EFFECTS OF REWARD LOSS ON C-FOS EXPRESSION:

BUILDING A NEURAL CONNECTOME

by

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EFFECTS OF REWARD LOSS ON C-FOS EXPRESSION:

BUILDING A NEURAL CONNECTOME

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ABSTRACT

Reward loss and the accompanying emotional responses have been the subject of extensive research in psychology and neuroscience. The experience of reward loss is associated with a range of negative emotions and stress responses, which have implications for substance use disorders, anxiety disorders, and depression. This investigation employs c-Fos as a marker of neuronal activation to examine the early responses of the brain to reward loss. A Pavlovian successive negative contract (pSNC) paradigm was implemented to induce anticipatory behavior and free choice influenced by reward loss. The results of the pSNC paradigm demonstrated altered neuronal patterns and significant correlations between brain regions in response to reward downshift. These findings contribute to the neural circuity underlying reward loss and the potential engagement of multiple brain areas in reward processing and emotional regulation.

Introduction

Reward loss occurs when there is a surprising reduction in the value of an expected reward or the absence of an expected reward. The experience of reward loss is a phenomenon intricately connected to psychological pain, stress, and a range of negative emotions (Papini et al., 2015). The study of reward loss holds significance as it is implicated in substance use disorders, anxiety disorders, and depression. The stress induced by the loss of rewards contributes to the initiation and development of addiction (Ortega et al., 2017). There is a neurobehavioral overlap between the stress response and reward brain systems, which can help us understand how a reward loss experience can increase an individual's vulnerability to developing a substance use disorder. Several areas of the brain are involved in the overlap between reward loss and addiction, such as the amygdala, striatum, ventral tegmental area (VTA), nucleus accumbens (NAc), anterior cingulate cortex (ACC), and insular cortex (IC). Similarly, opioids and certain drugs have been found to affect psychological pain related to reward loss, potentially contributing to addictive behaviors as well (Papini et al., 2006). The range of behavioral and physiological responses triggered by surprising reward downshifts is known as *frustrative nonreward*, which contributes to addiction as well as anxiety disorders (Papini et al., 2022). Moreover, abnormalities in reward-related brain structures, particularly in the VTA-NAc pathway, are associated with mood disorders such as depression (Russo & Nestler, 2013). Based on this research, the study of reward loss could have implications for understanding a variety of emotional disorders. However, little is known about the brain circuit activated by reward loss, a prerequisite to understanding what brain regions are of critical importance to develop therapeutic interventions. The present study explores the relationship between reward loss and increased neural activation in several brain regions associated with

emotional processing and reward-related functions. Emerging research themes highlight the connection between reward loss and addiction, encompassing neurotransmitters, genetic factors, and neural circuitry (Ortega et al., 2017).

The negative emotions tied to reward loss are triggered by tasks such as successive negative contrast and appetitive extinction (Papini et al., 2015). Successive negative contrast (SNC) disrupts behavior when the magnitude of a reward is reduced unexpectedly from a large to a small amount relative to a control group consistently receiving the small amount (Morillo-Rivero et al., 2020). SNC has been studied in instrumental (iSNC) and consummatory (cSNC) situations. iSNC assesses the animals' anticipatory responses to the incentive, whereas cSNC assesses the animals' interaction with the incentive (Torres & Papini, 2017). Animals tend to reject a reward when the value of it is lower than expected. There are two stages in the cSNC effect (Flaherty, 1996): the initial downshift characterized by behavioral suppression and the recovery of behavior that follows. Both of these stages elicit different emotional responses with the initial downshift associated with primary frustration (i.e., the unconditioned response to the downshift), and the recovery that follows associated with anxiety and conflict induced by secondary (i.e., anticipatory) frustration.

The effects of reward downshifts were studied using a Pavlovian conditioning procedure known as autoshaping. In this Pavlovian SNC (pSNC) paradigm, animals are trained in a forced-choice situation involving trials with one retractable lever signaling a large reward and a second retractable lever signaling a small reward. In these forced choice trials, only one lever is presented at a time so that the animals can only decide whether to respond or not to that lever (hence the label "forced choice"). Such Pavlovian processes are likely accountable for the diverse situations where a contrast arises between anticipated outcomes and actual results,

leading to contradictory biases and suboptimal choices made by animals (Zentall, 2022). In occasional free-choice trials, both levers are presented simultaneously, and now the animal can choose to respond to one or the other or not to respond to either lever. Animals prefer the lever associated with the largest reward; however, after a reward devaluation, their choice switches from the downshifted lever to the unshifted lever (Conrad & Papini, 2018). This paradigm is designed to elicit the pSNC effect, wherein the response to the small reward is negatively contrasted against the expectation of the large reward, leading to altered behavioral responses and emotional states. Virtually nothing is known about the neural circuit underlying the pSNC effect.

Understanding the neurobiology behind SNC will lead to a deeper understanding of the neural communication mechanisms involved in the response to unexpected reward loss. Studies have implicated the dopaminergic reward system in processing rewards and, potentially, responding to the emotional and motivational aspects of reward loss (Arias-Carrión et al., 2010). One study indicated that the SNC effect is, at least in part, mediated by dopamine signaling (Phelps et al., 2015). Behavioral studies show that dopamine projections to the striatum and frontal cortex play a central role in mediating the effects of rewards on behavior and learning (Arias-Carrión et al., 2010). In another study, expression levels of pCREB, a marker of synaptic plasticity, were higher in the prelimbic cortex, anterior cingulate cortex, and dorso-medial striatum after cSNC, suggesting an episode of reward devaluation may trigger memory consolidation (Glueck et al., 2015). Furthermore, evidence has demonstrated that activation of the central amygdala (CeA) can magnify incentive motivation towards rewards, indicating its potential involvement in reward circuitry (Warlow & Berridge, 2021), whereas inactivation of the CeA eliminates the cSNC effect (Guarino et al., 2020; Kawasaki et al., 2015). This research

has laid the groundwork for the exploration of these specific brain areas and other regions potentially involved in reward loss. I have examined the brain areas listed in Table 1, together with the A/P coordinate. I hypothesize that reward processing areas will be significantly correlated with emotional regulation areas when the animal experiences the reward downshift. I also hypothesize that areas whose level of activation is positively or negatively correlated during reward loss could be directly influencing each other, whether in terms of excitation or inhibition. Such correlation could reveal a portion of the neural circuitry.

Table 1. Abbreviations for brain areas, coordinates, and functions

Abbrev.	Area	A/P	Function
NAcS	Nucleus Accumbens Shell	1.7	Reward processing
NAcC	Nucleus Accumbens Core	1.7	Reward processing
ACC	Anterior Cingulate Cortex	1.7	Motivation, emotional regulation
IC	Insular Cortex	1.7	Emotional regulation, affective states
CPu	Caudate-Putamen	-2.12	Emotional processing, motor control
GPe	Globus Pallidus Externus	-2.12	Emotional processing, motor control
GPi	Globus Pallidus Internus	-2.12	Emotional processing, motor control
PVN	Paraventricular Nucleus of the Thalamus	-3.14	Emotional regulation, sensory processes
Pir	Piriform Cortex	-2.12	Olfactory memory and perception
DEn	Dorsal Endopiriform Nucleus	-2.12	Olfactory memory and perception
VEn	Ventral Endopiriform Nucleus	-2.12	Olfactory memory and perception
BMA	Basomedial Amygdala, Anterior Part	-2.12	Emotional processing
ACo	Anterior Cortical Amygdala	-2.12	Emotional processing
BAOT	Bed Nucleus of the Anterior Commissure	-2.12	Emotional processing
MeAV	Medial Amygdala, Anteroventral Part	-2.12	Emotional processing
IM	Intercalated Amygdala, Main Part	-2.12	Emotional processing
BLA	Basolateral Amygdala, Anterior Part	-2.12	Emotional processing
CeM	Central Amygdala, Medial Part	-2.12	Emotional processing
CeL	Central Amygdala, Lateral Part	-2.12	Emotional processing
CeC	Central Amygdala, Capsular Part	-2.12	Emotional processing
CA1	CA1 Field of the Hippocampus	-3.14	Memory processing and retrieval
CA2/3	CA2/3 Field of the Hippocampus	-3.14	Memory processing and retrieval
DG	Dentate Gyrus	-3.14	Memory processing and retrieval
PL	Prelimbic Cortex	2.2	Motivation and emotional regulation
IL	Infralimbic Cortex	2.2	Motivation and emotional regulation
MHb	Medial Habenula	-3.14	Reward processing
MedLHb	Lateral Habenula, Medial Part	-3.14	Reward processing
LatLHb	Lateral Habenula, Lateral Part	-3.14	Reward processing

Note. All values are in millimeters relative to bregma. Coordinates from Paxinos and Watson (2013). A/P: anterior/posterior coordinates.

To quantify neural activity, I use c-Fos as a marker of neuronal activation, consistent with the approaches used in related studies (Cruz-Mendoza et al., 2022; Alfonso-Gonzalez & Riesgo-Escovar, 2018). The protein product of the *c-fos* gene, the c-Fos protein, is commonly used as a marker of cellular responses to stimuli because of their widespread activation (Alfonso-Gonzalez & Riesgo-Escovar, 2018). The use of c-Fos as an indicator of recently activated neurons allows us to explore the immediate early responses of neural regions involved in reward loss, shedding light on the dynamics of neuronal communication (Alfonso-Gonzalez & Riesgo-Escovar, 2018). Based on significant relationships between brain areas and increased c-Fos activity, a hypothesized circuitry of neural activity involved in reward loss can be created (Figure 1).

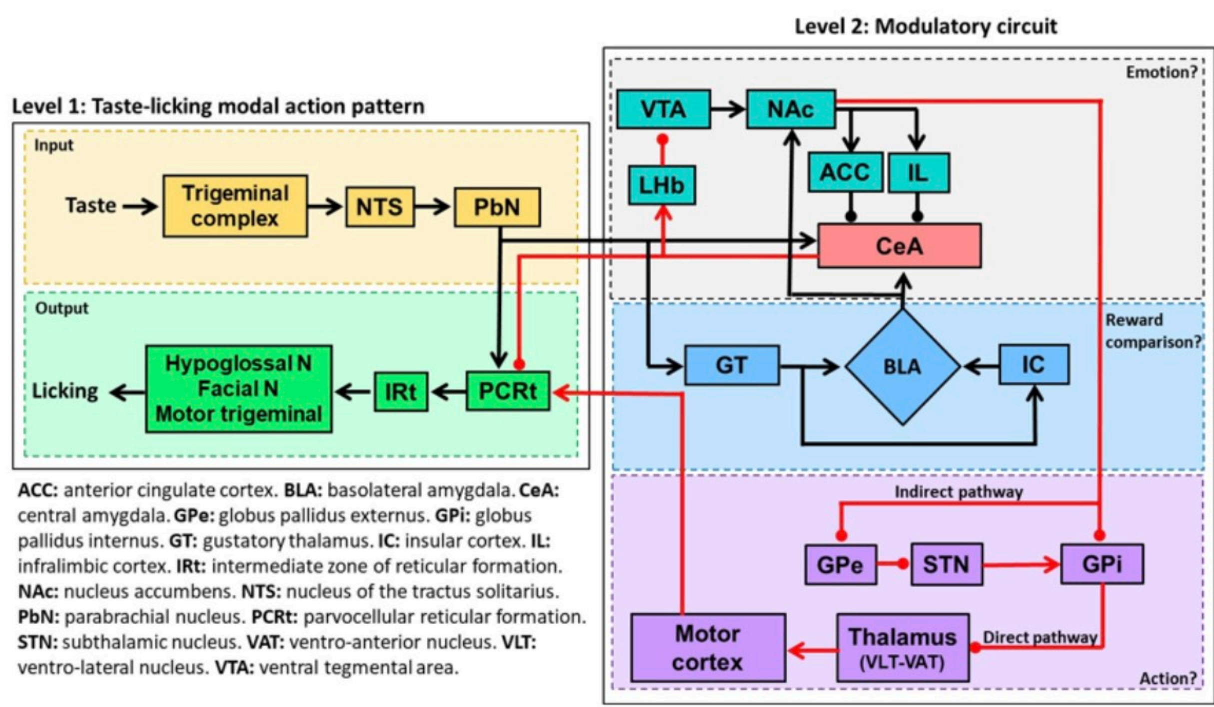

Figure 1. Hypothesized neural circuitry underlying reward downshift. Although this hypothetical circuit was originally proposed to explain the cSNC effect, it can be used as a guide to explore other effects involving reward loss, such as the pSNC paradigm used in the present experiment. (Modified from Ortega et al., 2017.)

Understanding the neural mechanisms that underlie the experience of reward loss within a circuitry holds the potential to provide valuable insights into emotional disorders. This investigation aims to build upon the existing body of literature and provide new data for the hypothesized neural circuitry. By expanding our understanding of how reward loss influences neural circuitry, this study contributes to the growing knowledge in the fields of psychology and behavioral neuroscience.

Current Study

The present study aimed to further identify brain regions activated selectively during reward loss. Specifically, I seek to establish a comprehensive map of brain activity, or connectome, underlying episodes of reward downshift in the pSNC task. An autoshaping procedure was used to implement the pSNC paradigm, incorporating a pellet downshift to examine the neural substrates involved in reward learning and the anticipatory responses to reward loss. In the pSNC task, the control conditions were built in the within-subject comparison between the downshifted and unshifted levers. However, c-Fos expression was studied in two groups of animals sacrificed either after the preshift test on reward magnitude (Group Pre) or after the postshift test on reward downshift (Group Post). Rats were perfused after each of these points in training, enabling us to capture their neural activity at different stages of the learning process. After perfusions, their brains were carefully extracted and sliced using cryostat equipment. To evaluate neuronal activation in the relevant brain regions, I employ immunohistochemistry staining techniques. Following the staining process, c-Fos-positive cells were counted in the target brain regions. The further control of untrained animals should provide a baseline for brain activity.

Method

Subjects

The subjects included 18 female Wistar rats, all of which were individually housed in wire-bottom cages with free access to water and an enrichment device providing space for hiding and a flat surface to stand on. All cages were kept in a colony room with constant temperature (22-23°C), humidity (45-65%), and a set light schedule of 12 h light/12 h dark. Animal care followed TCU vivarium-approved IACUC protocols. Animals were weaned at 21 days of age and group housed until 60 days of age. At 90 days of age, food was restricted until they reached 81-84% of their ad libitum body weight. The mean (±SEM) ad libitum weight for the selected animals was 278.9 g (±4.8). The average ad libitum weight was calculated by weighing them on two consecutive days while on free food.

Apparatus

Conditioning was conducted in four standard operant chambers (MED Associates, St. Albans, VT), each enclosed in a cabinet to attenuate unsystematic noises. These cabinets featured a GE 1820 house light, a fan for air circulation, and a speaker to produce masking white noise (80.1dB, SPS Scale C). The dimensions of the boxes were 20.1W × 28L × 20.5H cm, with a floor of stainless steel bars measuring 0.4 cm in diameter and spaced 1.6 cm apart. Underneath the floor bars, a container filled with corncob bedding collected feces and urine. A dispenser external to the chamber provided 45-mg precision food pellets (Bio-Serv, Flemington, NJ), releasing them into a food cup situated 2 cm from the floor and centered on the front wall. The food cup was equipped with photocells capable of detecting head entries, automatically recording goal-entry behavior. On either side of the food cup and situated 6 cm from the floor were two stainless steel retractable levers. A computer equipped with MED Notation software (MED Associates, St.

Albans, VT) and placed in an adjacent room was responsible for controlling the sequence and timing of events, administering pellets, and automatically logging each lever press and goal entry on each trial.

Behavioral Testing Procedure

The pSNC procedure involves anticipatory behavior with reward downshift and free choice. For the first 14 sessions, over 14 days, the animals were trained to associate one lever with 12 pellets and the second lever with 2 pellets. The assignment of each lever to a particular reward magnitude was counterbalanced across animals. There were three trials with each lever and reward magnitude per session. Starting on session 10, all animals experienced one free-choice trial on even numbered sessions (e.g., sessions 10, 12, etc.) in addition to the six forced-choice trials (three trials with each reward magnitude). These free-choice trials allowed the animals to exhibit lever preference as both levers were presented simultaneously. Free-choice trials were never reinforced. Half the animals (Group Pre) were perfused when showing a preference for the 12-pellet lever over the 2-pellet lever. For the other half of the animals, once they exhibited a preference for the 12-pellet lever, they proceeded to enter the downshift sessions (Group Post). During the downshift phase, both levers were associated with 2 pellets each, and the animals experienced six forced-choice trials per session. In addition, they also received one free-choice trial with both levers presented simultaneously on all even sessions after session 10. Once they exhibited a shift in preference from the lever previously paired with 12 pellets (and now paired also with 2 pellets) to the unshifted lever always paired with 2 pellets, animals were perfused and their brains extracted for c-Fos analysis.

Perfusion and Immunohistochemistry

The animals were perfused after the session in which they exhibited either a preference for the 12-pellet lever (Group Pre) or after the session in which they shifted preference away from the lever that used to be paired with 12 pellets (Group Post). Brains were immediately extracted and embedded in 4% paraformaldehyde for at least three days. Afterwards, the brains were embedded in 30% sucrose for at least two days. Once fixed, the brains were sliced in 40 μm sections using a cryostat. Sections were placed onto slides, and immunohistochemistry staining techniques were applied. Neural activation was assessed via microscopy and c-Fos counting.

Results

pSNC Free Choice

For free-choice trials, there were a large number of zero responses as animals typically responded to one of two levers. This violated normality, thus requiring the use of nonparametric tests to analyze behavior in free-choice trials. During preshift, both perfused, $Z=-2.67$, $p<0.01$, and nonperfused, $Z=-2.67$, $p<0.01$, animals showed a significant preference for the 12-pellet lever, with all animals exclusively pressing the 12-pellet lever. For animals that were not perfused and continued to downshift, they significantly showed a preference for the 2-pellet lever, $Z=-2.67$, $p<0.01$, with all animals exclusively choosing the 2-pellet, unshifted lever over the downshifted lever. Figure 2 shows free choice lever pressing for animals perfused during preshift, and Figure 3 shows free choice data (both preshift and downshift) for animals perfused during postshift. Given the behavioral criterion placed on the animals in preshift and postshift, these results were not unexpected, but they ensured that all animals were in the same behavioral state before they were perfused for c-Fos analysis.

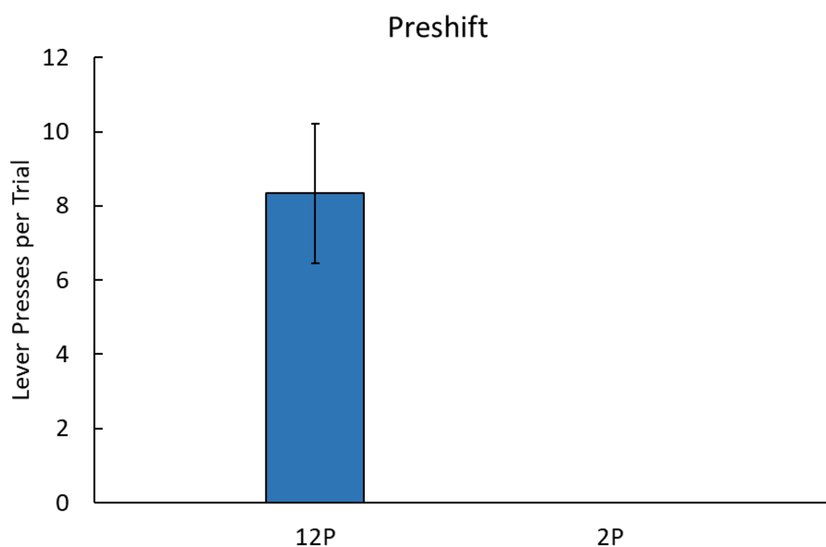

Figure 2. Free choice lever pressing data for animals perfused during preshift, showing a significant preference for the 12-pellet lever with all animals exclusively choosing the 12-pellet lever.

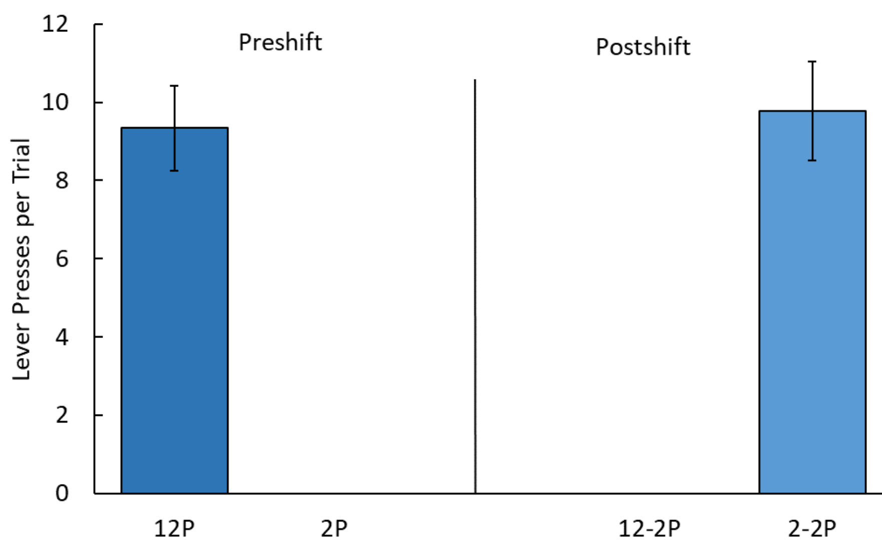

Figure 3. Free choice data (both preshift and downshift) for animals perfused during postshift, demonstrating a significant shift in preference from the 12-pellet lever to the 2-pellet lever, with all animals exclusively choosing the 2-pellet lever postshift.

c-Fos Correlations

All significant correlations are reported in Tables 2 and 3 for preshift and postshift, respectively. All other correlations were nonsignificant, *p*s>0.05. Areas that had no significant correlations with other areas were omitted from Tables 2 and 3. Correlations unique to either preshift or postshift are graphically represented in Figures 4 and 5. Of note are significant positive correlations between the prelimbic cortex (PL) and the lateral part of the habenula (latLHb), $r=0.025$, $p<0.05$, as well as the significant negative correlations between the PL and the medial habenula (MHb), $r=-0.003$, $p<0.05$, during postshift.

Table 2. Significant Pearson's correlations between pairs of brain areas during preshift sessions.

Area	NAcS	NAcC	ACC	IC	CPu	GPe	GPi	PVN	Pir	DEn	VEn	BMA	ACo	BAOT	MeAV	IM	BLA	CeM	CeL	CeC	CA1	CA2/3	DG	PL	IL	MHb	MedLHb	LatLHb
NAcS		0.007**			0.000**																							
NAcC				0.030*				0.013*																				
ACC																												
IC																												
CPu																												
GPe																												
GPi																												
PVN																												
Pir										0.014*					0.043*		0.023*											
DEn																												0.022*
VEn																												
BMA																												
ACo																										0.024*		
BAOT																	0.035*											
MeAV																												
IM																				0.044*							0.049*	
BLA																												
CeM																												
CeL																												
CeC																												
CA1																						0.000**	0.001**					
CA2/3																							0.000**					
DG																												
PL																									0.002**			
IL																												
MHb																												
MedLHb																												
LatLHb																												

Note. *: $p<0.05$. **: $p<0.01$.

Table 3. Significant Pearson's correlations between pairs of brain areas during postshift sessions.

Area	NAcS	NAcC	ACC	IC	CPu	GPe	GPi	PVN	Pir	DEn	VEn	BMA	ACo	BAOT	MeAV	IM	BLA	CeM	CeL	CeC	CA1	CA2/3	DG	PL	IL	MHb	MedLHb	LatLHb
NAcS		0.000**		0.006**				0.009**																				
NAcC				0.014*				0.005**																				
ACC																					0.043*							
IC																												
CPu						0.019*	0.034*																					
GPe							0.000**																					
GPi																												
PVN																							0.05					
Pir											0.032*																	
DEn											0.016*						0.023*			0.009**								
VEn												0.019*					0.000**	0.021*		0.015*								
BMA																	0.006**											
ACo															0.037*												0.047*	
BAOT																												
MeAV																												
IM																	0.047*	0.041*										
BLA																		0.016*	0.022*	0.014*								
CeM																												
CeL																				0.036*								
CeC																												
CA1																						0.009**	0.001**					
CA2/3																							0.036*					0.018*
DG																												0.05
PL																										0.003**		0.025*
IL																												
MHb																												0.010**
MedLHb																												
LatLHb																												

Note. *: $p<0.05$. **: $p<0.01$

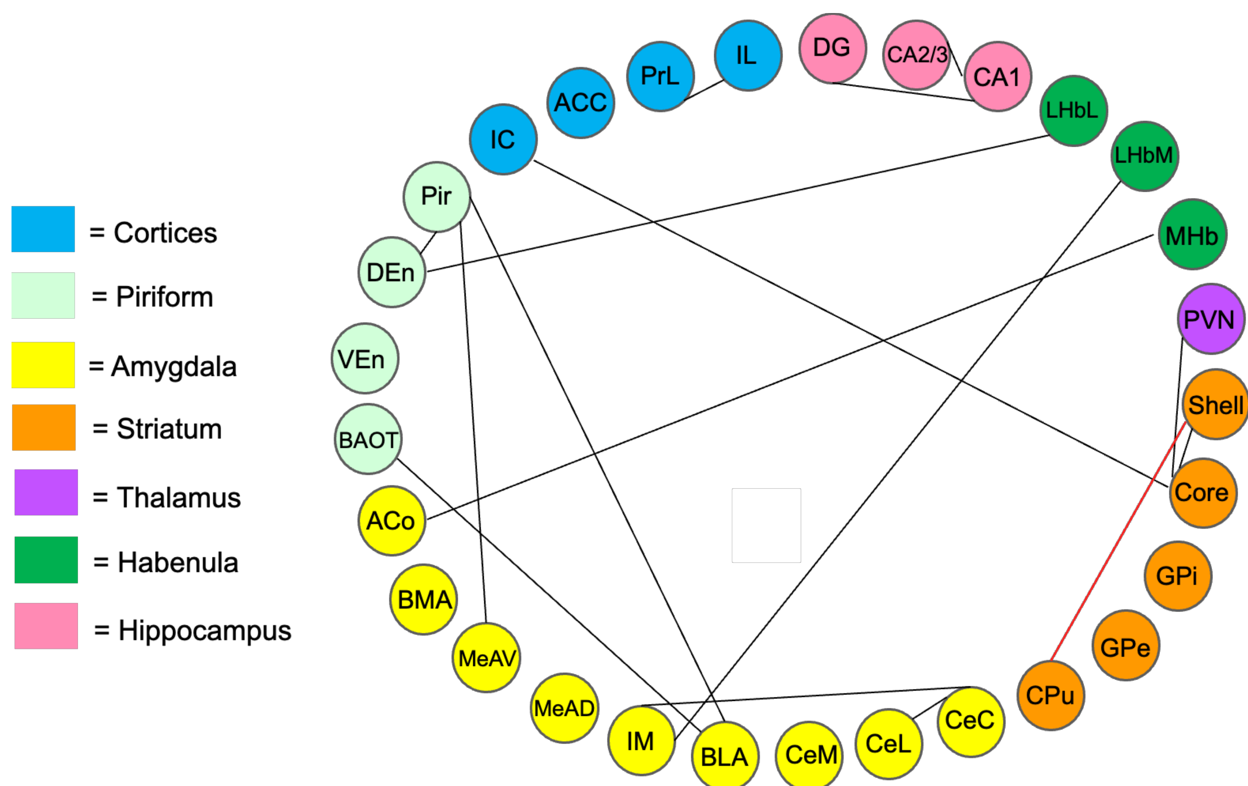

Figure 4. Illustration of significant correlations between brain areas observed in animals during preshift sessions. Black lines represent positive correlations, and the red line represents a negative correlation. Different colors are used to represent subsets of the 29 specific brain regions where c-Fos positive cells were counted.

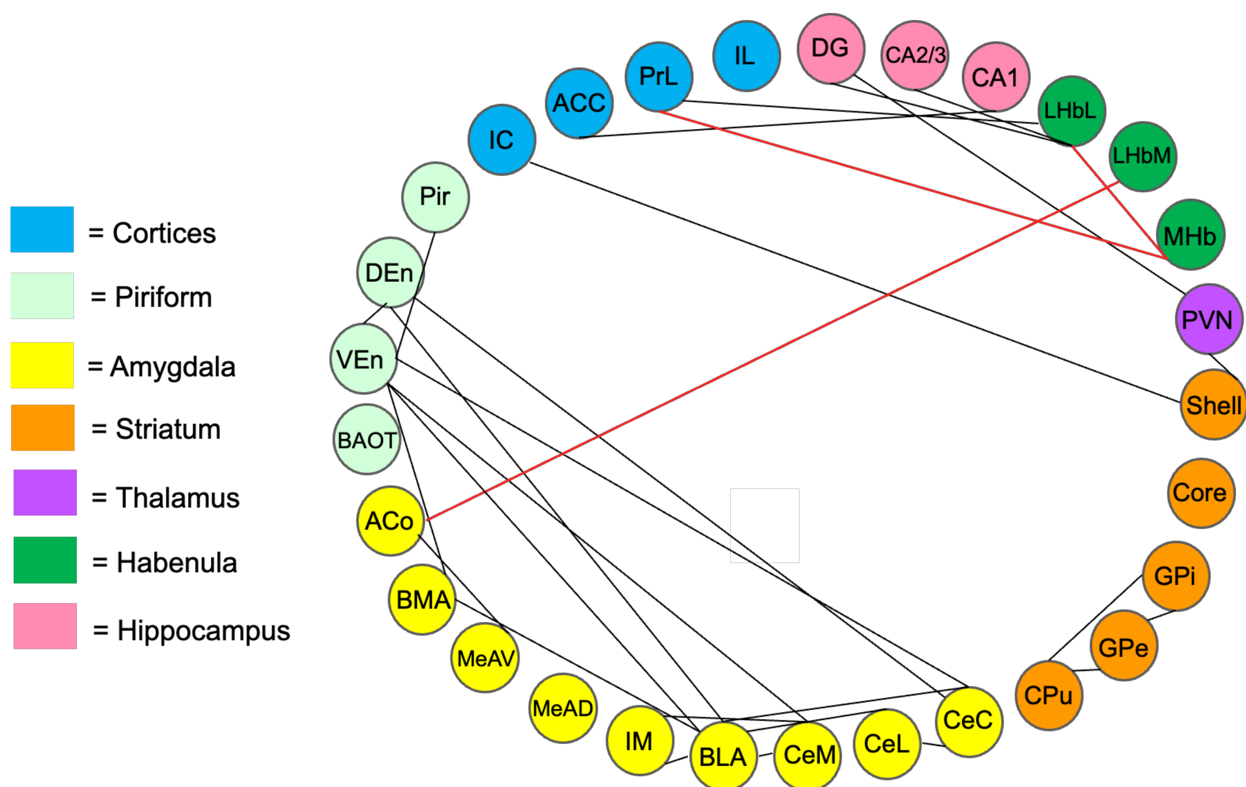

Figure 5. Illustration of significant correlations between brain areas observed in animals during postshift sessions. Black lines represent positive correlations, and the red lines represents negative correlations. Different colors are used to represent subsets of the 29 specific brain regions where c-Fos positive cells were counted.

Discussion

The present study sought to explore the effects of reward loss on neuronal activation in specific brain regions, and its potential implications for understanding emotional responses and addictive behaviors. Building on previous experimental results, I delved deeper into the effects of reward downshift using a Pavlovian successive negative contrast (pSNC) paradigm. This experiment involved a reduction in reward magnitude from 12 pellets to 2 pellets, accompanied by free-choice tests. The extensive data collection and analysis in this study allowed for a more

nuanced understanding of the neural and behavioral responses to reward loss. My analysis of experimental data revealed several key findings.

Frustrative nonreward was revealed in terms of a distinct shift in lever preference from the downshifted lever to the unshifted lever when both levers yielded a reward of 2 pellets. This behavioral response underscores the intricate nature of reward processing and the adaptive adjustments made by subjects in response to altered reward contingencies. Furthermore, my analysis of c-Fos positive cells revealed a noteworthy increase in correlations within the amygdala and basal ganglia during postshift tests compared to preshift conditions. These changes in the matrix of correlations suggest heightened emotional processing during reward loss, as both the amygdala and basal ganglia are implicated in emotional evaluation and action regulation, respectively.

My findings also unveiled a significant correlation pattern involving the prelimbic cortex (PL) and the lateral region of the lateral habenula (latLHb), as well as the PL and the medial habenula (MHb). Specifically, I observed a positive correlation between the PL and latLHb, indicative of an integration between emotional regulation and reward processing mechanisms. By contrast, a negative correlation was found between the PL and MHb, suggesting a nuanced modulation of emotional and reward-related responses. This differential functionality in the habenula indicates the specialized roles of its subregions in reward processing. In addition to these correlations, my analysis identified positive correlations between the anterior cingulate cortex (ACC) and the CA1 field of the hippocampus, as well as between the paraventricular nucleus of the thalamus (PVN) and dentate gyrus (DG). These correlations further underscore the interconnectedness of brain regions involved in emotional regulation, memory retrieval, and reward processing during postshift trials.

The integration of these correlational data provides valuable insights into the complex neural circuitry underlying emotional and reward-related responses during reward loss. These findings contribute significantly to understanding the interplay between motivational processes, emotional states, and neural dynamics in the context of reward modulation and its implications for behavioral outcomes.

Conclusion

In summary, this study, utilizing the Pavlovian successive negative contrast (pSNC) paradigm, sheds light on the complex interplay between reward loss, neuronal activation, and emotional regulation. Through behavioral observations and correlated neural analyses, I observed adaptive responses during reward downshift, indicating the dynamic nature of reward processes and emotional states. The pSNC paradigm revealed significant correlations within key brain regions involved in reward downshift and frustrative nonreward. Overall, the exploration of the pSNC paradigm provides valuable insights into how organisms navigate changing reward environments, highlighting the integration of emotional regulation and reward processing mechanisms in response to reward loss.

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