Place Preference Can Be Conditioned by Corticolimbic Glutamate Blockade of Accumbens Shell in a Familiar Environment

by

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Abstract

Desire and dread can be produced in medial nucleus accumbens shell in a rostrocaudal gradient along which hyperpolarizing microinjections of the AMPA receptor antagonist DNQX can generate appetitive behavior (in rostral shell) and fearful behaviors (in caudal shell). Additionally, environmental ambience retunes the valence of behavior elicited by glutamate receptor blockade in the accumbens, increasing positively motivated behavior in a home environment, and increasing defensive behavior in a stressful environment. Despite producing intense eating, rostral shell DNQX has previously been reported to produce a conditioned place aversion under standard laboratory conditions. The purpose of this study was to determine whether the comfortable, home environment could allow DNQX to illicit a conditioned place preference. Here, DNQX was shown to establish a conditioned place preference in rats with far rostral microinjection sites, but it did not establish a conditioned place preference in rats with more mid rostral microinjection sites. This effect contrasts with DNQX effects on unconditioned appetitive behavior (eating), which is increased by DNQX at both far and mid rostral locations. Appetitive behaviors produced by corticolimbic glutamate signals are influenced by emotional ambience, even in the case of conditioned behaviors.

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Different theories exist regarding what underlies emotion in the brain. Some researchers argue for a categorical theory of emotion, which would suggest that emotions – inherited and reflexive modules such as anger, happiness, fear, or disgust – could be classified into discrete groups (Eckman, 1972). People across the world are capable of categorizing particular expressions, and people blind from birth still generate particular emotional expressions, even though they have never visualized these expressions, providing evidence for universality in emotion (Eckman, 1972). This may mean that the brain is utilizing a separate systems approach to generating emotion in which a certain emotion is elicited and coded for by a discrete system in the brain. However, in disagreement with these notions, there is evidence that brain activations observed from fMRI occur in concurrence with more than one different emotion – such as the amygdala's role in not just fear, but also possibly reward (Barrett & Wager, 2006). Moreover, emotions have been linked to brain activations in more than one region such as linkage between sadness and not only the anterior cingulate cortex but also the medial prefrontal cortex (Phan, Wager, Taylor, & Liberzon, 2002; Murphy, Nimmo-Smith & Lawrence, 2003).

Interestingly, fMRI studies have consistently found common culprits involved in almost all emotions – amygdala, nucleus accumbens, orbitofrontal and cingulate cortex – including positive emotions in response to money or trust and negative emotions in response to pain or fear (Knutson et al, 2004; O'Doherty et al, 2004; Singer et al, 2004; Morris & Dolan, 2004). Posing another problem for the hypothesis that specific brain systems govern specific emotions or motivations is the neuronal plasticity observed for hypothalamic circuits: stimulation of the lateral hypothalamus can result in either feeding *or* drinking behavior, depending on the

availability of food (Valenstein, Cox & Kakolewski, 1969). Rats that typically eat following lateral hypothalamic electrode stimulation can be 'turned into' drinking rats if they are stimulated with free water availability but no food. When the food is returned, however, these rats will again eat following hypothalamic stimulation (Valenstein et al, 1969). This would seem that there is some sort of shared component in these neurons that is malleable. Could it be that affective components can be built flexibly into emotion?

For instance, appetitive and fearful motivation may share a form of motivational salience – incentive salience in the case of appetitive motivation and fearful salience in the case of fearful motivation (Berridge, 2004). A brain structure of particular interest due to its role in motivated behaviors is the nucleus accumbens (Reynolds & Berridge, 2002, 2003). Positive motivational behavior and defensive, fearful behavior can be generated by hyperpolarizations induced by either the stimulation of $GABA_A$ receptors or blockade of glutamate AMPA receptors in the nucleus accumbens shell (Maldonado-Irizarry, Swanson & Kelley, 1995; Stratford & Kelley, 1999; Reynolds & Berridge, 2001). There exists a rostrocaudal gradient in the accumbens shell in which varying rostrocaudal locations produce increased appetitive behavior (positive motivation) or increased defensive behavior when neurochemically altered with either a $GABA_A$ agonist or glutamate AMPA antagonist (Reynolds & Berridge, 2001, 2003). The rostrocaudal gradient is analogous to a limbic 'affective keyboard,' capable of producing many different combinations of appetitive and defensive behaviors, each of which correspond to the specific location of injection sites of neuron-hyperpolarizing drugs into the accumbens shell. Microinjections into rostral sites generate strong appetitive motivation, characterized by increased eating behavior and food intake, and injections into caudal sites generate negative fearful motivation (Reynolds & Berridge, 2001, 2003). The behavior most easily characterized as

fearful is 'defensive treading,' in which a rodent will attempt to bury a negative stimulus using rapid forward thrusts of the forepaws. In nature, the stimulus could be a predator; while in the lab, the stimulus could be a probe that sends a shock into the foot of the rat (Treit, Pinel & Fibiger, 1981).

When microinjected into the nucleus accumbens shell, the $GABA_A$ receptor agonist muscimol demonstrated a similar rostrocaudal gradient for hedonic impact – liking and disliking – and conditioned place preference/avoidance; yet the glutamate AMPA receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX) failed to modulate these hedonic reactions (Reynolds & Berridge, 2002, 2003; Faure, Richard & Berridge, 2010). This demonstrates a clear difference in the glutamatergic and GABAergic circuits and how they govern different behaviors. The glutamatergic circuit receives top-down input from structures such as the prefrontal cortex, basolateral amygdala and the hippocampus, while the GABAergic circuit receives bottom-up input from the ventral pallidum and ventral tegmentum area as well as other GABAergic neurons from the nucleus accumbens (Faure et al, 2010). This different type of processing involed in the glutamatergic and GABAergic circuits could explain why even though both circuits are shown to be equally responsible in generating motivated behaviors such as positive, appetitive behavior and negative, fearful behavior, these circuits differently govern emotion responses such as hedonics and place preference.

A pattern of conditioned place preference and avoidance could be observed in accordance with the rostrocaudal gradient of the nucleus accumbens following muscimol microinjection: rostral microinjection produces a positive place preference, whereas more caudal microinjections produce a negative place aversion (Reynolds & Berridge, 2002). However, microinjections of a glutamate blocker, DNQX, into accumbens shell resulted in only avoidance, which progressed,

on average, from mild to strong along the rostrocaudal gradient (Reynolds & Berridge, 2003). That DNQX did not demonstrate this rostrocaudal gradient is of particular interest, and we propose that the top-down processing that is sensitive to environmental change is impacting DNQX's ability to generate conditioned place preference. Environmental ambiance has also been shown to affect the valence of motivational behavior induced by DNQX microinjections into the accumbens shell (Reynolds & Berridge, 2008). The valence simply refers to whether the behavior elicited in a rat can be classified as positive (appetitive) or negative (fearful). The environmental influence on the rostrocaudal gradient of the accumbens shell can provide more insight into the mechanism that controls certain complex disorders of motivation. Reynolds and Berridge's (2008) study featured rats exposed to one of three different environments. Rats tested in the home environment were tested in the actual room in which they were housed, featuring familiar sounds, smells and lighting – intended to be of positive valence. A standard laboratory environment was used as a control. Finally, the stressful environment was both brightly lit and featured loud music, a stark contrast from the dimly lit and quiet, home environment (Reynolds & Berridge, 2008).

The nucleus accumbens can be divided into zones, based on the type of behavior that is elicited from a microinjection of a hyperpolarizing agent. The 'positive zone' exists rostrally in the nucleus accumbens shell and microinjections into this zone result in appetitive behavior. Conversely, the 'negative zone' exists caudally in the nucleus accumbens shell and microinjections into this zone result in fearful behavior. Changing the environment in which rats were tested for motivated behaviors showed that the 'positive zone' and 'negative zone' increased in size when rats were tested in the comfortable, home environment and in the stressful environment, respectively (Reynolds & Berridge, 2008). That is, when rats were placed in the

home environment, injection sites in the rostrocaudal gradient that previously resulted in either ambivalent or negative behavior (in the standard environment) now resulted in positive, appetitive behavior (Reynolds & Berridge, 2008). Testing in the home environment increased the appetitive behavior, especially that which is activated by neurons in the middle rostrocaudal zone (between +1.2 and 1.8 mm ahead of bregma) of the accumbens shell. Because hyperpolarization of these neurons usually elicits equal amounts of appetitive or defensive behavior in the standard environment, the middle of the accumbens shell features the greatest abundance of neurons that are likely to flip valence of behavior as a result of environmental influence (Reynolds & Berridge, 2008). In the home environment, glutamate blockade resulted in appetitive behavior elicited by a larger number of activation sites than the standard, control environment. In the stressful environment, glutamate blockade resulted in defensive behavior elicited by a larger number of activation sites than the standard environment, especially sites in the middle rostrocaudal zone (Reynolds & Berridge, 2008). Contrastingly, the GABAergic was less sensitive to environmental change (Richard & Berridge, 2011).

The testing paradigm for rats is, by nature, a stressful experience for rats. The microinjection procedure takes 8-10 minutes, and the testing chamber is unfamiliar. With glutamate circuits being so sensitive to the environment, this could explain why the rostral portion of the nucleus accumbens – which produces positively motivated eating behavior following DNQX microinjection – would still produce a conditioned place avoidance. Such an uncomfortable experience for rats does not allow the positive effects of DNQX to overcome the stress of the place preference test.

This brings about the question of whether neuronal plasticity could possibly change the observed place avoidance resulting from DNQX (glutamate antagonist) microinjection into

accumbens shell (Reynolds & Berridge, 2002, 2003, 2008). Following glutamate antagonist microinjection, emotional environments were shown to retune the valence of motivation in the nucleus accumbens, particularly with regards to those neurons in middle rostrocaudal zone. While DNQX microinjections in accumbens shells established a rostrocaudal gradient of negative conditioned place avoidance, rostral sites featured only 25% mean avoidance while caudal shell featured 55% mean avoidance (Reynolds & Berridge, 2003). That far rostral sites only demonstrated moderate conditioned place avoidance makes the neurons in that region of accumbens our target. The far rostral sites in the accumbens did not demonstrate the flip of valence that medial sites did, but this could be because these sites already produced robust eating effects following DNQX microinjection (Reynolds & Berridge, 2003, 2008). It would seem, however, that a soothing environment has the potential to make positive zones more positive, which would enable the far rostral sites of the nucleus accumbens to overcome the stress of testing.

The purpose of this study is to uncover whether the same neuronal plasticity for increasing appetitive zones in the comfortable, home environment could result in new, conditioned place preference following microinjections of glutamate into far rostral sites of the accumbens shell. To confirm the effects of DNQX microinjection into rostral shell on motivated behaviors, a group of rats were assigned to behavioral testing to examine whether these rodents demonstrated an increase in eating behavior. We found that the far rostral sites of the nucleus accumbens produced a conditioned place preference effect that was a result of rats avoiding the vehicle-paired chamber while their time spent in the DNQX-paired chamber remained the same. Rats with mid rostral microinjection sites did not demonstrate any conditioned place preference, and they largely avoided both chambers. Here, we saw the same retuned valence of motivational

salience that was observed in Reynolds and Berridge's (2008) study. While the testing procedure was still stressful, testing in a more familiar environment allowed DNQX's positive motivational effects to make the DNQX-paired chamber a preferred experience for at least some of the microinjection sites in medial shell.

Methods

Subjects

Male Sprague Dawley rats (N=28 [amphetamine validation group, N=10; DNQX place preference group, N=12; DNQX unconditioned motivation group, N=6] 280–350 g at the time of surgery) were group housed (21°C; 12 hr light/dark cycle) with *ad libitum* food (Purina Rat Chow) and water (tap water).

Microinjection cannula surgery

Rats (N=18) were pretreated with atropine sulfate (0.05 mg/kg) and anesthetized with a combination of ketamine (80 mg/kg, i.p.) and xylazine (5 mg/kg). To prevent damage to the lateral ventricles, rats were placed in a stereotaxic apparatus in a slanted position with the incisor bar set to 5.0 mm above interaural zero. Chronic microinjection guide cannulae (23 gauge) were implanted bilaterally to end 2 mm above rostral sites in the medial nucleus accumbens shell. Coordinates for rostral sites were chosen on the basis of the capacity of rostral sites to maximally evoke positively motivated behavior following DNQX microinjection (Reynolds and Berridge, 2003). Rats received cannulae targeted in the rostral half of the accumbens shell [targeted at anteroposterior (AP) +3.1-3.3 mm ahead of bregma, mediolateral (ML) ±1.0 mm from bregma, dorsoventral (DV) -5.7 mm below skull], although actual placements also included some rats with more intermediate sites. Microinjection cannulae were anchored to the skull with bone screws and acrylic cement. A stainless steel obturator was inserted into each microinjection

guide cannula to help prevent occlusions. After surgery, each rat received subcutaneous injection of cefazolin (75 mg/kg) to prevent infection and carprofen (5 mg/kg) for pain relief. Rats received carprofen again 24 hours later and were afforded at least 7 days to recover before testing.

Drugs and Microinjections

DNQX (6,7-dinitroquinoxaline-2,3(1H,4H)-dione), an AMPA/kainite receptor glutamate antagonist, was dissolved in 50% DMSO/50% 0.15 M saline, which was also used for vehicle control microinjections. The DNQX dosage (500 ng/0.5 µl per side) that was chosen was based on that used to produce rostrocaudal gradients of eating and defensive treading behaviors via microinjections into medial shell in recent studies (Faure et al, 2010; Reynolds & Berridge, 2008). Microinjection cannulae (29 gauge), extending 2 mm beyond the ventral tip of the guide, were attached to a syringe pump via PE-20 tubing, and rats were gently hand-held as they were bilaterally infused with a microinjection volume of 0.5 µl at a rate of 0.30 µl / min as used in prior studies (Faure et al, 2010; Reynolds & Berridge, 2008). After infusion, the injectors remained in place for an extra 60 seconds to ensure drug diffusion before they were withdrawn and replaced with the obturators. Immediately after microinjection, rats were placed into the behavioral testing chamber. DNQX and vehicle microinjections were spaced 48 hours apart, and counterbalanced across rats.

Place Preference Apparatus

Conditioned place preference training occurred in a three-compartment apparatus that was located in the rats' home environment in which they live, under the dim red lighting used for their reverse light cycle with minimal unfamiliar noise (Reynolds & Berridge, 2008). The apparatus featured two large side chambers (28 x 21 x 21 cm) surrounded a smaller central

compartment (12 x 21 x 21 cm) (Reynolds & Berridge, 2002). One side compartment was cleaned using Versa-Clean and had black-colored walls and a wire grid floor. The other side compartment was cleaned using 70% Ethanol and had white walls and a wire mesh floor. Before this experiment, the effectiveness of our place conditioning procedure was confirmed using a separate group of rats (N=10), successfully conditioned to have a place preference for a compartment paired with amphetamine administration (1 mg/kg, i.p) (Reynolds & Berridge, 2002).

Pre-exposure

For this test (day 0), rats were not given microinjections. They were taken from the home cage and placed into the central compartment of the place preference apparatus and allowed to freely explore the entire apparatus for 30 minutes. Their location during this session was videorecorded and scored for cumulative time (seconds) spent in each compartment. A camera was positioned above the testing chamber, mainly focused on the middle apparatus. The experimenter could see the entrance point to all three chambers. A rat was considered to be in a particular compartment whenever its head and both forelimbs were inside. Rats who exhibited a strong place preference (> 70% time spent on either side) prior to conditioning were excluded from further conditioning/testing.

Place Conditioning Training Procedure

Each rat (N=12) was assigned in a counterbalanced manner to have one side compartment paired with DNQX microinjection. Rats received six conditioning trials (spaced 48 hours apart) containing three DNQX microinjections paired with their assigned compartment alternating with three vehicle microinjections paired with the other compartment, counterbalanced for order. On conditioning days, rats received bilateral microinjections (0.5 µl),

as described above, before immediately being placed in the appropriate side compartment, where they were confined for 30 minutes.

Conditioned place preference test

Following the six days of conditioning, rats were tested for conditioned place preference (day 7) utilizing the same procedure from the pre-exposure.

Tests of spontaneous motivated behavior

On a test day, rats (N=6) received one of the microinjection conditions described above (either DNQX or vehicle) and were immediately placed in a transparent test chamber for behavioral testing. The floor was covered with granular bedding (crushed corn cob) spread 3 cm deep (to support defensive treading behavior), and the chamber contained pre-weighed food chow pellets (~20 g) and a water spout (to support eating and drinking behaviors). Spontaneous behavior was videotaped for 60 min for subsequent off-line analysis (Reynolds & Berridge, 2002, 2003). DNQX-induced motivated behaviors typically directed toward appropriate stimuli in the environment. Appetitive behavior is directed to food pellets or a waterspout in the chamber. Defensive treading behavior is typically directed toward light-reflecting corners and the most exposed transparent wall of the experimenters, open room, and glittering corners appear to be the most threatening stimuli in the chamber, and perhaps for that reason are most defended against by vigorous defensive treading behavior stimulated by DNQX (Reynolds & Berridge, 2002, 2003). Defensive treading stimulated by DNQX microinjection in caudal shell typically results in rats building a mound of the granular bedding placed in corners or between the rat and the transparent wall that reveals the outside experimenter (Reynolds & Berridge, 2002, 2003).

The videotaped behavior of each rat was scored in an analysis of eating, defensive treading, and other behaviors by an experimenter blind to drug treatment. Behavior was analyzed

for cumulative time (seconds) spent in (1) eating, (2) drinking, (3) defensive treading, (4) grooming, (5) burrowing (insertion of head under corn-cob bedding, with downward and forward thrust), and (6) burrow treading (combination of burrowing head thrust and paw-treading movements), and for the total number of occurrences of (7) rearing and (8) locomotion (crossing of line that divides the front and back of the cage) as seen in prior studies (Reynolds & Berridge, 2003, 2008)

Amphetamine Validation

In order to determine that our procedures could successfully condition a place preference, a subset of rats was conditioned with amphetamine. Rats (N=10) were pre-exposed to the testing chamber as described above. 2 rats were excluded from further conditioning because they demonstrated a strong place preference (> 70% time spent on either side) prior to conditioning. On conditioning days, rats received injections of amphetamine or saline (1 mg/kg, i.p.), counterbalanced for order and chamber in which amphetamine was received as described above in the procedure for DNQX conditioning. Following the six days of conditioning, rats were tested for conditioned place preference using the testing procedure and videoscoring methods as previously described.

Histology

Following all testing, rats were deeply anesthetized with an overdose of sodium pentobarbital and decapitated. Brains were removed and fixed in 10% paraformaldehyde overnight, and then cryoprotected in 25% sucrose solution for at least 2 days. Brains were then sliced at 60 μm on a freezing microtome, and stained with Cresyl violet for verification of microinjection sites. Bilateral microinjection sites were placed on coronal slices from a rat brain

atlas and were used to extrapolate placements in a sagittal view of medial shell (Paxinos & Watson, 2007)

Statistical analysis

Effects of amphetamine injections on conditioned place preference were analyzed by comparing duration(s) spent in each chamber using 2-way within-subjects ANOVA (test day [pre vs. post] X drug condition [saline vs. amphetamine]). Additionally duration spent in each chamber following conditioning was compared using paired-samples T-test (post-test time spent in saline-paired chamber vs. post-test time spent in amphetamine-paired chamber). Effects of DNQX microinjections on conditioned place preference were analyzed by comparing duration(s) spent in each chamber using 3-way mixed ANOVA (test day [pre vs. post] X drug condition [vehicle vs. DNQX] X placement [far rostral vs. mid rostral]). Rats were then split by placement (far rostral and mid rostral) and effects observed in each microinjection placement respectively were analyzed using 2-way within-subjects ANOVA (test day [pre vs. post] X drug condition [vehicle vs. DNQX]). Food intake behaviors were each analyzed using paired-samples T-test and were then analyzed to determine any differences in behavior based on placement using 2-way ANOVA (behavior [DNQX vs. Vehicle] X Placement).

Results

Intraperitoneal Amphetamine injection causes a conditioned place preference when tested in the familiar home environment

Intraperitoneal amphetamine injection successfully conditioned a place preference in the rats' home environment. Rats (N=8), on average, increased their time spent in the amphetamine-paired chamber following conditioning and decreased their time spent in the saline-paired chamber following conditioning (interaction of drug X pre vs. post test, $F_{(1,7)} = 20.03$, $p = 0.003$;

Fig. 1A). The rats' percentage of time spent in the saline-paired chamber decreased from 46.38% before conditioning to 30.30% following conditioning (Fig. 1A). In actual time, rats spent 836.15 seconds in the saline-paired chamber before conditioning. Following conditioning, rats spent 545.37 seconds in the saline-paired chamber, a decrease of 290.77 seconds. Percentage of time spent in the amphetamine-paired chamber increased from 36.36% before conditioning to 50.33% following conditioning (Fig. 1A). Rats spent 655.95 seconds in the amphetamine-paired chamber prior to conditioning, and they increased their time spent in the amphetamine-paired chamber following conditioning by 250.15 seconds to an average of 906.10 seconds. Following conditioning, rats avoided the saline-paired chamber and preferred the amphetamine-paired chamber, and they spent more time in the amphetamine-paired chamber than in the saline-paired chamber (post-conditioning time spent in amphetamine-paired chamber vs. post-conditioning time spent in saline-paired chamber, $T_{(7)} = 2.24$, $p = 0.060$; Fig. 1A).

Far rostral sites elicit place preference while mid rostral sites elicit no reaction

Microinjections of the glutamate AMPA receptor antagonist DNQX into the far rostral shell of the nucleus accumbens caused a stronger place preference than microinjections into the mid rostral shell (main effect of microinjection placement on place preference, $t_{(7)} = 2.75$, $p = 0.029$; difference in pre vs. post time, interaction of drug and placement, $F_{(1,7)} = 7.55$, $p = 0.029$; Fig 1B and 2B). The place preference score (calculated as the difference between the percentage of time spent in the DNQX-paired chamber [post – pre] and the percentage of time spent in the vehicle-paired chamber [post – pre]) of rats with far rostral microinjection sites was on average 13.96%, demonstrating a positive place preference; in comparison, rats with mid rostral microinjection sites (N = 5) had a place preference score of -2.95 %, indicating a slight aversion to the DNQX-paired chamber, or no preference (Fig. 1B and 2B). While the place preference

conditioned in the home environment differed in far rostral microinjection sites and mid rostral microinjection sites, there was no difference in the strength of the conditioned place preference between rats conditioned with intraperitoneal amphetamine and rats conditioned with far rostral microinjection of DNQX into accumbens shell (Amphetamine place preference score versus Far rostral place preference score, $t_{(10)} = 1.53$, $p = 0.156$; Fig. 1B and 2B). The place preference conditioned by intraperitoneal amphetamine was stronger than the place preference conditioned by mid rostral DNQX microinjection in accumbens shell (Amphetamine place preference score versus Mid rostral place preference score, $t_{(11)} = 3.69$, $p = 0.004$; Fig. 1B and 2B).

On average, rats with far rostral microinjection sites (N = 4) spent 846.83 seconds in the vehicle-paired chamber before conditioning with DNQX and 596.16 seconds in the vehicle-paired chamber following conditioning, a decreased of 250.675 seconds less in the vehicle-paired chamber after conditioning. The rats spent 47.04% of their time in the vehicle-paired chamber prior to conditioning, and they spent 34.20% of their time in the vehicle-paired chamber following conditioning (Fig. 1C and 2B). Differently from time spent in the vehicle-paired chamber, rats' time spent in the DNQX-paired chamber was unchanged (interaction of drug X pre vs. post test, $F_{(1,3)} = 5.662$, $p = 0.098$; Fig. 1C). The rats spent 615.66 seconds (34.20% of their time in the testing apparatus) in the DNQX-paired chamber prior to conditioning, and they spent 616.20 seconds (34.23% of their time in the testing apparatus) in the DNQX-paired chamber following conditioning, virtually no change (Fig 1C and 2B).

Rats with mid rostral microinjection sites spent, on average, 857.76 seconds (47.64% of time spent in the testing apparatus) in the vehicle-paired chamber before conditioning and 793.43 seconds (44.10% of time spent in the testing apparatus) in the vehicle-paired chamber following conditioning (Fig. 1D and 2B). These rats spent 630.50 seconds (35.02% of time spent in the

testing apparatus) in the DNQX-paired chamber prior to conditioning and 513.63 seconds (28.53% of time spent in the testing apparatus) in the chamber following conditioning. The rats spent 63.82 seconds less in the vehicle-paired chamber following conditioning, and they spent 116.87 seconds less in the DNQX-paired chamber following conditioning (time spent in the DNQX-paired chamber X time spent in the Vehicle-paired chamber, $F_{(1,4)} = 0.99$, $p = 0.377$; Fig. 1D and 2B).

Additionally, one rat with a caudal microinjection placement spent a great amount of time in the DNQX-paired chamber following conditioning (1523.29 seconds, or 84.62% of time in the testing apparatus) compared to just 44.52 seconds (2.47% of time spent in the testing apparatus) spent in the vehicle-paired chamber following conditioning (Fig. 1E and 2B). Compared to the rat's pre-conditioning numbers (the rat spent 884.7 seconds [49.14%] in the DNQX-paired chamber and 624.95 seconds [34.71%] in the Vehicle-paired chamber), this rat increased its time spent in the DNQX-paired chamber and decreased its time spent in the vehicle-paired chamber (Fig. 1E and 2B). With only one caudal rat, however, no comparisons could be made. Additionally, because of the nature of the testing chamber and where the video camera was situated, it was impossible to determine if the rat's locomotive activity was reduced. It is likely that this rat simply fell asleep on one side of the chamber, which would explain why it spent such a majority of its time there.

DNQX microinjections increased eating behavior but did not affect other behaviors.

On vehicle, rats (N=5) did not exhibit any appetitive behavior. They all ate for 0 seconds and consequently did not ingest or carry any food. On DNQX, rats ate for, on average, 146.2 seconds. They consumed an average of 1.24 grams of food and carried their food an average of 4 times. DNQX microinjection caused increases in all three of these appetitive behaviors (food

intake: main effect of DNQX, $t_{(4)} = 1.619$, $p = 0.181$; Fig. 2A and 3A; eating time: main effect of DNQX, $t_{(4)} = 2.239$, $p = 0.089$; Fig. 2A and 3B; food carries: main effect of DNQX, $t_{(4)} = 2.384$, $p = 0.076$). None of these tests carried any statistical significance, but the p-values for time spent eating and food carries demonstrate a trend of increased eating behavior. Moreover, since the rats did not exhibit any of these behaviors following vehicle microinjection, it is clear that these behaviors, on average, did increase. The only eating behavior that was largely unaffected by DNQX was the amount of time rats sniffed their food, which demonstrated a minimal increase from 6.6 food sniffs on vehicle to 8.8 food sniffs on DNQX (food sniffs: main effect of DNQX, $t_{(4)} = 0.648$, $p = 0.553$). Rats with far rostral microinjection sites (N = 2) did not exhibit any eating behavior that differed from rats with mid rostral microinjection sites (N = 3) (eating time: DNQX – Vehicle X Placement, $F_{(1,3)} = 0.016$, $p = 0.906$; food intake: DNQX – Vehicle X Placement, $F_{(1,3)} = 0.220$, $p = 0.671$; food carries: DNQX – Vehicle X Placement, $F_{(1,3)} = 2.751$, $p = 0.196$). That far rostral and mid rostral microinjection sites had no difference in appetitive behavior differs from what we observed in the conditioned place preference tests in which there was a difference in behavior between rats with far rostral and mid rostral microinjection sites. DNQX microinjection into rostral accumbens shell can produce increased appetitive behavior, but only the far rostral portion of accumbens shell is susceptible to environmentally influenced conditioned place preference.

No rats exhibited any defensive behavior (treads, burrows or burrow-treads). Additionally, rats did not demonstrate any change in general locomotor activities (drinking time: main effect of DNQX, $T_{(4)} = 0.049$, $p = 0.963$; sleeping time: main effect of DNQX, $T_{(4)} = 0.473$, $p = 0.661$; grooming: main effect of DNQX, $T_{(4)} = 0.910$, $p = 0.414$; cage crosses: main effect of DNQX, $T_{(4)} = 0.110$, $p = 0.918$; rearing: main effect of DNQX, $T_{(4)} = 0.096$, $p = 0.928$). Drinking time

was almost identical on both DNQX (21.60 seconds spent drinking) and vehicle (21.80 seconds spent drinking). Rats slept for an average of 210.8 seconds on vehicle, and they slept for 165.00 seconds on DNQX. Rats groomed on average 5.8 times on vehicle and 8.00 times on DNQX. The amount of times that rats crossed their cage (front to back or back to front) was also largely identical for both experimental conditions. Rats cross 29.8 times on vehicle and 30.8 times on DNQX. Finally, rats reared 74.2 times on vehicle and 76.6 times on DNQX. With a sample size of only 5 rats (one rat was eliminated from statistical testing due to a brain infection), it is expected that the difference in eating behaviors exhibited within rats following DNQX and vehicle microinjection would be greater if more rats were included for testing.

Discussion

In the comfortable, home environment, DNQX microinjection in the nucleus accumbens established a conditioned place preference in rats with far rostral microinjection sites, but failed to do so in rats with mid rostral microinjection sites. This finding is of great importance because previous study found that DNQX at all sites in medial shell (even at far rostral sites) produced a conditioned place aversion, and we have found that under the right conditions DNQX in far rostral sites of the nucleus accumbens can produce a conditioned place preference. Following conditioning, rats should be able to make a decision on which side of the chamber is more rewarding (i.e. made them feel good or perhaps ‘less bad’), and that would be the chamber that they would spend more time in following conditioning. The home environment ought to serve as a means of making the DNQX-paired chamber a more soothing experience, therefore more rewarding and worth returning to during the post-conditioning test. The conditioned place preference observed in the rats with far rostral microinjection sites was a product of rats avoiding the vehicle-paired chamber, while demonstrating no real change in time spent in the DNQX-

paired chamber. The microinjection experience is generally a stressful one for rats, and through conditioning, they learned to associate the vehicle-paired chamber as an unpleasant experience. In contrast, rats did not associate the DNQX-paired chamber as an unpleasant experience. This would indicate that when DNQX is injected into the far rostral portion of the nucleus accumbens shell, the experience is made less aversive. Rats with mid rostral microinjection sites were conditioned to have a slight aversion to the DNQX-paired chamber, or no preference at all. Despite the fact that far rostral microinjections of DNQX into the nucleus accumbens can successfully condition a place preference and mid rostral microinjections cannot, DNQX microinjections at both sites increased appetitive behaviors (food intake, time spent eating and food carries), regardless of particular rostral location.

Since amphetamine has been demonstrated to have rewarding properties to rats, we used intraperitoneal amphetamine injections to demonstrate that rats could be conditioned to prefer a drug-paired chamber to a saline-paired chamber in the same testing conditions that were used for the DNQX conditioned place preference procedure. Following conditioning, rats increased their time spent in the amphetamine-paired chamber and decreased their time spent in the saline-paired chamber. This proves that these experimental conditions could produce a conditioned place preference, provided that the drug-paired chamber is a rewarding enough experience to combat the stressors of the test – similar to our findings of the DNQX-induced conditioned place preference in rats with far rostral microinjection sites.

DNQX acts antagonistically on AMPA receptors, ligand-gated ion channels that require glutamate to bind to the receptor for proper functioning (Wang et al, 2006). Glutamatergic inputs into the nucleus accumbens come from the medial prefrontal cortex, orbitofrontal cortex, hippocampus, and basolateral amygdala (Kelley, Domesick & Nauta, 1982; Groenewegen et al,

1999; Kelley, Baldo, Pratt & Will, 2005; Cardinal, Parkinson, Hall & Everitt, 2002). The prefrontal cortex has been implicated in complex cognitive behavior, emotion and decision-making, and the orbitofrontal cortex is a cortical region with extensive connections to the basolateral amygdala as well as other cortical and subcortical regions that might act in reward (Miller, Freedman & Wallis, 2002; Cardinal et al, 2002). So, the prefrontal cortex could be sending down signals to accumbens shell that determine whether environmental cues (the chamber and the microinjection experience) are of a negative or aversive nature. This could explain why rats find the conditioning experience so aversive. Only in the home environmental conditions could the prefrontal cortex send signals to the nucleus accumbens that weren't predisposed to be fearful. The basolateral amygdala, receiving projections from the prefrontal cortex, sends projections heavily to the nucleus accumbens shell and is involved in mediating the effects of emotional arousal and memory (Cardinal et al, 2002). The amygdala has also been discovered to have a significant role in fear conditioning, linking external stimuli to defensive responses (LeDoux, 2002). With such a heavy involvement in sending fearful signals, the projections that the amygdala sends to the nucleus accumbens could be predisposing the accumbens to determine signals as fearful. The hippocampus monosynaptically projects to the nucleus accumbens shell and could play a role in the fear conditioning process as it converts information from short-term to long-term memory (French and Totterdell, 2002). In this case, the hippocampus would be playing a role similar to the amygdala, sending fearful signals to the nucleus accumbens and potentially negating the positive effects on emotion that DNQX could have.

The ability of changes in environmental ambience to modify conditioned behaviors produced by glutamate circuits indicates that top-down processing could have a distinct role in

why DNQX fails to produce a conditioned place preference under standard laboratory conditions, and instead produces an aversion (Reynolds & Berridge, 2003). The experience of receiving the microinjection could be so unpleasant that the normal appetitive bias of rostral DNQX cannot overcome this. Conditioning the rats in their home environment is an attempt to combat the top-down processing that could be making rats fearful. By testing the rats in their home environment, they do not have to experience any stressors (i.e. traveling from their home to the test site or being placed in an unfamiliar room) other than the microinjection and conditioning experience, which are both unavoidable. The home environment can make the microinjection process a less aversive experience because of its familiarity in sounds and smells utilizing the same top-down processing from structures such as a prefrontal cortex, basolateral amygdala and the hippocampus. However, the top-down signals that are sent to the accumbens in the home environment could be telling the nucleus accumbens that the experience is not an aversive one.

Similar to the effects of environment on fear and feeding, corticolimbic circuits involving the nucleus accumbens may be utilizing the malleable nature of affective-generating functions (Reynolds & Berridge, 2008). In prior experiments, the environment in which a rat was placed in was able to influence the valence of motivated behavior along the rostrocaudal gradient of the accumbens shell (Reynolds & Berridge, 2008). Medial sites in the nucleus accumbens which, in the standard lab environment, generally produced a combination of both appetitive and fearful behavior could ‘switch’ based on the environment in which the rats were tested in: the home environment caused an increase in appetitive zones in the nucleus accumbens and the stressful environment, in which loud music was played and bright lights were utilized, caused an increase in fearful (defensive treading) zones. We try to take advantage of that ‘switch’ from fearful to desirable in the far rostral zones of the nucleus accumbens shell.

There are many potential signals that may interact with glutamate in the accumbens shell in order to change the valence of behavior. Local dopamine is essential for the motivational functions that are observed in the rostrocaudal gradient of the accumbens shell, in that blocking local endogenous dopamine prevents DNQX from generating appetitive eating or defensive behaviors (Faure, Reynolds, Richard & Berridge, 2008; Richard & Berridge, 2011). Specifically, D_1 dopamine receptors are involved in the rostral generation of eating, but D_1 and D_2 dopamine receptors must be utilized simultaneously for the generation of fearful behavior from caudal shell (Richard & Berridge, 2011). Environmental manipulation that yields increased eating in the home environment and increased defensive behavior in the stressful environment caused the roles of the dopamine receptors to switch to match the motivational valence generated (Reynolds & Berridge, 2008; Richard & Berridge, 2011). This dynamic nature of dopamine signaling to the nucleus accumbens can perhaps influence the valence of the behavioral response and be involved in how the calm, home environment can produce a conditioned place preference in far rostral accumbens shell. It could be the case that there is a decrease of D_2 dopamine signaling in the home environment. If this was true, the fearful signals from D_2 dopamine receptors may not be influencing caudal shell, which generates defensive and fearful behavior.

Opioids have a role in the hedonics involved during the consummatory phase of eating, as indicated by paw licks and orofacial reactions (Baldo & Kelley, 2007). Since opioid signals can affect how rats respond to appetitive food stimuli, they could also have an effect on how rats respond to the drug-paired or vehicle-paired chambers both during and after conditioning. Opioids differently mediate how rats ‘like’ and ‘want’ reward. The nucleus accumbens features small hedonic hotspots and coldspots for reward, which affect how much rats ‘like’ a reward (Peciña, 2008). However, the entire medial shell receives stimulation of ‘wanting’ from opioid

signals (Peciña, 2008). The hedonic hotspots and coldspots for opioid signals could be factoring into whether rats are perceiving environmentally stimuli as positive or negative, and this could potentially explain any shifts from conditioned preference to aversion or vice versa, as well as changes in unconditioned behaviors such as eating and treading.

Norepinephrine that projects to the caudal accumbens shell from the hindbrain is differently facilitated by D_1 and D_2 dopamine receptors and could potentially play in role in motivational valence (Vanderschuren, Wardeh, De Vries, Mulder & Schoffelmeer, 1999; Richard & Berridge, 2011). This has great relevance to the stress induced by the microinjection experience and the subsequent aversion that results, because fear is generated in the caudal nucleus accumbens shell. When stressed, the hindbrain produces norepinephrine, which could potentially be activating a fearful motivational state once it projects to neurons in the caudal shell of nucleus accumbens, a region that can produce fearful and aversive behavior (Reynolds & Berridge, 2001, 2002, 2003; Faure et al, 2010). Finally, metabotropic glutamate receptor blockade, specifically of Group II metabotropic glutamate receptors (mglu2/3), has been shown to suppress positively motivated behaviors such as feeding and 'liking', shifting the valence of these behaviors toward fear and disgust (Richard & Berridge. 2011). There is a component of displeasure or disgust in rats avoiding one or both of the chambers following conditioning. It could be that decreased activity at mglu2/3 receptors is a potential source of aversion to the DNQX-paired chamber in a standard or more stressful lab environment. Perhaps some component of the environmental condition can limit this effect, possibly increasing activity at metabotropic glutamate receptors, which may enable rats to not avoid the DNQX-paired chamber following microinjection of DNQX at far rostral nucleus accumbens sites and allow DNQX to produce a positive place preference.

The nucleus accumbens shell's output structures include the ventral tegmental area, ventral pallidum and lateral hypothalamus (Heimer & Van Hoesen, 2006; Zahm, 2006) The ventral pallidum receives GABAergic inputs from the nucleus accumbens and projects to the thalamus, which projects to the prefrontal cortex in a limbic loop of the basal ganglia – a pathway involved in the regulation of motivated behaviors and emotion (Zahm, 2006). When the nucleus accumbens is hyperpolarized, it does not send GABA to the ventral pallidum, leaving it receptive to depolarization and allowing ventral pallidum to potentially increase appetitive motivation and 'liking'. Also part of the limbic loop, the ventral tegmental area receives inhibitory inputs from the nucleus accumbens and may have a role in avoidance and fear from the inputs it receives from the amygdala (Heimer & Van Hoesen 2006, Zahm, 2006). The fearful signals are processed in a similar limbic loop and eventually are projected back to the nucleus accumbens. Finally, the lateral hypothalamus receives inputs from the nucleus accumbens and releases endocrine hormones, many of which lead to changed appetitive function (Maldonado-Irizarry et al, 1995). A nucleus accumbens hyperpolarization prevents the hypothalamus from receiving inhibitory GABA signaling from the accumbens, potentially releasing lateral hypothalamic activity to produce robust eating. However, the expression of the feeding response to increase hypothalamic activity depends on an NMDA-receptor-mediated activation of lateral hypothalamic neurons and is not just the result of inhibiting the GABA transmission (Stratford & Kelley, 1999). So, glutamate inputs to the hypothalamus have an important role to increase appetitive motivation resulting from lateral hypothalamus stimulation just as the blocking of GABA inputs does.

There are a few potential shortcomings in our experiment. As previously mentioned, the testing paradigm is very stressful for rats. We have previously discussed how sensitive the nature

of the cortical glutamate circuit and how sensitive it is to top-down processing. One potential solution would be for the experimenter to handle the rats for a greater amount of time prior to conditioning. This could possibly eliminate or at least reduce the aversive nature of the experimental procedure. Another solution would be to uncover a method to make the microinjection process an easier one to endure. In addition to the microinjection experience being not being pleasurable, the chambers in which the rats are confined to are small and enclosed. The three-compartment chamber features the two microinjection-paired chambers (DNQX and vehicle, respectively) surrounding a smaller, third chamber. Many rats seem to generally avoid both microinjection-paired chambers. One way in which to force the rats to demonstrate which chamber is more pleasurable or aversive would be to either eliminate or minimalize the area in the middle in which rats tend to hover around. This would also help to rectify a problem that sometimes occurred during videoscoring. Rats would often rear (lift up their front paws) right at the edge of the middle chamber and a drug-paired chamber, making it difficult to decipher which chamber a rat should be classified in. This could also explain why the time spent in the middle increased following conditioning. Prior to conditioning, rats did not have any preference or aversion to either chamber, and that might be why they spent less time hovering around the middle. A common trend observed throughout testing was that rats generally preferred the chamber cleaned with Versa-clean featuring black-colored walls and a wire grid floor over the chamber cleaned with 70% Ethanol featuring white-colored walls and a wire mesh floor. When rats have a predisposition for one chamber or the other it could impact the effectiveness of the conditioning process. Finally, during the food intake experiment, rats did not eat under vehicle conditions. In general, rats eat small, but consistent amounts of approximately 1 gram of food following vehicle microinjection (Reynolds and Berridge, 2002, 2003, 2008;

Faure et al, 2008; Richard and Berridge, 2011). This is likely just a product of a small sample size of rats, but it could have impacted the change from vehicle experienced from DNQX. Moreover, DNQX did not cause as robust eating increases as in prior experiments (Reynolds & Berridge, 2003). It is possibly that there was some issue with the drug or microinjections themselves. More rats would increase the power of all statistical tests, but that would not necessarily change our results. Rats conditioned in the standard environment using a similar protocol were conditioned to have an aversion to the DNQX-paired chamber (Reynolds & Berridge, 2003). The rostrocaudal gradient of positive to negative motivational function seen in eating was somewhat observed as the far rostral sites of the accumbens had a smaller aversion than mid rostral sites or caudal sites (Reynolds & Berridge, 2003). The ability of a familiar environment to enable DNQX to produce a conditioned place preference is consistent with previous reports that a comfortable and familiar environment can retune the valence of unconditioned motivated behaviors; for example, in a home environment DNQX produces mostly eating behaviors rather than fearful behaviors, even in some zones that produce fearful behavior in the standard environment (Reynolds & Berridge, 2008).

We have shown that DNQX manipulation of the nucleus accumbens can produce a place preference under certain conditions even though it produces a place aversion under most conditions. DNQX may be producing a motivational salience that can be transformed into different states. Then, under the right conditions it could be turned into a conditioned place preference (incentive salience) such as in the home environment or a conditioned place aversion (fearful salience) such as in the standard – and likely, the stressful – environment. It would be expected that a place preference test in a stressful environment using DNQX manipulation of the nucleus accumbens would yield fearful salience. The conditioning experiment is already stressful

to rats, so conditioning in an environment with loud, bright and unfamiliar stimuli would be expected to produce an even greater aversion than conditioning in the standard environment produce. This would be consistent with the increase defensive treading behavior observed in rats that underwent unconditioned motivational behavior testing in a stressful environment (Reynolds & Berridge, 2008).

Motivational salience as a potential component of incentive and fearful salience has clinical implications as well. A person who has problems with motivational salience could have trouble balancing incentive and fearful salience. Past research stressing the involvement of the dopaminergic systems in both schizophrenia and the abuse of psychoactive drugs has shown that a high degree of comorbidity exists between addiction, potentially involving intense, incentive salience, and schizophrenia, potentially involving intense, fearful salience (Batel, 2000; Kapur, 2003). Additionally it is hypothesized that the shared vulnerability to different forms of intense motivational salience could be responsible for why many people with schizophrenia are more susceptible to develop addiction to medication (Batel, 2000). This effect was also seen in experiments that tested environmental manipulation on the valence of appetite and fear as some rats were observed to have bouts of stress-induced eating (Reynolds & Berridge, 2008; Richard & Berridge, 2011). Binge eating followed immediately by defensive treading is a demonstrating of motivational salience that is out of balance, and the brain would have difficulty managing the different signals of incentive and fearful salience.

A further example of conflicting motivational salience can be seen in ‘sign-tracking’ rats that attribute high incentive salience to appetitive cues have a propensity to attribute fearful salience to Pavlovian conditioning of fearful stimuli (Morrow, Maren & Robinson, 2011). While it makes sense that those who attribute high incentive salience to appetitive cues would be

susceptible to addiction, this implies that they also may be susceptible to disorders of fearful salience such as schizophrenia, Post-traumatic stress disorder (PTSD) or anxiety disorders. More research on the subject of comorbidity of disease has demonstrated that people who suffer from schizophrenia also have higher rates of obesity (Elman, Borsook & Lukas, 2006). This is an example of motivational salience that can flip in the opposite direction in which people who have fearful disorders of negative motivation can develop incentive disorders of too much positive motivation. The idea of a fragile state of motivational salience in individuals with disorders of too much positive or negative salience can begin to explain why those who suffer from schizophrenia can develop addiction and why those who suffer from addiction could develop paranoid tendencies. A further understanding of the comorbidity of these diseases would enable future research to uncover what neurobiological mechanisms may underlie the switch from positive to negative salience. We are starting to understand one variable that could contribute to the switching of motivational salience: the environment. Coupled with a future understanding of the mechanisms underlying the switch of affective valence, we could begin to develop new ideas for treatment, cure and prevention of motivational disorders both in individuals who have demonstrated to be susceptible to these diseases.

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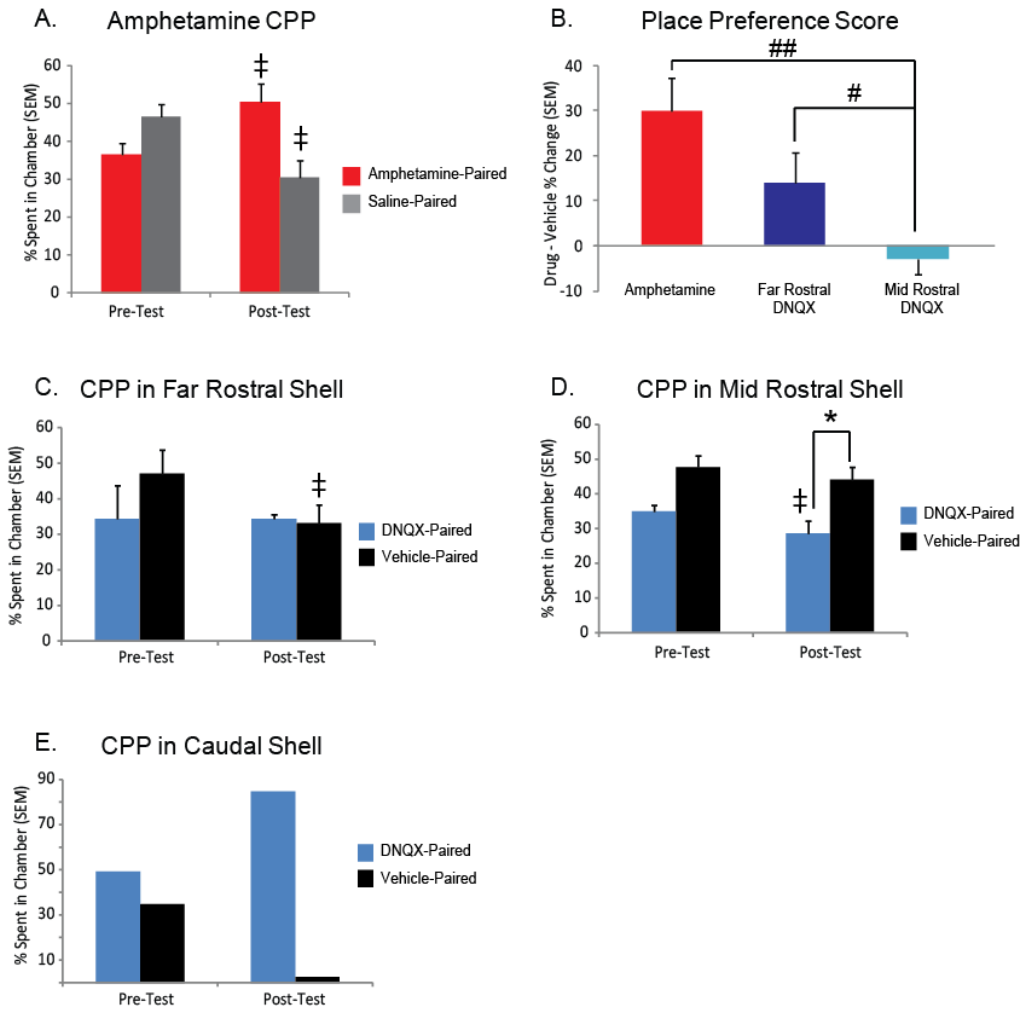

Figure 1. (A) The amphetamine validation in the familiar, home environment. (B) Place preference score in far rostral and mid rostral accumbens shell. (C) Conditioned place preference in far rostral shell comparing time spent in the DNQX-paired chamber and vehicle-paired chamber in the pre and post-test. (D) Conditioned place preference in mid rostral shell comparing time spent in the DNQX-paired chamber and vehicle-paired chamber in the pre and post-test. (E) Conditioned place preference in caudal shell comparing time spent in the DNQX-paired chamber and vehicle-paired chamber in the pre and post-test. ‡, $p < .05$ pre versus post test; ‡‡, $p < .01$ pre versus post test; *, $p < .05$ drug versus vehicle; **, $p < .01$ drug versus vehicle; #, $p < .05$ between group difference; ##, $p < .01$ between group difference.

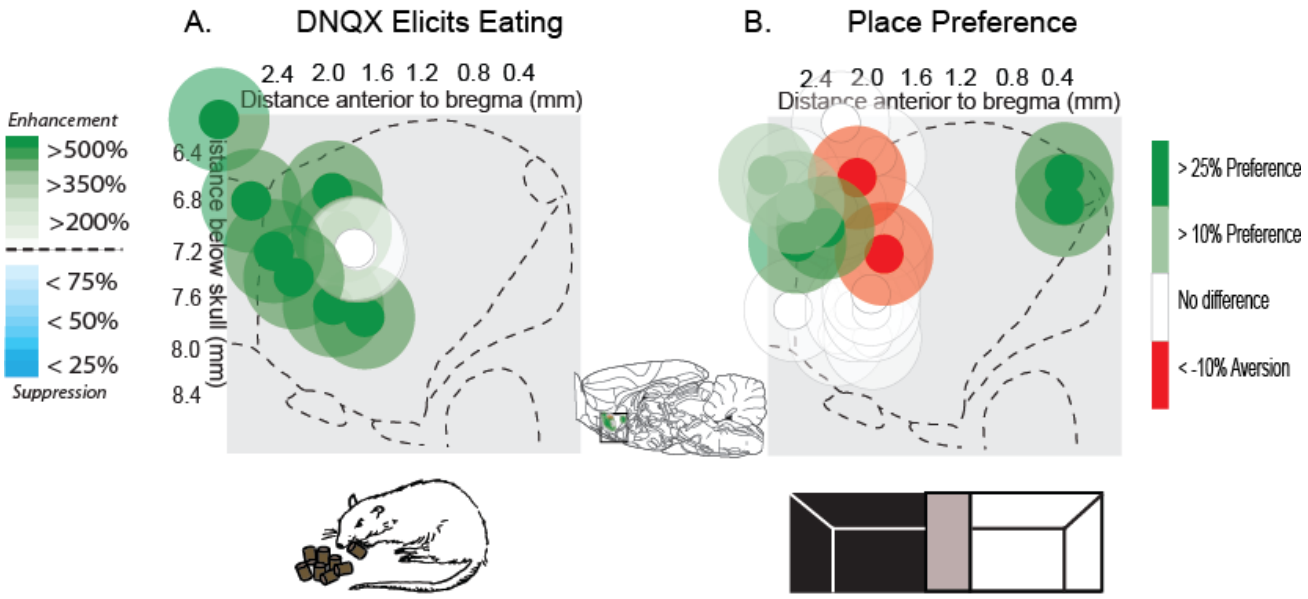

Figure 2. (A) Fos plume map indicating the amount of eating induced by DNQX by placement in nucleus accumbens shell. The colors represent the amount of percent change from vehicle of eating under DNQX and correspond to the placement of a microinjection site. (B) Fos plume map indicating the place preference following conditioning in the familiar environment. The colors represent the place preference score, calculated as DNQX – Vehicle % Change.

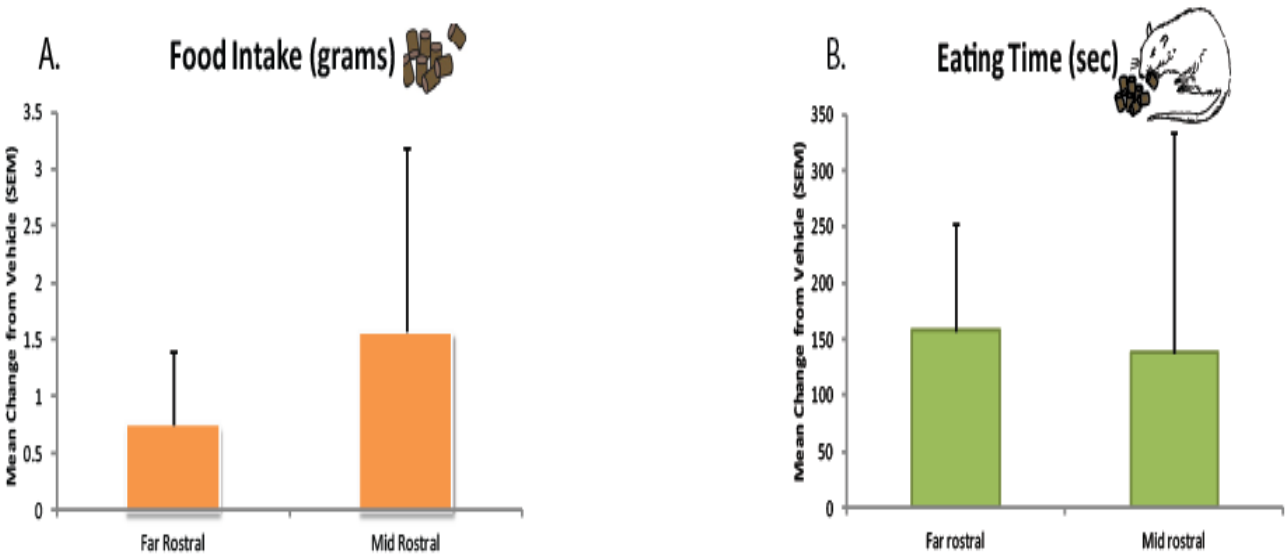

Figure 3. (A) The grams of food eaten calculated as the mean change from vehicle. (B) The time spent eating in seconds calculated as the mean change from vehicle.