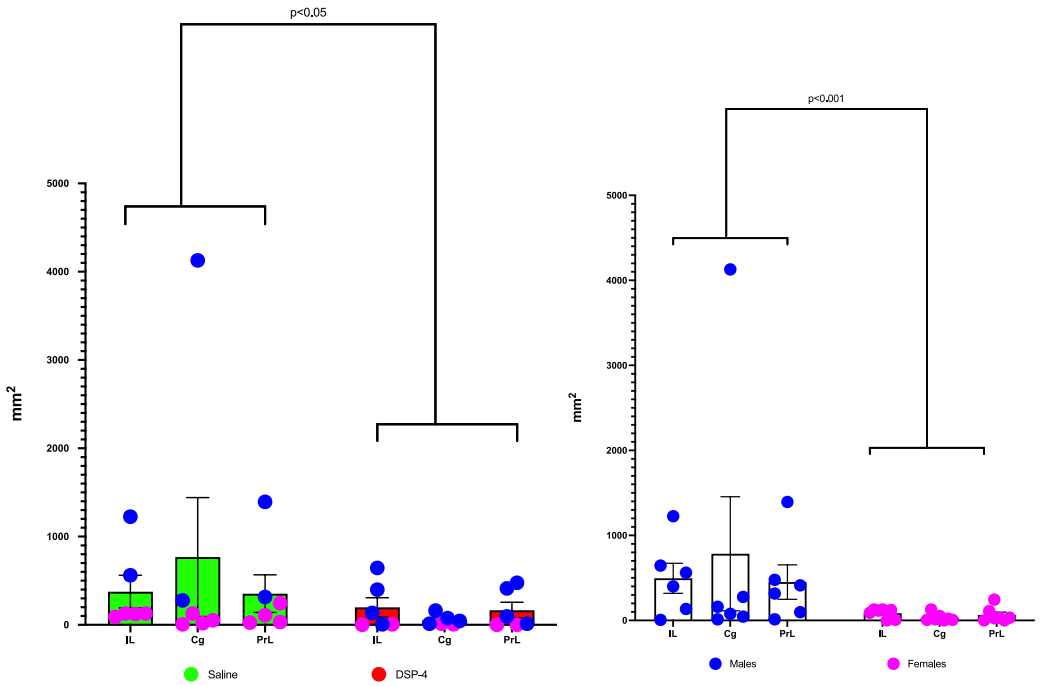

Fig. 5. DSP-4-treated rats and females have lower DβH: The area quantified for DβH by Immunofluorescence showed a reduction in rats given DSP-4 [Main effect of group: $F(1,27) = 9.7$, $p < 0.05$], and females tended to have lower DβH in the PFC [Main effect of Sex $F(1, 27) = 13.99$, $p < 0.01$. The males in the Saline group had higher DβH as well [Group × Sex Interaction: $F(1, 27) = 7.55$, $p < 0.05$].

using voluntary and involuntary abstinence paradigms [4]. Like using an extinction period, our method covers more scenarios where an individual cannot obtain the reward (for example, due to incarceration). However, situations where responses could give access to the reward or an alternative option (such as interaction with another rat) could be used to validate the effectiveness of natural reinforcers like sugar and further examine Fos expressing neurons. In future studies, it is recommended to use an extinction period and other relapse models, such as priming or stress-induced reinstatement, after providing access to palatable food while disrupting norepinephrine (NE) activity. Moreover, future research should broaden beyond the conventional relapse models, such as cue, stress, and priming-induced reinstatement, and encompass modifications to the re-exposure environment. The aim would be to increase the challenge for animals in recalling the context/cues of the self-administration box. Changes in cognitive demand have been reported to require NE [38], and our previous [26,39] work with odor conditioning [39] and spatial memory [26] corroborte this effect. We are investigating this phenomenon in the PFC with direct pharmacological manipulations of NE.

Several investigators have reported an incubation of craving effect with food [32] and drugs [40]. Incubation of craving is defined as a time-dependent increase in cue-induced responses to stimuli previously associated with reward. We did not observe such an effect

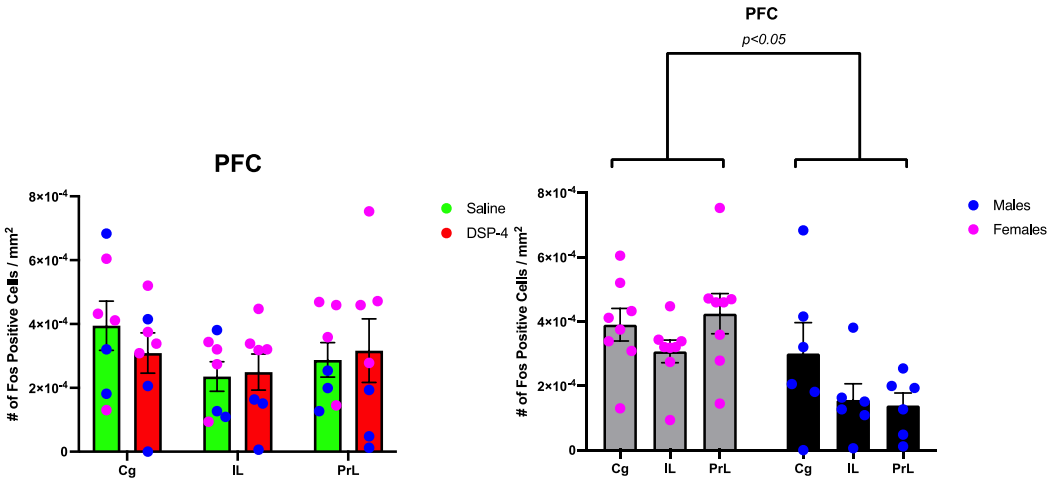

Fig. 6. Females have higher Fos in the prefrontal cortex [Main effect of Sex F(1, 27) = 8.77, $p < 0.01$] and a Sex × Group Interaction F(1,27) = 7.69, $p < 0.05$.

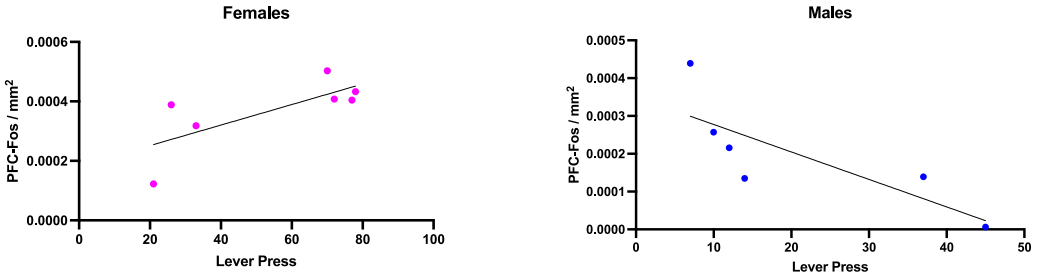

Fig. 7. PFC Fos Correlation with Lever-pressing on the re-exposure day. Males ($F([1,4] = [6.97]$, $p = [0.057]$. Females ($F([1,5] = [5.86]$, $p = [0.06]$.

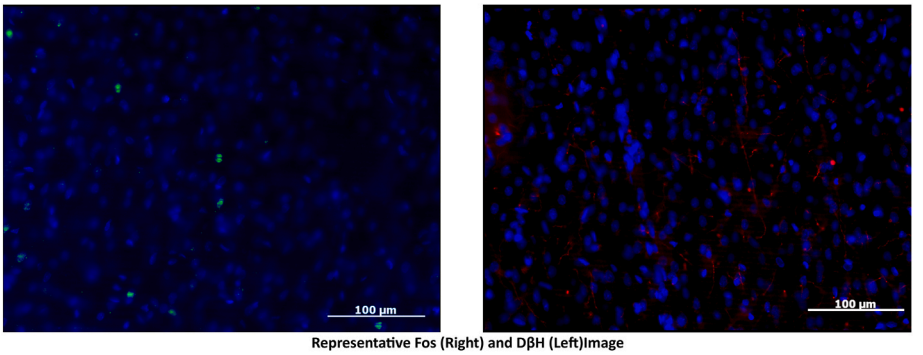

Fig. 8. Representative images of Fos and DβH. Green stained cells are Fos and Red stained ones are DβH. Blue cells are DAPI.

in our study as the number of lever presses during re-exposure had actually dropped. Two reasons may explain this: 1, We did not wait long enough to see an incubation effect. The sucrose incubation effect has been documented at 30 days or more after abstinence [41], and 2, our lever pressing during acquisition has been lower; compared to previous reports [9,32]. As an added complication, studies that normally examine an incubation of craving effect test the animals 1 day after the last day of training [42,43]. Without this 24-h test, we can only make our re-exposure day comparison to the last day of self-administration.

4.2. DSP-4 and DβH in the PFC

Despite having no effect on the number of lever presses on re-exposure day, DβH in the PFC were shown to be decreased, an effect that was predominately driven by females. The LC has been shown to be a very sexually dimorphic brain nucleus [44–46]. The female LC has more synaptic contacts, longer dendrites [44], and a larger soma [47]. The reduction in DβH levels in this study are consistent with our previous work with the same dosage and timeline showed decreases in the PFC [26]. Nevertheless, previous work has shown that the use of 50/mg/kg/ml/i.p of DSP-4 rapidly decreases NE but has a more delayed effect on DβH [25].

Despite the lack of a behavioral effect, we are confident that our injection of DSP-4 effectively attenuated NE in the PFC, as our previous work with the same dosage and timeline showed decreases in the PFC [26]. Another possibility is that a higher dose of DSP-4 or a longer waiting period may be required for DβH levels to decrease. This is because 50/mg/kg/ml/i.p of DSP-4 rapidly decreases NE but has a slower effect on DβH. Since our work was not sufficiently powered to perform group X sex comparisons, future studies should use a higher dose, multiple injections of DSP-4, and larger sample sizes. To our knowledge, DβH levels in the PFC have not been compared between sexes, and our results may be the first to show an effect. Although the PFC receives a large amount of input from the brain stem, anatomical innervation of the PFC by the LC is not a homogeneous process [48]; for instance, fiber volume is denser in the more ventral areas [48]. However, we did not observe any differences in DβH staining of the cingulate, prelimbic, or infralimbic regions.

4.3. PFC and fos expressing neurons

Several investigators have shown the upregulation of Fos-expressing neurons in the PFC as a result of food reinforcement learning [9,13,49]. Our experiment did not possess a non-trained group of animals; therefore, we cannot conclude if re-exposure to self-administration chambers increased Fos number in the PFC, as our design was not aiming to answer this question. Nevertheless, our results found no difference between the saline and DSP-4 groups in Fos-positive cell numbers within tested areas. Another open question is: what psychological processes are encoded in the Fos cells when confronted with the self-administration environment? Computation in this brain region could result from attention, the memory that the environment predicts reward availability, the environment now being imbued by incentive salience or a combination of all three. Single-neuron activity in the PFC has been shown to code for all three phenomena [50,51]. Future work should focus on what psychological process or a combination of processes the Fos expressing neurons within particular PFC sub-regions are performing.

The number of cells positive in the PFC of our study matches that of previous work (Quintana-Feliciano, 2021) in that less than 10 % of cells in the PFC are active during recall of a food-associated area. However, we did not find differences in the number of Fos in the three subregions (Cingulate, Prelimbic, and Infralimbic). Studies have shown that these regions may participate in different phases of reinforcement learning. For example, regional inactivation studies have shown PFC subregional specificity in food-seeking behavior [52,53]. Pharmacological inhibition of the infralimbic cortex reinstates food seeking (Peters & De Vries, 2013), while inhibition of the prelimbic cortex decreases reinstatement (Calu et al., 2013). Other studies have found that Fos-expressing neurons mediating extinction and reward are intermingled in the infralimbic cortex (Warren et al., 2016). Although significant Fos differences were observed for sex, the correlational design used here does not exclude their involvement in palatable food re-exposure responses. Therefore, it cannot be assumed that since Fos levels were not different between the DSP-4 and saline group, this excludes the *requirement* of Fos and *LC-NE* in the PFC. In future studies, experiments should be designed where the LC and PFC neuronal expressing neurons are ablated in the same animal. A surprising finding of the study was the trending negative correlation between the number of Fos and the frequency of lever pressing in males. The administration of stimulants [54], locomotor activity [55], and food-seeking [56] all increase Fos activity, which was indeed found in females in our study. Therefore, it is possible that Fos-expressing neurons in males may represent extinction learning during the re-exposure day. There is evidence for this notion as functional studies have revealed distinct Fos expressing neurons dedicated to conditioned learning and extinction [49]. Future work can test this possibility using correlational methods where ensembles for two distinct episodes of experience can be captured in the same animal [57].

4.4. Sex differences in food reinforcement

There are significant sex differences in humans' consumption of palatable foods. Women rate low-calorie food images higher (Legget et al., 2023) and report liking unhealthy foods less than men [58]. Images of food increase activation of the lateral dorsolateral prefrontal cortex in women more than in men [59]. Additionally, intranasal insulin increases PFC activity in both women and in men [60]. Differences in responding to palatable foods have also been reported in rodent studies. Intact females self-administer more palatable foods than males or ovariectomized females [61]. Females show greater reinstatement to palatable food-seeking, and in a food renewal test [62], the number of Fos-positive cells in the Prelimbic cortex decreases for females and increases for males [63]. We tracked the female estrus cycle in the acquisition phase of this study. By the last acquisition day, most female rats were in non-estrus, and the lever-pressing change was unrelated to the estrus cycle. Our study lacks the statistical power to draw any definitive conclusions regarding Sex by Group interactions; however, future work should benefit from using both sexes and higher numbers to provide sufficient power for analysis.

5. Conclusion

In summary, although we did not observe a DSP-4 effect on lever pressing after forced abstinence in the same self-administration

context, we did observe several sex differences in both Fos-positive counts and NE DβH levels. Further exploration of these differences, along with other models of relapse to rewards involving drugs or natural stimuli, will undoubtedly aid in therapeutic options for disorders of consumption.

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CRediT authorship contribution statement

L.N. Callan: Writing – review & editing, Project administration, Methodology, Investigation, Data curation, Conceptualization. **A. J. Caroland-Williams:** Writing – review & editing, Supervision, Methodology, Data curation. **G. Lee:** Writing – review & editing, Methodology. **J.M. Belflower:** Methodology. **J.T. Belflower:** Methodology. **U.A. Modi:** Writing – review & editing, Methodology. **C. V. Kase:** Software, Resources. **A.D. Patel:** Methodology. **N.A. Collins:** Methodology. **A. Datta:** Methodology. **S. Qasi:** Methodology. **A. Gheidi:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest. The animal portion of this manuscript was approved under protocol #. A220814.

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References

[1] NIDA, Treatment and Recovery, 2023.
[2] U.E. Ghitza, et al., The anxiogenic drug yohimbine reinstates palatable food seeking in a rat relapse model: a role of CRF1 receptors, Neuropsychopharmacology 31 (10) (2006) 2188–2196.
[3] A. Markou, et al., Cue-induced nicotine-seeking behavior after withdrawal with or without extinction in rats, Addiction Biol. 23 (1) (2018) 111–119.
[4] I. Fredriksson, et al., Animal models of drug relapse and craving after voluntary abstinence: a review, Pharmacol. Rev. 73 (3) (2021) 1050–1083.
[5] K.T. Ball, et al., Dopamine D(1)-like receptors in prelimbic, but not infralimbic, medial prefrontal cortex contribute to chronic stress-induced increases in cue-induced relapse to palatable food seeking during forced abstinence, Behav. Brain Res. 417 (2022) 113583.
[6] S.Y. Khoo, K.J. Clemens, G.P. McNally, Palatable food self-administration and reinstatement are not affected by dual orexin receptor antagonism, Prog. Neuro-Psychopharmacol. Biol. Psychiatry 87 (Pt A) (2018) 147–157.
[7] Y.W. Chen, et al., Effect of yohimbine on reinstatement of operant responding in rats is dependent on cue contingency but not food reward history, Addiction Biol. 20 (4) (2015) 690–700.
[8] D. Hebb, The Organization of Behavior: A Neuropsychological Theory, John Wiley & Sons Inc, 1949.
[9] R. Quintana-Feliciano, et al., Food-seeking behavior is mediated by fos-expressing neuronal ensembles formed at first learning in rats, eNeuro 8 (2) (2021).
[10] L. Kane, et al., Fos-expressing neuronal ensemble in rat ventromedial prefrontal cortex encodes cocaine seeking but not food seeking in rats, Addiction Biol. 26 (3) (2021) e12943.
[11] B.L. Warren, et al., Separate vmPFC ensembles control cocaine self-administration versus extinction in rats, J. Neurosci. 39 (37) (2019) 7394–7407.
[12] E. Nikolaev, et al., c-fos protooncogene expression in rat hippocampus and entorhinal cortex following tetanic stimulation of the perforant path, Brain Res. 560 (1–2) (1991) 346–349.
[13] L.R. Whitaker, et al., Bidirectional modulation of intrinsic excitability in rat prelimbic cortex neuronal ensembles and non-ensembles after operant learning, J. Neurosci. 37 (36) (2017) 8845–8856.
[14] S. Cole, et al., Medial prefrontal cortex neural plasticity, orexin receptor 1 signaling, and connectivity with the lateral hypothalamus are necessary in cue-potentiated feeding, J. Neurosci. 40 (8) (2020) 1744–1755.
[15] L.S. Brebner, et al., Extinction of cue-evoked food-seeking recruits a GABAergic interneuron ensemble in the dorsal medial prefrontal cortex of mice, Eur. J. Neurosci. 52 (7) (2020) 3723–3737.
[16] C.A. Schiltz, et al., Food-associated cues alter forebrain functional connectivity as assessed with immediate early gene and proenkephalin expression, BMC Biol. 5 (2007) 16.
[17] M. Condes-Lara, Different direct pathways of locus coeruleus to medial prefrontal cortex and centrolateral thalamic nucleus: electrical stimulation effects on the evoked responses to nociceptive peripheral stimulation, Eur. J. Pain 2 (1) (1998) 15–23.
[18] Y. Lu, et al., Differential distribution patterns from medial prefrontal cortex and dorsal raphe to the locus coeruleus in rats, Anat. Rec. 295 (7) (2012) 1192–1201.
[19] A. Marzo, et al., Unilateral electrical stimulation of rat locus coeruleus elicits bilateral response of norepinephrine neurons and sustained activation of medial prefrontal cortex, J. Neurophysiol. 111 (12) (2014) 2570–2588.
[20] Y. Wang, et al., Noradrenergic lesion of the locus coeruleus increases the firing activity of the medial prefrontal cortex pyramidal neurons and the role of alpha2-adrenoceptors in normal and medial forebrain bundle lesioned rats, Brain Res. 1324 (2010) 64–74.
[21] H. Beldjoud, et al., Chronic administration of a norepinephrine antagonist prevents and partially reverses escalation of cocaine self-administration, Addiction Biol. 28 (9) (2023) e13316.
[22] R. Ventura, et al., Norepinephrine in the prefrontal cortex is critical for amphetamine-induced reward and mesoaccumbens dopamine release, J. Neurosci. 23 (5) (2003) 1879–1885.

[23] N.E. Rowland, M. Marshall, J.D. Roth, Comparison of either norepinephrine-uptake inhibitors or phentermine combined with serotonergic agents on food intake in rats, Psychopharmacology (Berl) 149 (1) (2000) 77–83.
[24] A. Bresch, et al., Hunger and disinhibition but not cognitive restraint are associated with central norepinephrine transporter availability, Appetite 117 (2017) 270–274.
[25] S.B. Ross, C. Stenfors, DSP4, a selective neurotoxin for the locus coeruleus noradrenergic system. A review of its mode of action, Neurotox. Res. 27 (1) (2015) 15–30.
[26] A. Gheidi, et al., Norepinephrine depletion in the brain sex-dependently modulates aspects of spatial learning and memory in female and male rats, Psychopharmacology 240 (2023) 2585–2595.
[27] J.M. Fritschy, R. Grzanna, Selective effects of DSP-4 on locus coeruleus axons: are there pharmacologically different types of noradrenergic axons in the central nervous system? Prog. Brain Res. 88 (1991) 257–268.
[28] S.B. Ross, Long-term effects of N-2-chlorethyl-N-ethyl-2-bromobenzylamine hydrochloride on noradrenergic neurones in the rat brain and heart, Br. J. Pharmacol. 58 (4) (1976) 521–527.
[29] T. Archer, G. Jonsson, S.B. Ross, A parametric study of the effects of the noradrenaline neurotoxin DSP4 on avoidance acquisition and noradrenaline neurones in the CNS of the rat, Br. J. Pharmacol. 82 (1) (1984) 249–257.
[30] A.C. McLean, et al., Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification, J. Vis. Exp. (67) (2012) e4389.
[31] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordiantes, Academic Press, 1998.
[32] R. Madangopal, et al., Incubation of palatable food craving is associated with brain-wide neuronal activation in mice, Proc. Natl. Acad. Sci. U. S. A. 119 (45) (2022) e2209382119.
[33] K.T. Schmidt, et al., Norepinephrine regulates cocaine-primed reinstatement via α1-adrenergic receptors in the medial prefrontal cortex, Neuropharmacology 119 (2017) 134–140.
[34] J.P. Schroeder, et al., The selective dopamine β-hydroxylase inhibitor nepicastat attenuates multiple aspects of cocaine-seeking behavior, Neuropsychopharmacology 38 (6) (2013) 1032–1038.
[35] S. Erb, et al., Alpha-2 adrenergic receptor agonists block stress-induced reinstatement of cocaine seeking, Neuropsychopharmacology 23 (2) (2000) 138–150.
[36] B. Lee, et al., Pharmacological blockade of alpha2-adrenoceptors induces reinstatement of cocaine-seeking behavior in squirrel monkeys, Neuropsychopharmacology 29 (4) (2004) 686–693.
[37] J.P. Schroeder, et al., The selective dopamine beta-hydroxylase inhibitor nepicastat attenuates multiple aspects of cocaine-seeking behavior, Neuropsychopharmacology 38 (6) (2013) 1032–1038.
[38] S.L. Grella, et al., Norepinephrine as a spatial memory reset signal, Behav. Pharmacol. 32 (7) (2021) 531–548.
[39] A.M. Shakhawat, et al., Arc-expressing neuronal ensembles supporting pattern separation require adrenergic activity in anterior piriform cortex: an exploration of neural constraints on learning, J. Neurosci. 35 (41) (2015) 14070–14075.
[40] M. Venniro, et al., Factors modulating the incubation of drug and non-drug craving and their clinical implications, Neurosci. Biobehav. Rev. 131 (2021) 847–864.
[41] J.H. Harkness, S. Webb, J.W. Grimm, Abstinence-dependent transfer of lithium chloride-induced sucrose aversion to a sucrose-paired cue in rats, Psychopharmacology (Berl) 208 (4) (2010) 521–530.
[42] D.S. Counotte, et al., Time-dependent decreases in nucleus accumbens AMPA/NMDA ratio and incubation of sucrose craving in adolescent and adult rats, Psychopharmacology (Berl) 231 (8) (2014) 1675–1684.
[43] A.L. Nugent, et al., Incubation of cue-induced reinstatement of cocaine, but not sucrose, seeking in C57BL/6J mice, Pharmacol. Biochem. Behav. 159 (2017) 12–17.
[44] D.A. Bangasser, et al., Sexual dimorphism in locus coeruleus dendritic morphology: a structural basis for sex differences in emotional arousal, Physiol. Behav. 103 (3–4) (2011) 342–351.
[45] H. Pinos, P. Collado, M. Rodriguez-Zafra, C. Rodriguez, S. Segovia, A. Guillamón, The development of sex differences in the locus coeruleus of the rat, Brain Res. Bull. 56 (1) (2001) 73–78.
[46] B. Mulvey, et al., Molecular and functional sex differences of noradrenergic neurons in the mouse locus coeruleus, Cell Rep. 23 (8) (2018) 2225–2235.
[47] H. Pinos, et al., The development of sex differences in the locus coeruleus of the rat, Brain Res. Bull. 56 (1) (2001) 73–78.
[48] J.C. Cerpa, A.R. Marchand, E. Coutureau, Distinct regional patterns in noradrenergic innervation of the rat prefrontal cortex, J. Chem. Neuroanat. 96 (2019) 102–109.
[49] B.L. Warren, et al., Distinct fos-expressing neuronal ensembles in the ventromedial prefrontal cortex mediate food reward and extinction memories, J. Neurosci. 36 (25) (2016) 6691–6703.
[50] L. Duong, et al., A normalization circuit underlying coding of spatial attention in primate lateral prefrontal cortex, eNeuro 6 (2) (2019).
[51] S.L. Cowen, B.L. McNaughton, Selective delay activity in the medial prefrontal cortex of the rat: contribution of sensorimotor information and contingency, J. Neurophysiol. 98 (1) (2007) 303–316.
[52] D.J. Calu, et al., Optogenetic inhibition of dorsal medial prefrontal cortex attenuates stress-induced reinstatement of palatable food seeking in female rats, J. Neurosci. 33 (1) (2013) 214–226.
[53] J. Peters, T.J. De Vries, D-cycloserine administered directly to infralimbic medial prefrontal cortex enhances extinction memory in sucrose-seeking animals, Neuroscience 230 (2013) 24–30.
[54] A.M. Graybiel, R. Moratalla, H.A. Robertson, Amphetamine and cocaine induce drug-specific activation of the c-fos gene in striosome-matrix compartments and limbic subdivisions of the striatum, Proc. Natl. Acad. Sci. U. S. A. 87 (17) (1990) 6912–6916.
[55] L. Albrechet-Souza, K.G. Borelli, M.L. Brandão, Activity of the medial prefrontal cortex and amygdala underlies one-trial tolerance of rats in the elevated plus-maze, J. Neurosci. Methods 169 (1) (2008) 109–118.
[56] S.G. Nair, et al., Role of dorsal medial prefrontal cortex dopamine D1-family receptors in relapse to high-fat food seeking induced by the anxiogenic drug yohimbine, Neuropsychopharmacology 36 (2) (2011) 497–510.
[57] J.F. Guzowski, P.F. Worley, Cellular compartment analysis of temporal activity by fluorescence in situ hybridization (catFISH), Curr Protoc Neurosci (2001) **(Chapter 1)**: p. Unit 1.8.
[58] M. Hartman-Petrycka, et al., Who likes unhealthy food with a strong flavour? Influence of sex, age, body mass index, smoking and olfactory efficiency on junk food preferences, Nutrients 14 (19) (2022).
[59] M.A. Cornier, et al., Sex-based differences in the behavioral and neuronal responses to food, Physiol. Behav. 99 (4) (2010) 538–543.
[60] L. Wagner, et al., Sex differences in central insulin action: effect of intranasal insulin on neural food cue reactivity in adults with normal weight and overweight, Int. J. Obes. 46 (9) (2022) 1662–1670.
[61] A. Contini, et al., Comparison between male and female rats in a model of self-administration of a chocolate-flavored beverage: behavioral and neurochemical studies, Behav. Brain Res. 344 (2018) 28–41.
[62] K.T. Ball, et al., Effects of chronic stress on reinstatement of palatable food seeking: sex differences and relationship to trait anxiety, Physiol. Behav. 221 (2020) 112900.
[63] L.C. Anderson, G.D. Petrovich, Sex specific recruitment of a medial prefrontal cortex-hippocampal-thalamic system during context-dependent renewal of responding to food cues in rats, Neurobiol. Learn. Mem. 139 (2017) 11–21.