

NMDA Stimulation and AMPA Blockade in the Nucleus Accumbens

Shell Generate Appetitive and Fearful Motivation

by

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## Abstract

Glutamate signals in the nucleus accumbens shell play an important role in generating appetitive and fearful motivation. Previous studies have indicated that AMPA/kainate blockade generates motivation along a rostrocaudal gradient that shifts from appetitive motivation in rostral shell to fearful motivation in caudal shell. This study compared the effects of AMPA/kainate blockade and NMDA stimulation in rostral and caudal regions of shell. Microinjections of the AMPA/kainate receptor antagonist CNQX produced intense eating throughout shell, and active fear in the form of defensive treading or burying in caudal shell. NMDA receptor activation was achieved using NMDA and D-serine, an agonist at the glycine site on NMDA receptors. Stimulation of NMDA receptors increased eating only in caudal shell, but produced freezing throughout shell. In addition to producing different types of fear behaviors, CNQX also increased eating significantly more than NMDA activation did. These results indicate that while both AMPA/kainate receptor blockade and NMDA stimulation in the nucleus accumbens shell are capable of producing appetitive and fearful motivation, they affect feeding and fear behaviors differently.

## NMDA Stimulation and AMPA Blockade in the Nucleus Accumbens

### Shell Generate Appetitive and Fearful Motivation

The nucleus accumbens shell plays an important role in the generation of motivated behaviors. For instance, dopamine, glutamate, and GABA in the nucleus accumbens have been associated with both appetitive and aversive behaviors (Berridge, Mitton, Clark, & Roth, 1999; Horvitz, 2000; Salamone, 1994; Reynolds & Berridge, 2001, 2002, 2003; Faure, Reynolds, Richard, & Berridge, 2008; Faure, Richard, & Berridge, 2010). Enhancing GABAergic signals via muscimol or disrupting glutamatergic signals via DNQX in the nucleus accumbens shell both produce a rostrocaudal gradient of motivation that shifts from appetitive motivation in rostral shell to fearful motivation in caudal shell (Reynolds & Berridge, 2001, 2002, 2003; Faure et al., 2008, 2010). It is important to understand how the nucleus accumbens generates motivated behavior because dysregulated motivation may lead to mental health conditions such as schizophrenia or drug addiction, which are of great cost to both individuals and society. The cost of schizophrenia in the United States was 62.7 billion dollars in 2002 (Wu et al., 2005). Substance abuse in the United States costs 534 billion dollars annually (National Institute on Drug Abuse, 2008). Drug addiction creates persistent changes in nucleus accumbens circuitry, which alters the way the brain attributes salience (Robinson & Berridge, 2003). By making drugs and their associated cues especially salient, neuronal alterations involving the nucleus accumbens may be responsible for the excessive motivation to seek and take drugs that is present in addiction (Robinson & Berridge, 2003). Dysfunction of salience attribution and motivational systems may also be involved in schizophrenia (Kapur, 2003). Patients with schizophrenia may attribute excessive motivational salience to non-threatening and irrelevant stimuli leading to the delusions and hallucinations that are characteristic of schizophrenia (Kapur, 2003).

The nucleus accumbens shell receives glutamatergic projections from cortex and cortical-like structures, such as the medial prefrontal cortex, anterior cingulate cortex, basolateral amygdala, and hippocampus (Morgane, Galler, & Mokler, 2005; Faure et al., 2010). The nucleus accumbens, therefore, receives information from a variety of structures involved in motivation, emotion, and executive control. Glutamate from these sources can act at AMPA, kainate, or NMDA receptors in accumbens shell (Meredith, Baldo, Andrezjewski, & Kelley, 2008; Tarazi, Campbell, Yeghiayan, & Baldessarini, 1998b). An increased release of glutamate from the prefrontal cortex to the nucleus accumbens may be responsible for the unmanageable motivation associated with drug seeking in addiction (Kalivas, Volkow, & Seamans, 2005). Additionally, drug seeking and plasticity, which may be related to behavioral sensitization, depend on glutamate transmission in the nucleus accumbens (Morgane et al., 2005). Sensitization occurs due to increased activation of NMDA receptors, which can affect dopamine release in the nucleus accumbens (Morgane et al., 2005), and NMDA antagonists can inhibit amphetamine-induced sensitization (Vanderschuren & Kalivas, 2000). Drug addiction and schizophrenia may also be related to abnormal AMPA to NMDA ratios (Thomas, Beurrier, Bonci, & Malenka, 2001; Wolf et al., 2005). Decreased AMPA receptor binding and reduced NMDA receptor activity in the nucleus accumbens has been found in patients with schizophrenia (Coyle, 2006; Noga & Wang, 2002). People with schizophrenia also typically have reduced glutamate innervation to the nucleus accumbens (Aparicio-Legarza, Cutts, Davis, & Reynolds, 1997). Yet, the particular roles of AMPA versus NMDA receptors in the generation of aberrant motivation remain relatively unknown.

The nucleus accumbens also plays a clear role in eating and appetitive behavior. The nucleus accumbens receives taste information from the nucleus of the solitary tract and gustatory

thalamus (Kelley, 2004). It also has reciprocal projections with the lateral hypothalamus, which is important for regulating food intake (Kelley, 2004; Meredith et al., 2008). Activation of GABA receptors in the anterior nucleus accumbens shell elicits robust increases in food intake (Stratford & Kelley, 1997; Pulman, Somerville, & Clifton, 2010; Reynolds & Berridge, 2001; Faure et al., 2010). Since the nucleus accumbens is composed primarily of medium spiny neurons that use GABA as a neurotransmitter (Meredith et al., 2008), it is possible that GABA's effect on food intake is due to reduced activity of the nucleus accumbens shell, thereby, releasing a downstream structure, such as the lateral hypothalamus, from tonic GABAergic inhibition (Echo et al., 2001). If a release of inhibition to a downstream structure is the mechanism responsible for the effects of GABA stimulation within accumbens shell, then glutamate blockade would be expected to have a similar effect (Echo et al., 2001). Blocking the excitatory influence of glutamate or increasing the inhibitory influence of GABA should both reduce activity in the nucleus accumbens shell (Echo et al., 2001).

Consistent with this hypothesis, blockade of AMPA/kainate receptors with DNQX, CNQX, or NBQX in the anterior portion of the nucleus accumbens shell greatly increases eating (Maldonado-Irizarry, Swanson, & Kelley, 1995). DNQX in the anterior nucleus accumbens shell increases intake of both liquid and solid food, but not gnawing or water intake (Stratford, Swanson, & Kelley, 1998). Reynolds and Berridge (2003) observed that DNQX generated a rostrocaudal gradient of motivation with the rostral shell proving especially important for eating. Echo and colleagues (2001), however, found that AMPA in the nucleus accumbens shell, which would be expected to increase activity of the inhibitory medium spiny neurons, also increased eating. Although the nucleus accumbens shell and lateral hypothalamus do interact to regulate eating, it is more likely due to an indirect glutamatergic projection as opposed to the direct

GABAergic projections from the nucleus accumbens shell to the lateral hypothalamus (Stratford & Kelley, 1999). Stratford and Kelley (1999) found that GABA blockade in the lateral hypothalamus, which should perform a similar action to the reduced firing of GABAergic projections originating in the nucleus accumbens shell, failed to alter eating. NMDA receptor blockade in the lateral hypothalamus, however, negated the enhanced eating caused by nucleus accumbens shell inhibition (Stratford & Kelley, 1999).

NMDA blockade in the nucleus accumbens shell, instead of enhancing eating in a manner similar to AMPA/kainate blockade, failed to increase food intake (Maldonado-Irizarry et al., 1995), and at high doses the NMDA antagonist MK-801 decreased eating in rostral and caudal shell (Reynolds & Berridge, 2003). Echo and colleagues (2001) found that NMDA activation in the nucleus accumbens shell increased eating; however, they also observed some differences between the eating that was evoked by AMPA compared to NMDA. AMPA increased eating more than NMDA did, and NMDA-induced eating was shorter in duration and latency (Echo et al., 2001).

In addition to its widely accepted role in appetitive behavior, the nucleus accumbens has also been linked to fear responses, such as freezing in response to an electric shock (Saulskaya & Marsden, 1995; Saulskaya, Fofonova, & Sudorgina, 2010). These fear responses are associated with altered glutamate and citrulline levels in the nucleus accumbens (Saulskaya & Marsden, 1995; Saulskaya et al., 2010). Aversive stimuli, such as tail pinch, tail shock, foot shock, and restraint stress, elevate dopamine levels in the nucleus accumbens (Berridge et al., 1999; Horvitz, 2000; Salamone, 1994). Aversive stimuli also result in the release of glucocorticoids, which can elevate dopamine levels as well as increase AMPA to NMDA ratios in the nucleus accumbens (Campioni, Xu, & McGehee, 2009). Both GABA stimulation (via muscimol) and AMPA

blockade (via DNQX) generate a motivational gradient with the rostral region of the nucleus accumbens shell greatly increasing food intake, but the caudal region producing active fear (Reynolds & Berridge, 2001, 2002, 2003; Faure et al., 2010). Microinjections of muscimol or DNQX in caudal shell result in bite attempts, escape attempts, distress vocalizations, and defensive treading (Reynolds & Berridge, 2001, 2002, 2003). Defensive treading, or burying, is an active defensive behavior performed by rodents in the presence of predators; it involves a vigorous back and forth motion of the forepaws resulting in sand or bedding being sprayed (De Boer & Koolhaas, 2003; Treit, Pinel, & Fibiger, 1981). Rodents will also perform defensive treading in a laboratory setting in order to bury an electric shock prod (De Boer & Koolhaas, 2003). Defensive treading has frequently been used as a model in the preclinical study of human fear, and it can be suppressed by several anxiolytic drugs (De Boer & Koolhaas, 2003).

Both GABA and DNQX cause defensive treading when microinjected into the caudal region of the nucleus accumbens shell (Reynolds & Berridge, 2003). While the NMDA antagonist MK-801 also produces defensive treading, it does so in both rostral and caudal shell, and it does not produce distress vocalizations or escape attempts (Reynolds & Berridge, 2003). This pattern may indicate that NMDA glutamate receptors are less capable of generating robust appetitive or aversive motivation in comparison to AMPA glutamate receptors. Yet, the role of *increased* activity at NMDA receptors in the generation of fear and feeding remains unknown.

The purpose of this experiment was to examine and compare the effects that AMPA/kainate blockade and NMDA stimulation have on motivated behavior, and to see if they would produce similar rostrocaudal gradients of eating to defensive behavior in nucleus accumbens shell. The AMPA/kainate receptor antagonist CNQX was tested in the nucleus accumbens shell in order to compare its effects on motivated behavior to DNQX, which

generates a rostrocaudal gradient of motivational behavior (Reynolds & Berridge, 2003). DNQX in rostral shell enhanced the amount of feeding, but DNQX in caudal shell increased the active fear measure of defensive treading. Here, CNQX in the nucleus accumbens shell greatly enhanced appetitive motivation, and, similarly to DNQX, only increased defensive treading in caudal shell.

D-serine, an agonist at the glycine site of NMDA receptors, was originally used alone to test the effects of NMDA receptor activation in the nucleus accumbens shell on motivated behavior. However, it failed to produce significant effects on motivated behavior. Since D-serine alone failed to significantly alter motivated behavior and 0.5  $\mu\text{g}$  NMDA produced intense, but very short, reactions (J. M. Richard, unpublished observations), lower doses of NMDA alone and in combination with 10  $\mu\text{g}$  D-serine, which appeared to be the most effective D-serine condition, were tested. While NMDA + D-serine increased feeding, consistent with the eating enhancement reported by Echo and colleagues (2001), it had no effect on defensive treading, even in caudal shell. It did, however, increase the passive fear measure of freezing. In order to fully characterize the active defensive fear produced by CNQX, and the seemingly passive fear produced by NMDA activation, it was important to compare the generation of motivated behavior by AMPA antagonist and NMDA agonist microinjections in the same animals. Although both CNQX and NMDA + D-serine enhanced eating, CNQX generated a much more robust increase in comparison to the effects of NMDA + D-serine, which were limited to the caudal region of the shell. These results indicate that AMPA/kainate receptor blockade and NMDA receptor activation may alter eating via distinct mechanisms.

## **Method**

### **Experiment Design**



The roles of AMPA/kainate blockade and NMDA stimulation, at the glycine site and the NMDA site, in the nucleus accumbens shell were investigated using four different sets of microinjections. AMPA/kainate blockade and NMDA stimulation were initially tested in separate groups of rats to determine the effectiveness of a variety of drug doses. The doses of NMDA with D-serine (NMDA agonists) and CNQX (an AMPA/kainate antagonist) that most effectively elicited motivated behavior were then tested for direct comparison in another group of rats. This setup allowed us to compare the effects of AMPA/kainate blockade and NMDA activation at the same microinjection sites in the same rats, while reducing the amount of site damage by limiting the number of microinjections to 5 per rat. Additional rats were used to examine the Fos expression caused by AMPA/kainate blockade and NMDA stimulation. Fos analysis provides a reasonable estimate of drug spread, which can be used to map the area where NMDA stimulation and AMPA/kainate blockade had the largest impact on appetitive and fearful motivation.

### **Animals**

Male Sprague Dawley rats (n = 46, 270-540 g at time of surgery; behavioral testing, n = 35; Fos plume, n = 11) were housed on a 12 h light/dark reverse cycle (lights on at 9:00 pm) in a climate-controlled room (~ 21 °C) with *ad libitum* access to food and water. UCUCA approved the use of these rats and the protocol for this experiment.

### **Surgery**

Each rat was handled for a total of 20 min spread over 2 days prior to surgery. Rats were anesthetized with a ketamine (80 mg/kg) and xylazine (5 mg/kg) mixture and injected with atropine (0.04 mg/kg) to prevent respiratory distress. Rats were placed in a stereotaxic device with the incisor bar set at 5.0 mm above interaural zero to avoid hitting the lateral ventricles.

Stainless steel microinjection guide cannulae (14 mm, 23 gauge) were implanted bilaterally, aimed 2 mm above target sites in the nucleus accumbens shell because the microinjection cannulae extend 2 mm beyond the guide cannulae. The coordinates for the cannulae placements were anteroposterior (AP) = +2.1-3.1 mm anterior to bregma, mediolateral (ML) =  $\pm$ 0.8-1.0 mm lateral to midline, and dorsoventral (DV) = -5.6-5.7 mm below the skull. A variety of coordinates were used in an attempt to examine the full extent of medial nucleus accumbens shell.

Placements that fell outside the nucleus accumbens shell were used as anatomical controls. The anatomical controls included bilateral placements in the nucleus accumbens core (n = 1) and lateral septal nucleus (n = 2) as well as unilateral placements in the lateral septal nucleus (n = 2), lateral ventricle (n = 1), navicular nucleus (n = 1), and nucleus accumbens core (n = 2).

Microinjection guide cannulae were stabilized with four bone screws and acrylic cement.

Immediately following surgery, rats were injected with chloramphenicol sodium succinate (60 mg/kg) to prevent infection, and a stainless steel stylet was placed in the cannulae to prevent occlusion. Carprofen (5 mg/kg) was administered immediately after surgery and 24 h post-surgery for analgesia. Rats were given 7 days to recover from surgery before behavioral testing began.

### **Drugs and Microinjections**

Pilot testing with microinjections of 0.5  $\mu$ g NMDA, which had previously been shown to produce enhanced eating (Echo et al., 2001), resulted in intense fear-like/panic behavior when microinjections were given at caudal sites (J.M. Richard, unpublished observations). These reactions consisted of intense distress vocalizations (rats engaged in prolonged and repeated high-pitched vocalizations) and escape attempts (rats ran rapidly up the experimenter or sides of the cage, and occasionally jumped out of the cage), and they were both short in latency

(occurring during the microinjection) and short in duration. We, therefore, wanted to examine the effects of what we thought would be a more subtle manipulation, stimulation of the glycine site of the NMDA receptors. Binding at the glycine site increases the recovery rate from desensitization and lengthens the decay time constant of EPSPs (Yang & Svensson, 2008), so we thought it may produce a more suitable timing of effects. For this reason, one group of rats was tested with varying doses of D-serine, an agonist at the glycine site of NMDA receptors (Sigma, St. Louis, MO). Each rat in this group (n = 12; nucleus accumbens shell, n = 9; lateral septal nucleus, n = 2; nucleus accumbens core and lateral septal nucleus, n = 1) received bilateral microinjections of D-serine dissolved in 0.15 M saline (0.5, 2, 10, and 50  $\mu\text{g}/0.5 \mu\text{l}$  per side) as well as a vehicle microinjection consisting only of 0.15 M saline. One of the rats in this group was euthanized after 3 testing sessions due to an abdominal infection and did not receive the 0.5  $\mu\text{g}$  or the 50  $\mu\text{g}$  dose of D-serine.

Reynolds and Berridge (2003) previously found that blocking AMPA/kainate receptors in the nucleus accumbens shell with DNQX generated appetitive and fearful motivation along a rostrocaudal gradient. DNQX-induced eating showed a rostral shell advantage and DNQX-induced fear showed a caudal shell advantage. We used a different AMPA/kainate receptor antagonist, CNQX (6-Cyano-7-nitroquinoxaline-2,3-dione, Sigma, St. Louis, MO), on a second group of rats to see if it would yield similar effects on motivated behavior. Each rat in this group (n = 6; nucleus accumbens shell, n = 5; nucleus accumbens core, n = 1) received bilateral microinjections of CNQX dissolved in 50% DMSO/50% 0.15 M saline vehicle (0.1, 0.25, 0.5, and 1.5  $\mu\text{g}/0.5 \mu\text{l}$  per side) as well as a vehicle microinjection consisting only of 50% DMSO/50% 0.15 M saline. The 0.5  $\mu\text{g}$  CNQX dose appeared to have the biggest impact on

motivated behavior and was used in the last group of rats for comparison with NMDA and D-serine.

Since D-serine did not produce strong effects on motivated behavior on its own (results discussed below) and the 0.5  $\mu\text{g}$  NMDA dose from the pilot study produced effects that were short in latency and duration, a third group of rats ( $n = 6$ ; nucleus accumbens shell,  $n = 6$ ) received bilateral microinjections of the following combinations of D-serine and lower doses of NMDA: NMDA dissolved in 0.15 M saline (0.125 and 0.25  $\mu\text{g}/0.5 \mu\text{l}$  per side), NMDA with D-serine dissolved in 0.15 M saline (0.125  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine/ $0.5 \mu\text{l}$  per side and 0.25  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine/ $0.5 \mu\text{l}$  per side), and a vehicle microinjection of 0.15 M saline. The 10  $\mu\text{g}$  dose of D-serine was chosen because it seemed to be the most effective dosage used in the D-serine group. Because microinjections of 0.25  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine proved to be most effective in eliciting motivated behavior, they were used in the last group of rats, for comparison with CNQX.

In order to compare behavior elicited by AMPA/kainate blockade and NMDA stimulation at the same microinjection sites within medial nucleus accumbens shell, a fourth group of rats ( $n = 11$ ; nucleus accumbens shell,  $n = 9$ ; nucleus accumbens core and navicular nucleus,  $n = 1$ ; lateral septal nucleus and lateral ventricle,  $n = 1$ ) received bilateral microinjections of CNQX dissolved in 50% DMSO/50% 0.15 M saline (0.5  $\mu\text{g}/0.5 \mu\text{l}$  per side), NMDA with D-serine dissolved in 0.15 M saline (0.25  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine/ $0.5 \mu\text{l}$  per side), and two vehicle microinjections. One vehicle microinjection was 0.15 M saline because the NMDA with D-serine was dissolved in 0.15 M saline, and the other vehicle microinjection was 50% DMSO/50% 0.15 M saline because the CNQX was dissolved in 50% DMSO/50% 0.15 M saline. The 0.5  $\mu\text{g}$  CNQX dose and the 0.25  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine dose were chosen

because they seemed to be the most effective doses in their respective groups. This combination of microinjections allowed direct comparisons of AMPA/kainate blockade and NMDA stimulation at the same microinjection site, in the same rats.

After surgery, each rat was handled for a total of 20 minutes spread over 2 days and habituated to the test cages for 3 days. Habituation ensured that any observed behavioral effect was not due to being in a novel environment. The rats were given a vehicle microinjection of 0.15 M saline 1 day after habituation was completed. During microinjection sessions, rats were gently held while microinjection cannulae (16 mm, 29 gauge) were inserted into the guide cannulae. The microinjection cannulae extended 2 mm beyond the guide cannulae into the nucleus accumbens shell and were attached to a syringe pump by PE-20 tubing. Rats received microinjections of 0.5  $\mu$ l at a rate of 0.3  $\mu$ l/min. The microinjection cannulae were left in for 60 sec following the microinjection to allow for drug diffusion. The stylets were cleaned and inserted back into the guide cannulae. The rat was then placed into a testing cage. The drug order was counterbalanced for each group of rats, and microinjection sessions were spaced 48 h apart.

### **Behavioral Testing**

Prior to microinjections, transparent testing cages were set up with corncob bedding, an accessible water bottle, and pre-weighed food ( $20 \pm 2$  g). Each rat received one of the possible microinjections and was placed in a testing cage for 60 min. The rat was videotaped while in the testing cage for future behavioral analysis. After 60 min, the rats were returned to their home cages and the food in the testing cages was re-weighed. The rats in the NMDA group (0.15 M saline, 0.125  $\mu$ g NMDA, 0.25  $\mu$ g NMDA, 0.125  $\mu$ g NMDA + 10  $\mu$ g D-serine, and 0.25  $\mu$ g NMDA + 10  $\mu$ g D-serine) and the rats in the NMDA and CNQX group (0.15 M Saline, 50% DMSO/50% 0.15 M saline, 0.5  $\mu$ g CNQX, and 0.25  $\mu$ g NMDA + 10  $\mu$ g D-serine) were also

videotaped during the microinjections in order to score the distress vocalizations and escape attempts that rats typically made during NMDA microinjections.

### **Behavioral Analysis**

All rats had their testing cage videotape analyzed for the number of treading bouts (shoving the bedding by a rapid backward and forward movement of one or both forepaws), as well as appetitive behaviors, such as the amount of food eaten (in grams), time spent eating (a 5 sec break was counted as a new bout), time spent drinking (a 5 sec break was counted as a new bout), number of food sniffs (rat sniffs near the food), and the number of food carries (food must be picked up in the mouth and moved). A variety of other behaviors were also scored, including time spent sleeping (rat must remain motionless in a resting position for at least a minute, and a 30 sec break was considered a new bout), number of grooming bouts (action chain characterized by bilateral paw strokes over the snout, unilateral paw strokes over the snout, paw strokes at the top of the head, and flank licks), number of cage crosses (crossing the midline of the cage), number of burrows (rat buries its head in the bedding and pushes forward), number of burrow-treads (burrowing and treading occurring simultaneously), and number of rears (rat raises its forepaws at least an inch in the air for at least 0.5 sec). This set of behaviors, and the criteria for their coding was based on previous work examining appetitive and defensive behavior in the same session (Reynolds & Berridge, 2001, 2002, 2003).

Because some rats who received NMDA microinjections exhibited fear-like behaviors not normally captured in our previous scoring system, the rats in the NMDA group (0.15 M saline, 0.125  $\mu$ g NMDA, 0.25  $\mu$ g NMDA, 0.125  $\mu$ g NMDA + 10  $\mu$ g D-serine, and 0.25  $\mu$ g NMDA + 10  $\mu$ g D-serine) and the rats in the NMDA and CNQX group (0.15 M Saline, 50% DMSO/50% 0.15 M saline, 0.5  $\mu$ g CNQX, and 0.25  $\mu$ g NMDA + 10  $\mu$ g D-serine) also had the

first 10 minutes of their testing cage videotapes analyzed for freezing without head movement (rat must remain immobile in a rigid stance for at least 5 sec) and freezing with head movement (rat must remain immobile, with the exception of head movement, in a rigid stance for at least 5 sec). These two groups also had their microinjection videotapes scored for vocalizations and escape attempts.

### **Histology**

**Behavioral Rats:** Rats were deeply anesthetized with sodium pentobarbital and decapitated upon completion of behavioral testing. Their brains were extracted and placed in a 10% paraformaldehyde solution for 1 day; then, they were transferred to a 30% sucrose solution until they sunk (~4 days). Brains were coronally sectioned in 60  $\mu\text{m}$  slices, which were mounted on slides and stained with cresyl violet. Slides were analyzed and cannulae placements were mapped onto a brain atlas. Cannulae placements were considered rostral if they were anterior to AP = +1.6 mm, and they were considered caudal if they were posterior to AP = +1.6 mm.

**Fos plume analysis:** Rats received microinjections of one of the drug conditions (0.15 M Saline, 50% DMSO/50% 0.15 M saline, 0.5  $\mu\text{g}$  CNQX, and 0.25  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine/0.5  $\mu\text{l}$  per side) and were placed back in their cages for 90 minutes. Rats were deeply anesthetized with sodium pentobarbital and transcardially perfused 90 minutes after microinjection. Rats were allowed to remain in their cage for 90 minutes prior to perfusion to provide enough time for the expression of the Fos protein. Fos activation was performed using the immunohistochemistry and immunofluorescence techniques described by Reynolds and Berridge (2008). After perfusion, the rats underwent decapitation and brain extraction. Brains were placed in 4% paraformaldehyde for 1 day; then, they were transferred to a 30% sucrose solution until they sunk (~4 days). Brains were coronally sectioned in 40  $\mu\text{m}$  slices with every

other slice being collected and stored in a 0.1 M sodium phosphate buffer. Sections were immersed in succession, with gentle agitation and intervening rinses, in 0.1 M sodium phosphate buffer with 0.2% Triton containing (1) 5% normal donkey serum for 30 min, (2) 5% normal donkey serum and goat anti-c-Fos (1:500) overnight at 4 °C, (3) 5% normal donkey serum, and (4) 5% normal donkey serum and donkey anti-goat Alexa Fluor 488 (excitation, 488 nm; emission, 519 nm; Invitrogen) for 2 h. The slices were mounted on to slides and allowed to dry. The slides were coverslipped with ProLong Gold antifade reagent (Invitrogen).

Sections were analyzed for Fos activation using a Leica microscope equipped for both brightfield and fluorescence microscopy. Fluorescence was visualized using a filter with an excitation band of 480-505 and an emission band of 505-545. The largest Fos plumes were usually located on the most caudal section that still had damage from the microinjection tip. A grid was placed over the image of this section in order to count the number of cells expressing Fos (10x magnification). The grid consisted of a central square, which was lined up over the microinjection damage, with 8 radial arms emanating from it. The arms were spaced 45° apart and were composed of 10 squares each. Cells expressing Fos were counted in each square except the central square.

### **Statistical Analysis**

Drug effects in the D-serine group (0.5, 2, 10, 50 µg D-serine, and 0.15 M saline), the CNQX group (0.1, 0.25, 0.5, 1.5 µg CNQX, and 50% DMSO/50% 0.15 M saline), and the NMDA and CNQX group (0.15 M Saline, 50% DMSO/50% 0.15 M saline, 0.5 µg CNQX, and 0.25 µg NMDA + 10 µg D-serine) were analyzed with a two-way ANOVA (drug X placement, rostral or caudal). The effects of NMDA in the NMDA group (0.15 M saline, 0.125 µg NMDA, 0.25 µg NMDA, 0.125 µg NMDA + 10 µg D-serine, and 0.25 µg NMDA + 10 µg D-serine)



were tested with a two-way ANOVA (NMDA X placement, rostral or caudal), and the effects of D-serine were tested with a three-way ANOVA (NMDA X D-serine X placement, rostral or caudal). If a significant effect was found, a one-way ANOVA and pairwise comparisons with Bonferroni corrections were run. A Pearson's correlational test was also run on the amount of behavioral change induced by CNQX versus NMDA in the NMDA and CNQX group (0.15 M Saline, 50% DMSO/50% 0.15 M saline, 0.5  $\mu$ g CNQX, and 0.25  $\mu$ g NMDA + 10  $\mu$ g D-serine).

## Results

### **D-serine alone does not significantly impact motivated behavior**

Although NMDA in the nucleus accumbens shell increases eating (Echo et al., 2001), pilot testing of 0.5  $\mu$ g of NMDA in caudal shell created short, intense fear reactions (J.M. Richard, unpublished observations); therefore, NMDA receptor activation via the glycine site alone was tested. D-serine in the nucleus accumbens shell seemed to cause a slight increase in food intake and eating time with the 10  $\mu$ g dose more than doubling both the amount of food eaten and the time spent eating compared to vehicle (food intake, average of  $2.1 \pm 0.60$  g SEM compared to  $1.0 \pm 0.38$  g SEM under vehicle; eating time, average of  $305.9 \pm 90.7$  sec SEM compared to  $141.44 \pm 57.9$  sec SEM under vehicle, see Figure 1). These increases were not significant, though (food intake, main effect of D-serine,  $F_{(4,24)} = 0.696$ ,  $p = 0.602$ ; eating time, main effect of D-serine,  $F_{(4,24)} = 0.670$ ,  $p = 0.619$ ). Other appetitive measures, such as food sniffs, food carries, and drinking time, were not significantly affected by D-serine ( $F$  values  $< 1$ ). Fear was similarly unaffected and no significant difference was found in defensive treading, which remained near zero (main effect of D-serine,  $F_{(4,24)} = 1.300$ ,  $p = 0.298$ , see Figure 1). D-serine also failed to alter the measures of normal activity, including cage crosses ( $F_{(4,24)} = 0.870$ ,

$p = 0.496$ ), sleeping ( $F_{(4,24)} = 0.636, p = 0.642$ ), burrowing ( $F_{(4,24)} = 0.857, p = 0.504$ ), rearing ( $F_{(4,24)} = 1.077, p = 0.390$ ), and grooming ( $F_{(4,24)} = 1.016, p = 0.419$ ).

### **NMDA + D-serine in caudal shell increases food intake**

Since D-serine alone did not affect motivated behavior, low doses of NMDA with D-serine were tested. Combined NMDA receptor activation at both the glycine site and the NMDA site produced more robust increases in food intake and eating time, especially in the caudal region of the nucleus accumbens shell. Although the lower doses of NMDA and D-serine (0.125  $\mu\text{g}$  NMDA, 0.25  $\mu\text{g}$  NMDA, and 0.125  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine) failed to produce significant effects on food intake or eating time ( $F$  values  $< 1$ ), the 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine condition more than doubled food intake and eating time over vehicle, which was significant (food intake, average of  $3.6 \pm 0.61$  g SEM compared to  $1.4 \pm 0.36$  g SEM under vehicle; eating time, average of  $428.7 \pm 76.2$  sec SEM compared to  $163.9 \pm 40.6$  sec SEM under vehicle; food intake, main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 10.116, p = 0.007$ ; eating time, main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 7.913, p = 0.015$ ). There was a significant interaction between the 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine condition and placement (drug X site interaction,  $F_{(1,13)} = 6.567, p = 0.024$ ) as microinjections in caudal shell more than tripled food intake (food intake, average of  $4.71 \pm 0.68$  g SEM compared to  $1.55 \pm 0.50$  g SEM under vehicle; caudal shell, main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,9)} = 18.074, p = 0.002$ , see Figure 2, 3, and 4). The 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine condition failed to significantly alter food intake when injected in rostral shell (food intake, average of  $1.48 \pm 0.36$  g SEM compared to  $1.14 \pm 0.49$  g SEM under vehicle; rostral shell, main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,4)} = 0.840, p = 0.411$ ).

Microinjections of NMDA and D-serine did not seem to affect the anatomical controls (nucleus

accumbens core and navicular nucleus,  $n = 1$ ; lateral septal nucleus and lateral ventricle,  $n = 1$ ), which never ate more than 1.7 g of food. Food sniffs (main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 1.519$ ,  $p = 0.240$ ), food carries ( $F$  values  $< 1$ ), and drinking time ( $F$  values  $< 1$ ) were not significantly affected by any dose of NMDA or D-serine.

### **NMDA + D-serine increases passive, but not active fear**

NMDA receptor activation did not have any effect on the active fear measure of defensive treading (treading, 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine condition, average of  $0.47 \pm 0.24$  bouts SEM compared to  $0.8 \pm 0.30$  bouts SEM under vehicle; main effect of NMDA,  $F_{(2,8)} = 1.531$ ,  $p = 0.274$ ; main effect of D-serine,  $F_{(1,4)} = 0.000$ ,  $p = 1.000$ ; main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 1.806$ ,  $p = 0.202$ , see Figure 2, 3, and 5). The 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine dose did significantly affect the passive fear measure of freezing with head movement, though (freezing with head movement, average of  $52.40 \pm 10.53$  sec SEM compared to  $9.53 \pm 3.23$  sec SEM under vehicle; main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 11.576$ ,  $p = 0.005$ , see Figure 3 and 6). The anatomical controls also seemed to produce similar increases in freezing with head movement, so this effect may not be localized to the nucleus accumbens shell (30 sec compared to 5 sec under vehicle, nucleus accumbens core and navicular nucleus,  $n = 1$ ; 165 sec compared to 0 sec under vehicle, lateral septal nucleus and lateral ventricle,  $n = 1$ ). Additional rats would need to be tested to determine if the increases at control placements are significant. Freezing without head movement also seemed to increase slightly, but this was not significant (main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 3.939$ ,  $p = 0.069$ ). Measures of escape attempts and vocalizations during microinjections did not yield significant results (escape attempts, main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,11)} = 0.788$ ,  $p = 0.394$ ; vocalizations, main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-

serine,  $F_{(1,11)} = 0.644$ ,  $p = 0.439$ ); however, one rat receiving the 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine condition had a particularly intense reaction that resulted in 9 escape attempts and 29 vocalizations.

### **NMDA + D-serine has no effect on other behaviors**

The measures of general activity were unaffected by NMDA activation, including rears (main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 0.178$ ,  $p = 0.680$ ; main effect of D-serine,  $F_{(1,4)} = 3.773$ ,  $p = 0.124$ ; main effect of NMDA,  $F_{(2,8)} = 1.132$ ,  $p = 0.369$ ), sleeping ( $F$  values  $< 1$ ), burrowing (main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 2.167$ ,  $p = 0.165$ ; main effect of NMDA,  $F_{(2,8)} = 2.667$ ,  $p = 0.130$ ), grooming (main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 0.748$ ,  $p = 0.403$ ; main effect of NMDA,  $F_{(2,8)} = 0.258$ ,  $p = 0.779$ ; main effect of D-serine,  $F_{(1,4)} = 4.737$ ,  $p = 0.095$ ), cage crosses (main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 2.634$ ,  $p = 0.129$ ; main effect of NMDA,  $F_{(2,8)} = 1.769$ ,  $p = 0.231$ ; main effect of D-serine,  $F_{(1,4)} = 0.033$ ,  $p = 0.865$ ), and burrow-treads (main effect of 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine,  $F_{(1,13)} = 2.167$ ,  $p = 0.165$ ; main effect of NMDA,  $F_{(2,8)} = 2.667$ ,  $p = 0.130$ ). This indicates that the effects of combined NMDA and D-serine microinjection were relatively specific to motivated appetitive behavior (eating) and passive fear.

### **CNQX increases appetitive motivation**

The motivational effects of CNQX were tested because it was previously found that DNQX, another AMPA/kainate antagonist, in the nucleus accumbens shell generated appetitive and fearful motivation along a rostrocaudal gradient (Reynolds & Berridge, 2003). Blockade of AMPA/kainate receptors in the nucleus accumbens shell with CNQX increased appetitive motivation. The 0.5  $\mu\text{g}$  dose of CNQX created a five-fold increase in food intake and significantly increased eating time as well (food intake, average of  $7.17 \pm 0.50$  g SEM compared

to  $1.36 \pm 0.30$  g SEM under vehicle; food intake, main effect of 0.5  $\mu$ g of CNQX,  $F_{(1,12)} = 110.428$ ,  $p = 0.000$ ; eating time, main effect of 0.5  $\mu$ g of CNQX,  $F_{(1,12)} = 44.400$ ,  $p = 0.000$ , see Figure 3, 7, and 8). Food intake and average eating time were non-significantly higher in rostral shell in comparison to caudal shell, especially with the 1.5  $\mu$ g dose (food intake, rostral shell, 1.5  $\mu$ g CNQX condition, average of  $8.4 \pm 1.75$  g SEM compared to  $1.73 \pm 1.17$  g SEM under vehicle; food intake, caudal shell, 1.5  $\mu$ g CNQX condition, average of  $6.4 \pm 0.8$  g SEM compared to  $0.45 \pm 0.45$  g SEM under vehicle; drug X site interaction,  $F_{(4,12)} = 0.875$ ,  $p = 0.507$ ; eating time, rostral shell, 1.5  $\mu$ g CNQX condition, average of  $1135 \pm 397$  sec SEM compared to  $181.3 \pm 116.6$  sec SEM under vehicle; eating time, caudal shell, 1.5  $\mu$ g CNQX condition, average of  $533 \pm 99$  sec SEM compared to  $45 \pm 45$  sec SEM under vehicle; drug X site interaction,  $F_{(4,12)} = 1.430$ ,  $p = 0.283$ ; food intake, rostral shell, 0.5  $\mu$ g CNQX condition, average of  $7.67 \pm 0.86$  g SEM compared to  $1.82 \pm 0.54$  g SEM under vehicle; food intake, caudal shell, 0.5  $\mu$ g CNQX condition, average of  $6.8 \pm 0.59$  g SEM compared to  $1.03 \pm 0.32$  g SEM under vehicle; drug X site interaction,  $F_{(1,12)} = 0.005$ ,  $p = 0.947$ ; eating time, rostral shell, 0.5  $\mu$ g CNQX condition, average of  $892.2 \pm 135.5$  sec SEM compared to  $203.2 \pm 53.8$  sec SEM under vehicle; eating time, caudal shell, 0.5  $\mu$ g CNQX condition, average of  $660.9 \pm 93.5$  sec SEM compared to  $154.8 \pm 59.8$  sec SEM under vehicle; drug X site interaction,  $F_{(1,12)} = 1.040$ ,  $p = 0.328$ ). Although the rostral shell advantage for eating was not significant, the pattern is consistent with the gradient produced by DNQX (Reynolds & Berridge, 2003; Faure et al., 2008, 2010). CNQX also increased the amount of time spent drinking (main effect of 0.5  $\mu$ g of CNQX,  $F_{(1,12)} = 8.757$ ,  $p = 0.012$ ). Food sniffs were increased primarily in caudal shell (caudal shell, main effect of 0.5  $\mu$ g of CNQX,  $F_{(1,7)} = 13.460$ ,  $p = 0.008$ ). Food carries, though, were not

significantly affected by CNQX (main effect of 0.5  $\mu\text{g}$  of CNQX,  $F_{(1,12)} = 4.061$ ,  $p = 0.067$ ).

CNQX microinjections into the nucleus accumbens core and navicular nucleus ( $n = 1$ ) as well as the lateral septal nucleus and lateral ventricle ( $n = 1$ ) did not seem to affect appetitive behaviors since these rats only ate a total of 0.1 g of food when given the 0.5  $\mu\text{g}$  dose of CNQX. Bilateral microinjection of the 0.5  $\mu\text{g}$  dose of CNQX into the nucleus accumbens core ( $n = 1$ ) did cause a rat to eat 8.2 g of food, though. These placements were partially in the nucleus accumbens shell, so it is possible this effect was still due to a drug effect in the nucleus accumbens shell. Since the other anatomical control rat that had a placement in the nucleus accumbens core only ate 0.1 g of food, additional placements in the nucleus accumbens core would be necessary to determine if CNQX in the nucleus accumbens core truly enhances food intake.

#### **CNQX in caudal shell increases defensive treading, but not at the 0.5 $\mu\text{g}$ dose**

The original CNQX group (0.1, 0.25, 0.5, and 1.5  $\mu\text{g}$ ) significantly increased defensive treading; an effect which seemed to be driven mainly by the 1.5  $\mu\text{g}$  CNQX dose since it produced the greatest treading (treading, 1.5  $\mu\text{g}$  CNQX condition, average of  $2.60 \pm 0.93$  bouts SEM compared to  $0.40 \pm 0.24$  bouts SEM under vehicle; main effect of CNQX,  $F_{(4,12)} = 4.094$ ,  $p = 0.026$ , see Figure 7). There was also a significant interaction between drug and placement (drug X site interaction,  $F_{(4,12)} = 3.790$ ,  $p = 0.032$ ), as caudal shell seemed especially important for CNQX-induced treading (caudal shell, 1.5  $\mu\text{g}$  CNQX condition, average of  $4.50 \pm 0.50$  bouts SEM compared to  $0.50 \pm 0.50$  bouts under vehicle; caudal shell, main effect of CNQX,  $F_{(4,4)} = 37.667$ ,  $p = 0.002$ , see Figure 3, 7, and 9). This caudal shell advantage for defensive treading is consistent with previous findings on treading induced by AMPA/kainate blockade (Reynolds & Berridge, 2003). The 0.5  $\mu\text{g}$  dose of CNQX did not show any effect on treading (main effect of 0.5  $\mu\text{g}$  CNQX,  $F_{(1,12)} = 2.882$ ,  $p = 0.115$ ). One rat did tread 21 times, so it is possible that more

rats would have yielded a significant result. It is also possible that these rats failed to tread because of their comfort level. Regions in moderately caudal areas of shell, typically responsible for DNQX-induced treading, can shift valence and generate appetitive motivation when rats are placed in a comfortable environment (Reynolds & Berridge, 2008). CNQX also may have produced a more robust effect on defensive treading if additional testing had been done with the 1.5  $\mu\text{g}$  dose. CNQX did not have any effect on freezing with or without head movement (freezing with head movement, main effect of 0.5  $\mu\text{g}$  CNQX,  $F_{(1,7)} = 0.011$ ,  $p = 0.921$ ; freezing without head movement, main effect of 0.5  $\mu\text{g}$  CNQX,  $F_{(1,7)} = 2.869$ ,  $p = 0.134$ , see Figure 3 and 10). Anatomical controls (nucleus accumbens core,  $n = 1$ ; nucleus accumbens core and navicular nucleus,  $n = 1$ ; lateral septal nucleus and lateral ventricle,  $n = 1$ ) never treaded more than one time in any drug condition.

#### **CNQX increases rears and crosses, but not at the 0.5 $\mu\text{g}$ dose**

The original CNQX group (0.1, 0.25, 0.5, and 1.5  $\mu\text{g}$ ) displayed a significant CNQX-induced increase in rears and cage crosses and a decrease in time spent sleeping (rears, main effect of CNQX,  $F_{(4,12)} = 6.723$ ,  $p = 0.004$ ; cage crosses, main effect of CNQX,  $F_{(4,12)} = 14.386$ ,  $p = 0.000$ ; sleep, main effect of CNQX,  $F_{(4,12)} = 3.716$ ,  $p = 0.034$ ). There was a significant interaction between CNQX and placement (rears, drug X site interaction,  $F_{(4,12)} = 8.536$ ,  $p = 0.002$ ; cage crosses, drug X site interaction,  $F_{(4,12)} = 14.234$ ,  $p = 0.000$ ) with a caudal shell advantage for the increases in both rears and cage crosses (caudal shell, rears,  $F_{(4,4)} = 7.164$ ,  $p = 0.041$ ; caudal shell, cage crosses,  $F_{(4,4)} = 13.843$ ,  $p = 0.013$ ), as reported previously with the AMPA/kainate antagonist DNQX (Faure et al., 2008). The 0.5  $\mu\text{g}$  CNQX condition, however, did not significantly alter sleeping (main effect of 0.5  $\mu\text{g}$  CNQX,  $F_{(1,12)} = 4.152$ ,  $p = 0.064$ ), rears (main effect of 0.5  $\mu\text{g}$  CNQX,  $F_{(1,12)} = 0.105$ ,  $p = 0.751$ ), or cage crosses (main effect of

0.5  $\mu\text{g}$  CNQX,  $F_{(1,12)} = 1.407$ ,  $p = 0.259$ ), so it is unlikely that the observed eating results were simply the result of enhanced motor activity.

### **NMDA + D-serine vs. CNQX impact food intake differently**

The 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine and 0.5  $\mu\text{g}$  CNQX were injected into the same rats, so that their effects could be compared at the same microinjection sites. The effects of NMDA + D-serine and CNQX were compared using two-way ANOVAs and Pearson's correlational tests. The CNQX-induced increase in food intake was significantly greater than the NMDA + D-serine-induced increase in food intake ( $F_{(1,7)} = 9.030$ ,  $p = 0.020$ , see Figure 3), and the amount of food intake generated by CNQX and NMDA + D-serine in the same rats was not correlated ( $r = -0.109$ ,  $n = 9$ ,  $p = 0.780$ ), indicating that the rats which ate the most following CNQX microinjection, were not the same rats who ate the most following NMDA + D-serine. The increases in food intake, therefore, may have been the result of two separate mechanisms, but food intake was the only appetitive measure for which CNQX and NMDA + D-serine produced significantly different changes from vehicle. CNQX and NMDA + D-serine failed to produce significantly different changes in eating *time*, food sniffs, and food carries (eating time,  $F_{(1,7)} = 2.900$ ,  $p = 0.132$ ; food sniffs,  $F_{(1,7)} = 0.611$ ,  $p = 0.460$ ; food carries,  $F_{(1,7)} = 0.055$ ,  $p = 0.822$ ).

The 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine and 0.5  $\mu\text{g}$  CNQX had similar, and limited, effects on the active fear measure of defensive treading. There was a significant correlation between the change in treading induced by 0.25  $\mu\text{g}$  NMDA with 10  $\mu\text{g}$  D-serine and 0.5  $\mu\text{g}$  CNQX ( $r = 0.820$ ,  $n = 9$ ,  $p = 0.007$ ) and there was not a significant difference between the change in treading induced by CNQX versus NMDA + D-serine ( $F_{(1,7)} = 1.869$ ,  $p = 0.214$ ). It is not surprising that the change in treading induced by the two drug conditions is correlated given



the low levels of treading present under each drug; however, this result does not necessarily mean that CNQX and NMDA + D-serine have the same effect on active fear. As previously noted, the 1.5  $\mu$ g CNQX dose evoked the most defensive treading of the CNQX doses tested, especially in caudal shell. This is consistent with previous findings that AMPA/kainate receptor blockade in caudal shell induces defensive treading (Reynolds & Berridge, 2003). It is possible that additional testing of the 1.5  $\mu$ g CNQX dose in comparison to NMDA activation would have resulted in a more robust treading effect and potentially revealed a significant difference between CNQX and NMDA + D-serine. There also was not a significant difference between the change in freezing with head movement induced by CNQX versus NMDA + D-serine ( $F_{(1,7)} = 3.376, p = 0.109$ ); however, they were not significantly correlated either ( $r = -0.002, n = 9, p = 0.996$ ). This lack of a correlation means that a rat's change in freezing under one drug condition was in no way predictive of its change in freezing under the other condition; therefore, it is likely that NMDA + D-serine and CNQX do not affect freezing in the same way. There was a significant correlation between the change in cage crosses induced by 0.25  $\mu$ g NMDA with 10  $\mu$ g D-serine and 0.5  $\mu$ g CNQX ( $r = 0.687, n = 9, p = 0.041$ ) with no significant difference between the change in cage crosses caused by the two drugs ( $F_{(1,7)} = 0.167, p = 0.695$ ). The significant correlation and lack of a significant difference for the change in cage crosses under the two drug conditions indicates that the two drugs had similar effects on locomotion, and any difference between the food intake or freezing induced by the two drug conditions cannot be attributed to a difference in motor activity. Additional testing, perhaps with the 1.5  $\mu$ g dose of CNQX in comparison to NMDA + D-serine, would be needed to fully compare the active defensive behavior produced by AMPA blockade, and the passive freezing produced by NMDA activation.

### **Fos plume analysis**

The amount of Fos expression induced by microinjections of NMDA + D-serine and CNQX in comparison to their respective vehicles was analyzed in order to determine how far from the microinjection site the drug effects spread. Microinjections of the 0.5  $\mu\text{g}$  CNQX dose resulted in intense activation of Fos within a small area surrounding the microinjection center of 0.025 mm radius (0.00007  $\text{mm}^3$  sphere volume), where CNQX created a five-fold increase in Fos levels in comparison to DMSO/saline. This center was surrounded by a band with a 0.1375 mm radius (0.01  $\text{mm}^3$  sphere volume) of three times Fos elevation over DMSO/saline and a larger band with a 0.19375 mm radius (0.03  $\text{mm}^3$  sphere volume) of two times Fos elevation over DMSO/saline. There was an outer ring with a radius of 0.30625 mm (0.12  $\text{mm}^3$  sphere volume) that induced mild Fos elevation of 1.5 times over DMSO/saline. In comparison to CNQX, NMDA + D-serine produced a smaller, less intense plume of Fos activation. Microinjections of 0.25  $\mu\text{g}$  NMDA + 10  $\mu\text{g}$  D-serine induced a three-fold increase in Fos activation compared to saline microinjections within a small center of 0.01875 mm radius (0.00003  $\text{mm}^3$  sphere volume). There was an intermediate band with a 0.15625 mm radius (0.016  $\text{mm}^3$  sphere volume) of two times Fos elevation and an outer ring with a 0.2375 mm radius (0.056  $\text{mm}^3$  sphere volume) of 1.5 times Fos elevation. The ability of both NMDA + D-serine and CNQX to elevate Fos activation, but the enhanced intensity of the CNQX Fos plume is consistent with the finding that both NMDA + D-serine and CNQX increase eating, but CNQX produces especially strong effects.

### **Discussion**

NMDA stimulation and AMPA blockade in the nucleus accumbens shell both produced feeding and fear; however, notable differences in the magnitude of their effects on eating, the nature of the fear behaviors produced, and the anatomical localization of their effects indicates

that they may act via different pathways or mechanisms to generate motivation. AMPA/kainate blockade throughout shell produced voracious eating. This intense desire to eat seemed especially strong when CNQX was administered in rostral shell, which resulted in non-significantly greater eating on average in comparison to caudal shell. In caudal shell, CNQX produced defensive treading as well as eating. The production of defensive treading indicates that AMPA/kainate blockade in caudal shell induces a state of extreme fear similar to what a rodent may experience when it encounters a predator (De Boer & Koolhaas, 2003). Although it did not produce a significant rostral shell advantage for eating, CNQX seemed to produce a similar rostrocaudal gradient to that produced by DNQX (Reynolds & Berridge, 2003, 2008; Faure et al., 2008, 2010). In contrast, NMDA activation only increased eating in caudal shell, and it was less effective than CNQX. NMDA stimulation also produced freezing, a more passive fear behavior compared to defensive treading, throughout shell. Despite their shared ability to increase eating and fearful behaviors, NMDA stimulation and AMPA/kainate blockade in the nucleus accumbens shell affect feeding and fear differently.

We found that the ability of NMDA receptor stimulation to affect motivated behavior required binding at the NMDA site, as activation of NMDA receptors via the glycine site alone was incapable of significantly altering motivated behavior. NMDA receptors require binding at both the NMDA site as well as the glycine site for activation (Yang & Svensson, 2008). Glycine levels are typically found to be very high in vivo leading some to believe that the glycine site is saturated or near saturated in some brain areas (Danysz & Parsons, 1998). If glycine levels were already high, it is possible that the microinjections of D-serine only slightly increased NMDA receptor activity corresponding to the slight increase in eating that was observed. High glycine levels imply that under normal conditions NMDA receptor activation is primarily regulated by

the amount of glutamate that is available to bind to the NMDA site. Significant effects on motivated behavior were not observed, therefore, until NMDA was used in combination with D-serine.

AMPA receptors and NMDA receptors have some similarities that may explain the fact that they both participate in the regulation of appetitive and fearful motivation. AMPA receptors and NMDA receptors are both ligand-gated receptors that require the binding of glutamate (Wang et al., 2006; Yang & Svensson, 2008). They are ionotropic receptors with tetrameric structures that are involved in fast excitatory synaptic transmission (Wang et al., 2006; Yang & Svensson, 2008). It is also possible that they are equally affected by the glutamatergic inputs originating in the cortex and cortical-like structures that innervate the nucleus accumbens shell. If these sources are providing vital information regarding whether or not motivation should be generated as well as what type of motivation is appropriate, and they affect both NMDA and AMPA receptors, it is reasonable that the general effects of NMDA and AMPA receptors would be similar.

Although NMDA receptors and AMPA receptors are both involved in the regulation of feeding and fear, stimulation of NMDA receptors generated eating and fear responses that were very different from those produced by AMPA/kainate receptor blockade. AMPA/kainate blockade generated a much greater eating enhancement and produced treading, which is an active defensive behavior. In contrast, NMDA activation produced passive fear in the form of freezing. AMPA and NMDA receptors differ in synaptic location and in their interactions with other neurotransmitters, which may explain the different roles they play in the generation of appetitive and fearful motivation. Only NMDA and kainate receptors are present on presynaptic terminals of projections from the hippocampus to the nucleus accumbens (Tarazi, Campbell, &

Baldessarini, 1998a). NMDA, AMPA, and kainate receptors are all found postsynaptically on neurons in the nucleus accumbens; however, only NMDA receptors are found on afferent projections originating in cortex (Tarazi et al., 1998b). AMPA receptors are also believed to be more important than NMDA receptors in generating postsynaptic currents (Coyle, 2006). The ability of both AMPA and NMDA receptors to act postsynaptically may explain their shared ability to alter feeding and fear; however, AMPA's greater importance in generating postsynaptic currents may also explain why it mediates a more active form of motivation characterized by intense eating and defensive treading. NMDA's more mild eating enhancement and production of freezing may be the result of a reduced influence on postsynaptic currents, or NMDA may modulate information from afferent inputs via its presynaptic receptors to switch motivation to a more passive form.

It is also possible that the effects of AMPA and NMDA receptors are due to interactions between glutamate and other neurotransmitters in the nucleus accumbens shell that are important for generating motivation. Metabotropic glutamate receptors in the nucleus accumbens shell, for example, are capable of altering dopamine and GABA levels (Richard & Berridge, 2011). Different interactions between NMDA and AMPA receptors and other motivationally important neurotransmitters in the nucleus accumbens shell may play a role in the different effects generated by AMPA/kainate blockade and NMDA stimulation. NMDA receptors in the nucleus accumbens shell are typically co-localized with  $\mu$ -opioid receptors (Echo et al., 2001). Injection of AMPA with an opioid agonist in the nucleus accumbens shell significantly increases eating compared to the opioid agonist alone (Echo et al., 2001). Injection of NMDA with the same opioid agonist, though, significantly reduces the eating induced by the opioid agonist alone (Echo et al., 2001). Since opioids in the nucleus accumbens shell are capable of enhancing

appetitive motivation and reward (Peciña & Berridge, 2005), the different motivational roles of AMPA and NMDA receptors may be due to different interactions with opioids in the nucleus accumbens shell. Since both AMPA and opioid receptors are capable of generating intense eating, but AMPA also significantly increases eating induced by opioids, it is possible that they are acting on overlapping circuits via separate physiological and psychological mechanisms, so that their effects alone on eating are similar, but together they are cumulatively greater. NMDA's ability to reduce opioid-induced eating and its co-localization with  $\mu$ -opioid receptors may indicate that NMDA receptors are capable of more directly interacting with the opioid system.

AMPA and NMDA receptors also interact with dopamine, which is also believed to play an important role in motivation (Berridge, 2007). D1 receptor activation is necessary for the eating increases caused by DNQX, and both D1 and D2 receptor activation is needed for the defensive treading generated by DNQX (Richard & Berridge, 2010). D1 activation enhances NMDA currents, but D2 activation reduces AMPA currents (Echo et al., 2001). Additionally, NMDA receptors may also alter dopamine release in the nucleus accumbens shell because they are typically co-localized with D1 receptors, and they are found presynaptically on dopamine projections (Meredith et al., 2008). The ability of both NMDA and AMPA receptors to increase eating, therefore, may be due in part to their mutual interaction with D1 receptors, which is important for appetitive motivation. A greater interaction between AMPA receptors and D2 receptors, which are necessary for active fear, may explain why AMPA blockade tends to generate defensive treading and not freezing.

In addition to enhancing food intake differently and creating different phenotypes of fear, NMDA stimulation and AMPA/kainate blockade also interact differently with shell placement. While CNQX generates eating throughout shell with a slight rostral shell advantage, NMDA

activation only produces eating in caudal shell. CNQX only produces fear in caudal shell, but NMDA activation increases freezing throughout shell. It is not unusual for the rostral and caudal region of the nucleus accumbens shell to play different roles in generating motivation and reward. Blockade of AMPA/kainate receptors with DNQX and activation of GABA receptors in rostral shell both increase eating (Reynolds & Berridge, 2001, 2002, 2003). In caudal shell, however, they both produce active fear (Reynolds & Berridge, 2001, 2002, 2003). Rostral shell dopamine transmission is important for generating motivation to maintain pair bonds (Aragona et al., 2006). The generation of pair bonds is consistent with the appetitive role of rostral shell in DNQX and GABA-induced motivation. Caudal shell, however, is capable of producing positively valenced motivation as well. Histamine blockade in the caudal shell more than the rostral shell is able to produce rewarding effects, including creating a conditioned place preference (Zimmermann, Privou, & Huston, 1999). It is not surprising, then, that a caudal shell advantage for appetitive motivation is created by NMDA stimulation, while a slight rostral shell advantage for appetitive behaviors is created by CNQX.

Rostral and caudal shell have different anatomical features, which may account for the different results they produce. Caudal shell has an especially strong noradrenergic innervation from the nucleus of the solitary tract (Berridge, Stratford, Foote, & Kelley, 1997; Delfs, Zhu, Druhan, & Aston-Jones, 1998), which may partially underlie the caudal shell advantage for CNQX-induced treading. Rostral and caudal shell also differ in their efferent projections (Usuda, Tanaka, & Chiba, 1998). Both rostral and caudal shell project to ventral pallidum, lateral hypothalamus, substantia nigra, and ventral tegmental area (Usuda et al., 1998). Since these are structures that have been implicated in motivation, reward, and eating (Kelley, 2004; Berridge, Ho, Richard, & DiFeliceantonio, 2010), these shared projections may explain why AMPA

blockade can produce eating throughout shell. Additionally, rostral shell projects to lateral preoptic area, and there is some evidence that caudal shell might project to parabrachial nucleus, substantia innominata, and retrorubral area (Usuda et al., 1998). Caudal shell, therefore, may have similar efferent projections to those of the extended amygdala, which also projects to the retrorubral area and parabrachial nucleus (Zahm, Jensen, Williams, & Martin, 1999). Since the extended amygdala also plays an important role in fear (Walker & Davis, 2008), the projections to the retrorubral area and parabrachial nucleus may be especially important for the treading produced by CNQX in caudal shell. Caudal shell's projection to the parabrachial nucleus may also be important in producing the caudal shell advantage for eating induced by NMDA stimulation since the parabrachial nucleus is associated with eating and reward (Soderpalm & Berridge, 2000; Berridge et al., 2010).

The effects of NMDA + D-serine and CNQX indicate that rostral and caudal shell are both capable of producing appetitive and fearful motivation. Rostral shell enhanced appetitive motivation when AMPA/kainate receptors were blocked, but it also increased freezing when NMDA receptors were activated. Caudal shell was similarly capable of generating bivalent motivation as NMDA receptor stimulation increased freezing and AMPA/kainate blockade increased defensive treading, and both increased eating. These results are very different from those produced by GABA and DNQX, which both generated eating in the rostral shell and fear in the caudal shell (Reynolds & Berridge, 2001, 2002, 2003). Although DNQX and GABA seem to generate similar rostrocaudal gradients, there are some important distinctions. DNQX tends to generate a more ambiguous motivation and produces a greater mixture of feeding and fear (Reynolds & Berridge, 2003). Additionally, the motivation generated under DNQX is flexible and can be altered by environmental influences (Reynolds & Berridge, 2008). A comforting



home environment can turn the majority of the nucleus accumbens shell into an appetitive-generating zone (Reynolds & Berridge, 2008). A loud, fearful environment, though, can convert the majority of the nucleus accumbens shell into a fear-generating zone (Reynolds & Berridge, 2008). There are other neurotransmitters that generate motivation without a gradient indicating that both rostral and caudal shell are capable of producing both appetitive and fearful motivation. Metabotropic glutamate blockade throughout the nucleus accumbens shell reduces appetitive motivation, increases defensive treading, and creates a hedonic shift from liking to disliking in response to sucrose (Richard & Berridge, 2011). NMDA blockade also creates a negatively valenced motivation that reduces eating and increases defensive treading throughout shell (Reynolds & Berridge, 2003). In contrast, opioids generate eating throughout the nucleus accumbens shell (Peciña & Berridge, 2005). This indicates that rostral and caudal shell are not strictly set as positively and negatively valenced. The difference in placement interaction demonstrated by AMPA and NMDA, therefore, may be explained by a different degree of interaction between the relevant neurotransmitters or a different interaction with inputs carrying environmental information.

Given the important inputs it receives from a variety of structures associated with emotion and executive control and its established role as an important area for generating motivation, the nucleus accumbens glutamate system is an important area of research. The excess motivation present in addiction and schizophrenia have both been linked to improper salience attribution (Robinson & Berridge, 2003; Kapur, 2003). In the case of addiction, excess incentive salience is attributed to drugs of abuse and stimuli associated with drugs creating a pathological wanting of the drug (Robinson & Berridge, 2003). In schizophrenia, salience is attributed to harmless stimuli making them appear frightening and producing delusions and

hallucinations (Kapur, 2003). AMPA/kainate blockade and NMDA stimulation in the nucleus accumbens have proven to be capable of generating both appetitive and fearful motivation potentially linking them to both addiction and schizophrenia. Blockade of AMPA/kainate receptors with CNQX increases active fear in caudal shell and induces an intense desire to eat throughout shell. NMDA receptor activation increases eating only in caudal shell, but increases passive fear throughout shell. Although AMPA/kainate blockade and NMDA stimulation in the nucleus accumbens shell both produce appetitive and fearful motivation, they produce different effects on eating and they create different types of fear.

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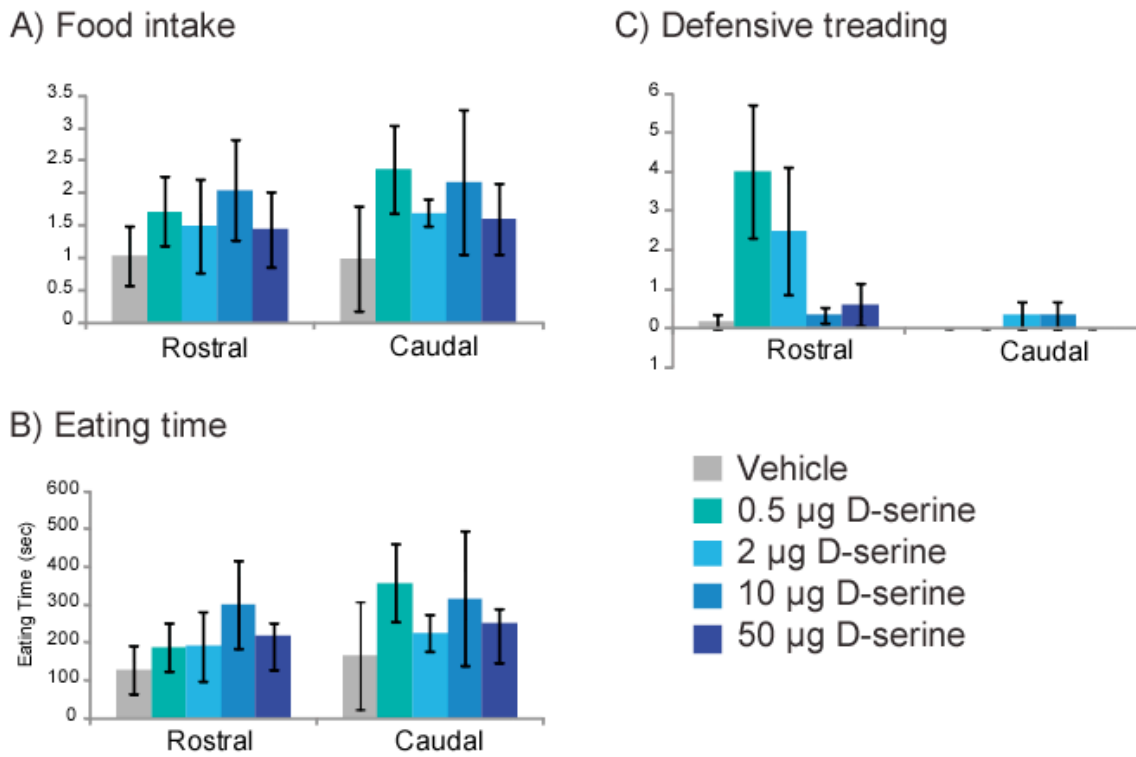
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## Author Note

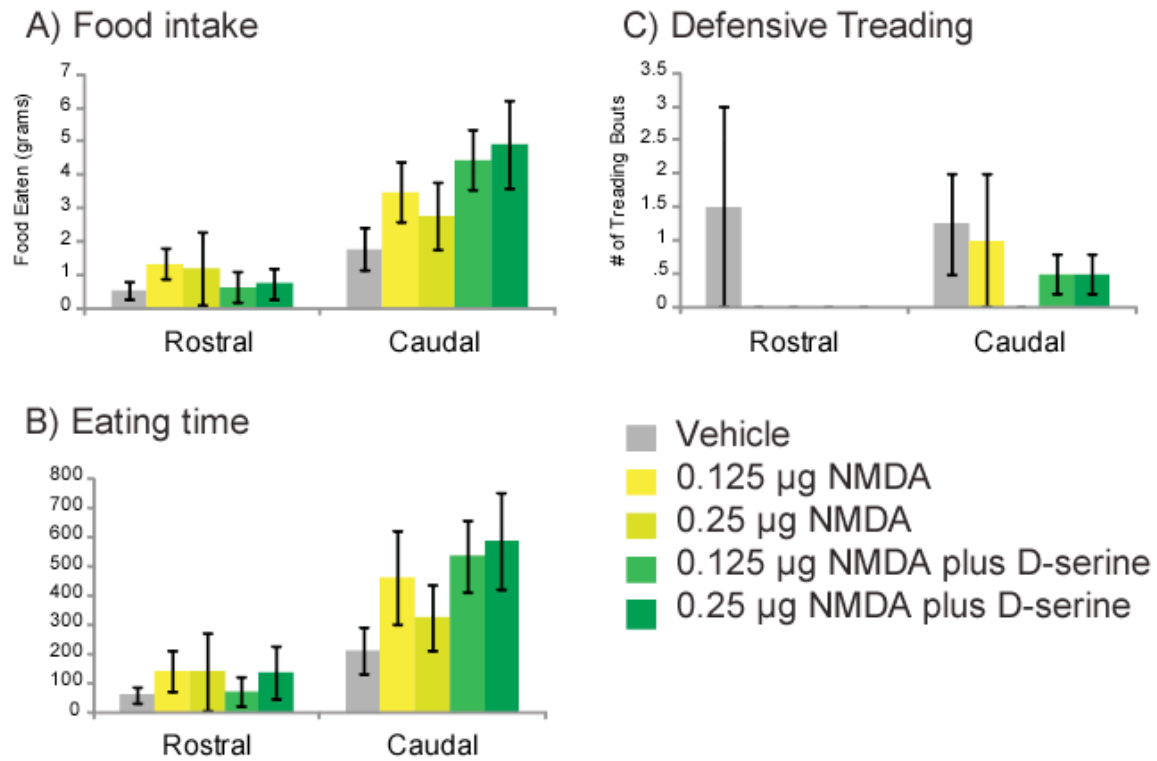
Aaron F. Garcia, Department of Psychology, University of Michigan, Ann Arbor

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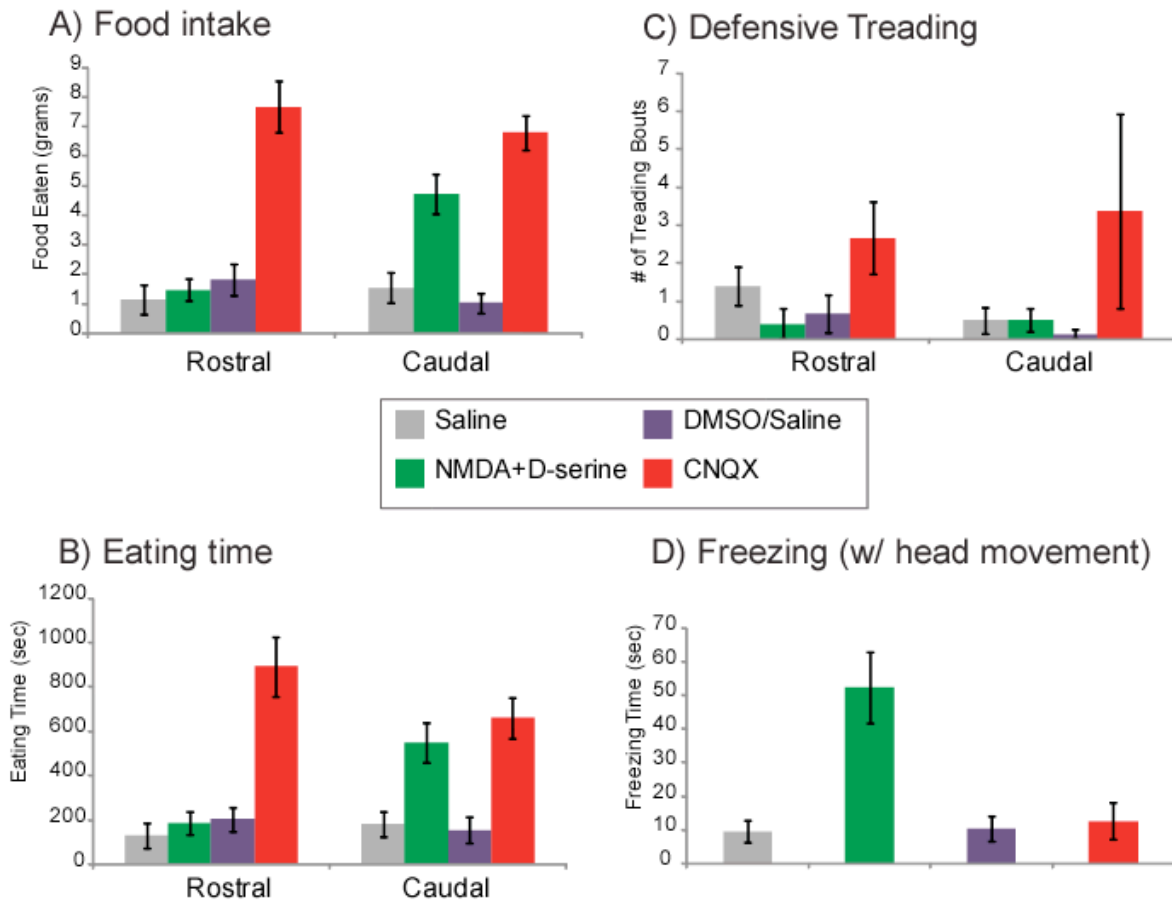
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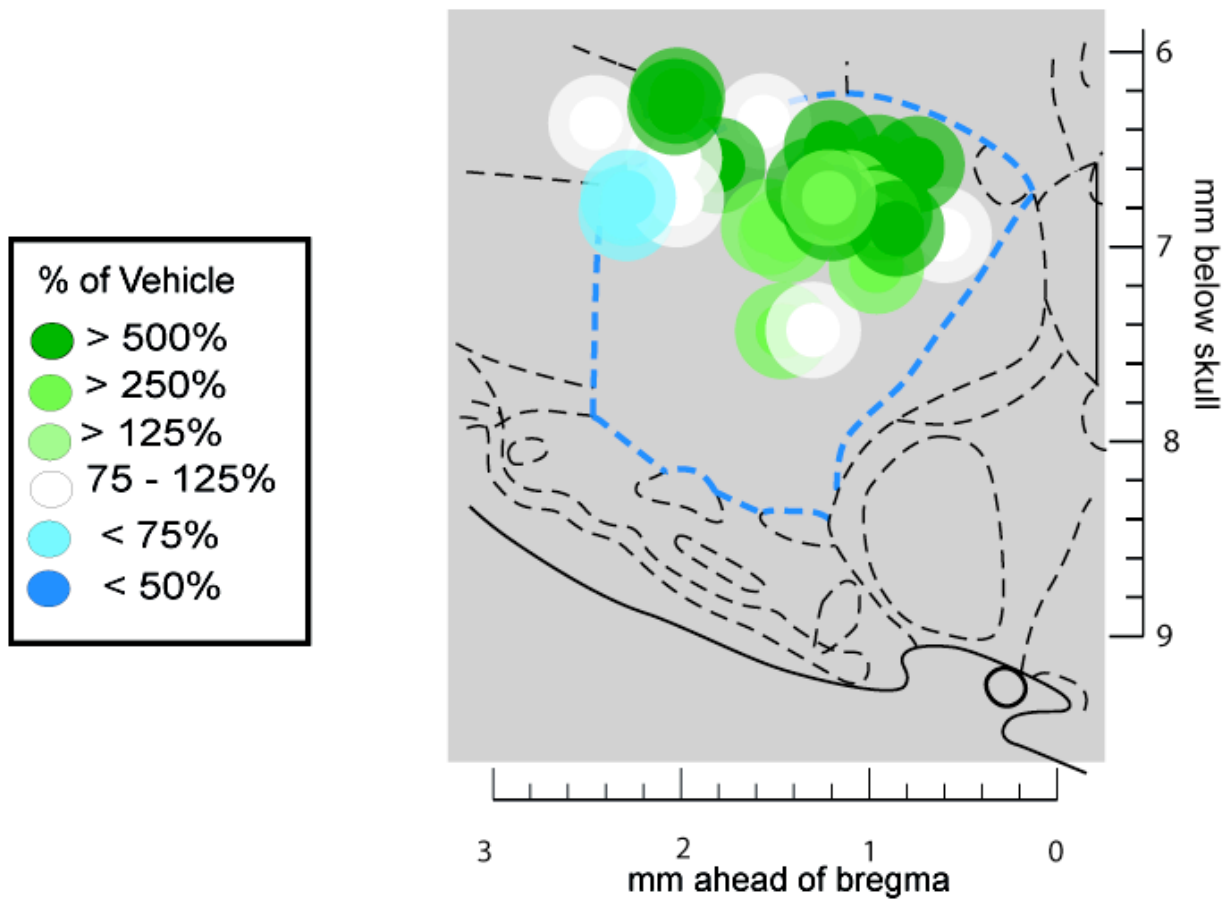
*Figure 1.* (A) The grams of food eaten under a saline vehicle or D-serine in rostral and caudal shell. The error bars represent SEM. (B) The time spent eating in seconds under a saline vehicle or D-serine in rostral and caudal shell. The error bars represent SEM. (C) The number of treading bouts under a saline vehicle or D-serine in rostral and caudal shell. The error bars represent SEM.



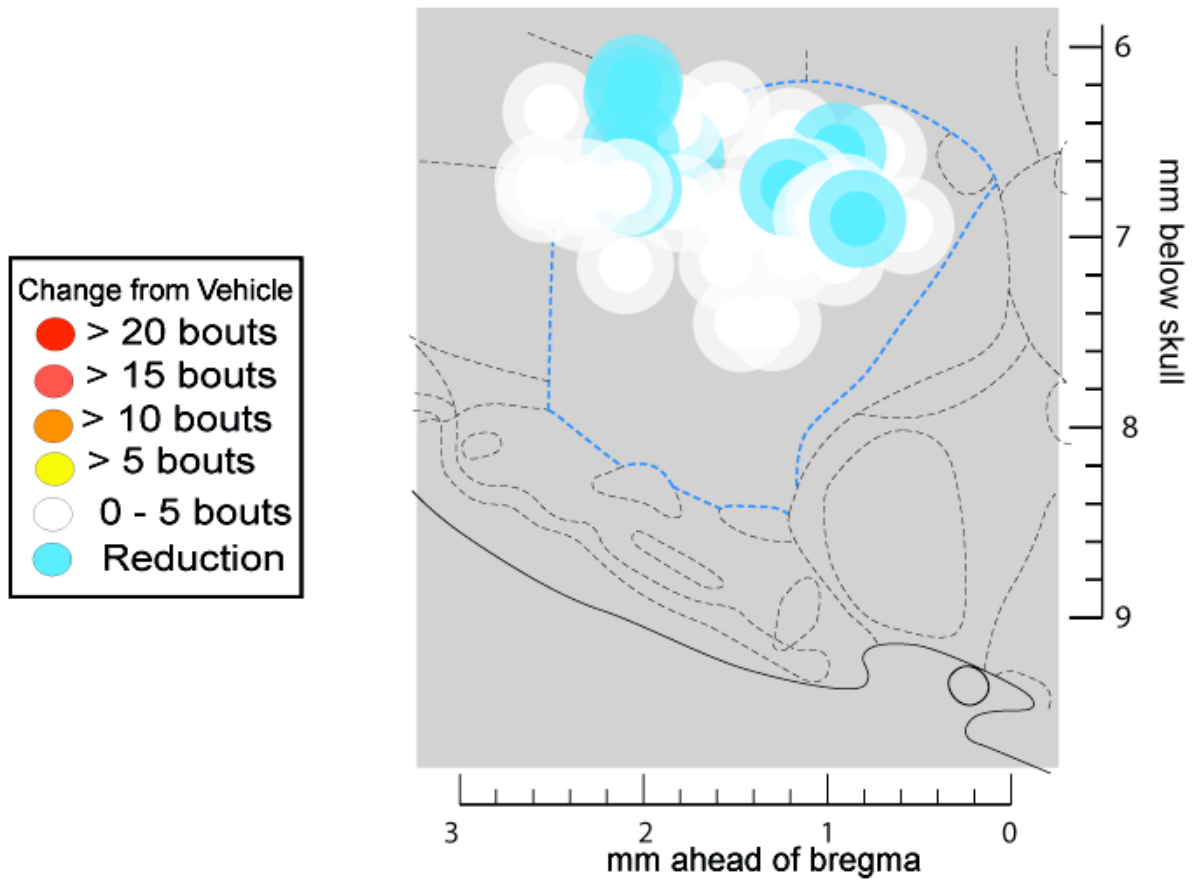
*Figure 2.* (A) The grams of food eaten under a saline vehicle, NMDA, or NMDA + D-serine in rostral and caudal shell. The error bars represent SEM. (B) The time spent eating in seconds under a saline vehicle, NMDA, or NMDA + D-serine in rostral and caudal shell. The error bars represent SEM. (C) The number of treading bouts under a saline vehicle, NMDA, or NMDA + D-serine in rostral and caudal shell. The error bars represent SEM.



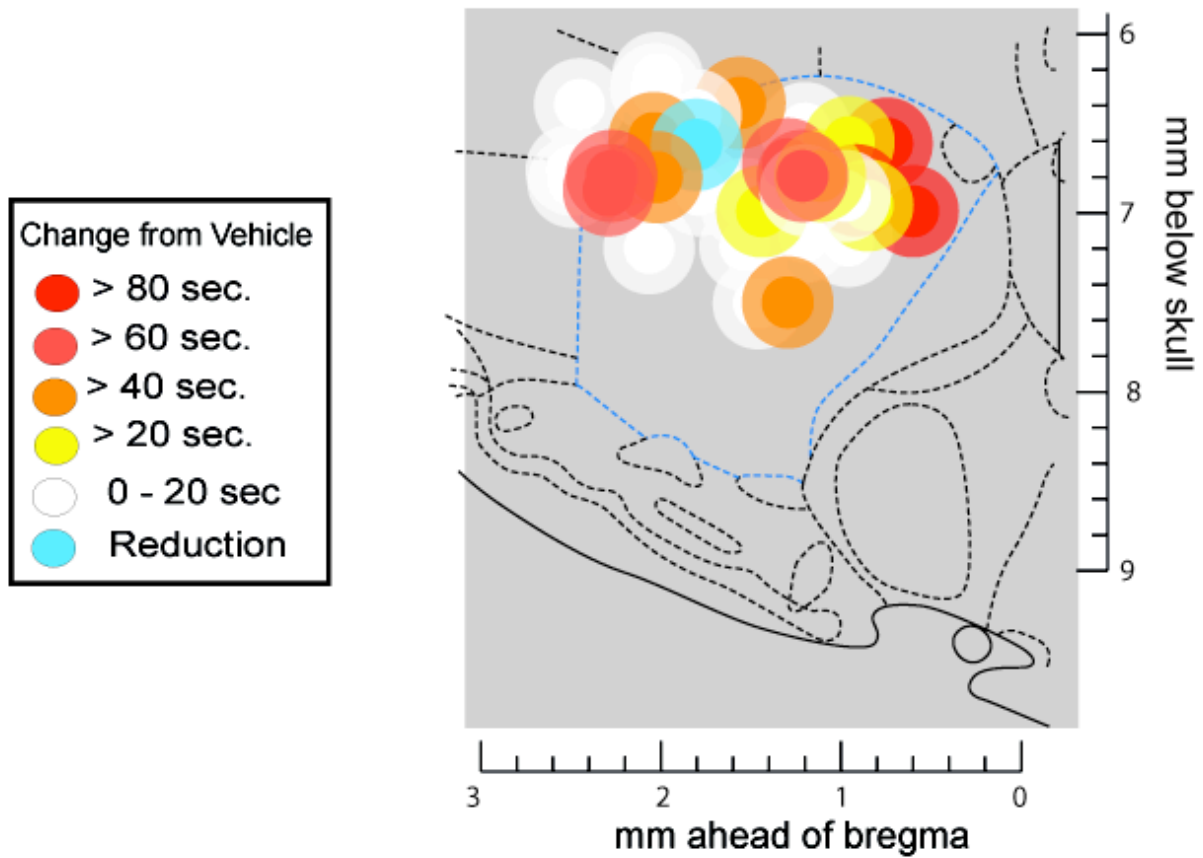
*Figure 3.* (A) The grams of food eaten under a saline vehicle, NMDA + D-serine, a DMSO/saline vehicle, or CNQX in rostral and caudal shell. The error bars represent SEM. (B) The time spent eating in seconds under a saline vehicle, NMDA + D-serine, a DMSO/saline vehicle, or CNQX in rostral and caudal shell. The error bars represent SEM. (C) The number of treading bouts under a saline vehicle, NMDA + D-serine, a DMSO/saline vehicle, or CNQX in rostral and caudal shell. The error bars represent SEM. (D) The time spent freezing (with head movement) in seconds under a saline vehicle, NMDA + D-serine, a DMSO/saline vehicle, or CNQX. The error bars represent SEM.



*Figure 4.* Fos plume map indicating the amount of eating induced by NMDA + D-serine. The colors represent the amount of eating induced by NMDA + D-serine as a percentage of eating under a saline vehicle. The inner circle represents 2 times Fos elevation over vehicle (0.15625 mm radius). The outer ring represents 1.5 times Fos elevation over vehicle (0.2375 mm radius). The map shows a sagittal view of the nucleus accumbens shell (dotted blue outline).

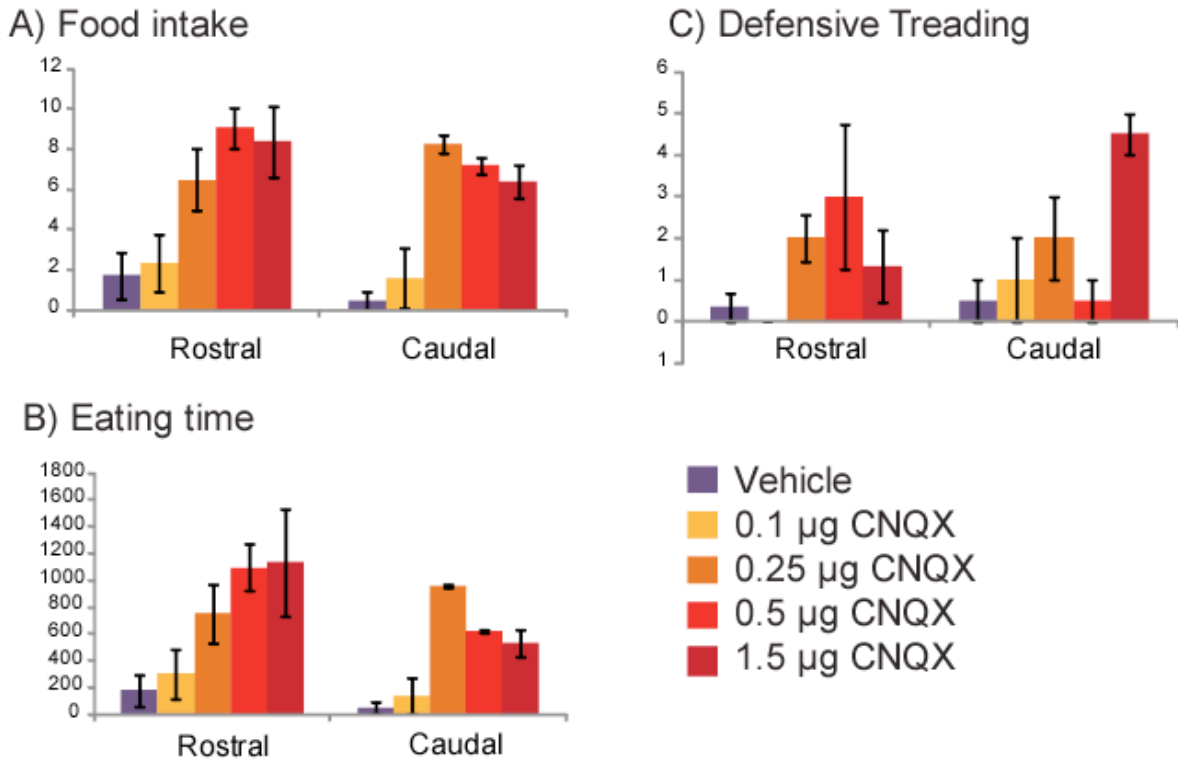


*Figure 5.* Fos plume map indicating the amount of defensive treading induced by NMDA + D-serine. The colors represent the number of treading bouts induced by NMDA + D-serine in comparison to a saline vehicle. The inner circle represents 2 times Fos elevation over vehicle (0.15625 mm radius). The outer ring represents 1.5 times Fos elevation over vehicle (0.2375 mm radius). The map shows a sagittal view of the nucleus accumbens shell (dotted blue outline).

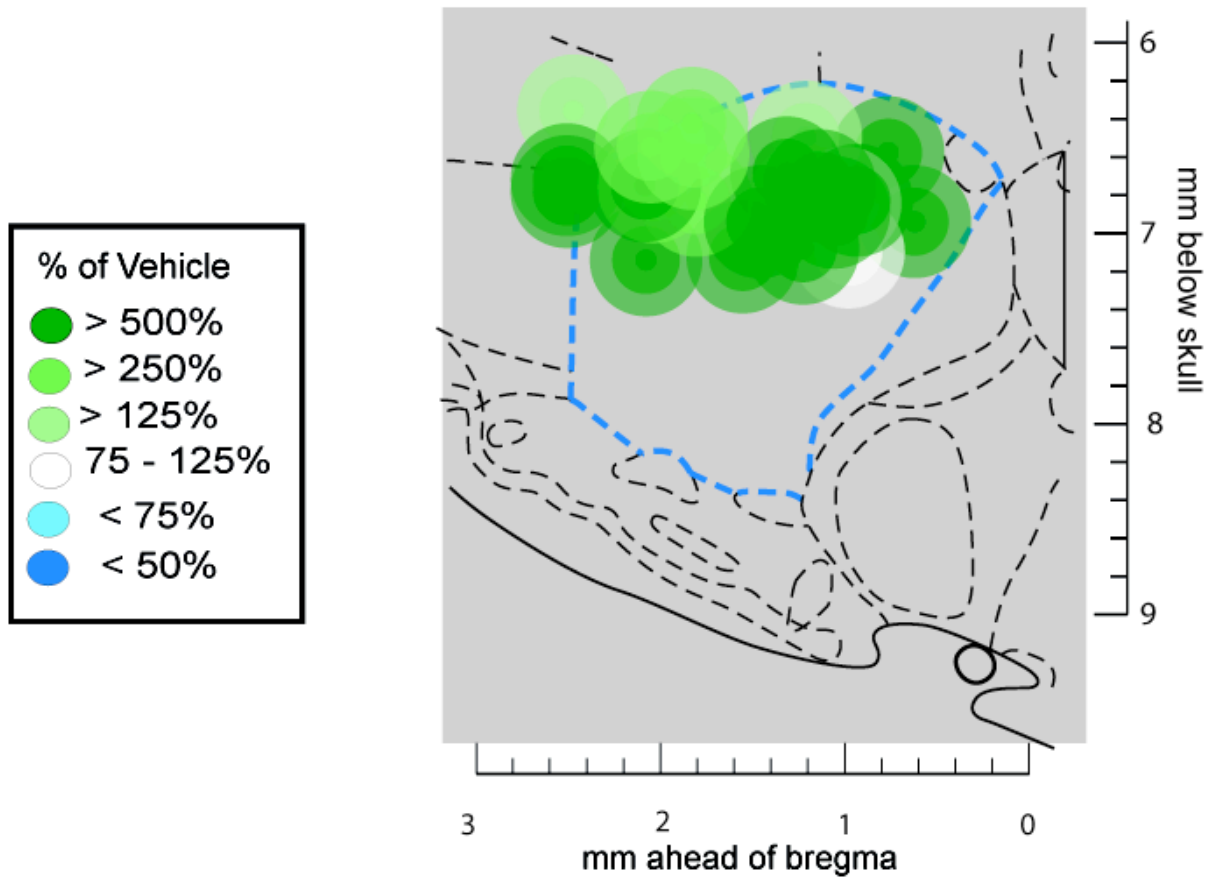


*Figure 6.* Fos plume map indicating the amount of freezing induced by NMDA + D-serine. The colors represent the time spent freezing induced by NMDA + D-serine in comparison to a saline vehicle. The inner circle represents 2 times Fos elevation over vehicle (0.15625 mm radius). The outer ring represents 1.5 times Fos elevation over vehicle (0.2375 mm radius). The map shows a sagittal view of the nucleus accumbens shell (dotted blue outline).

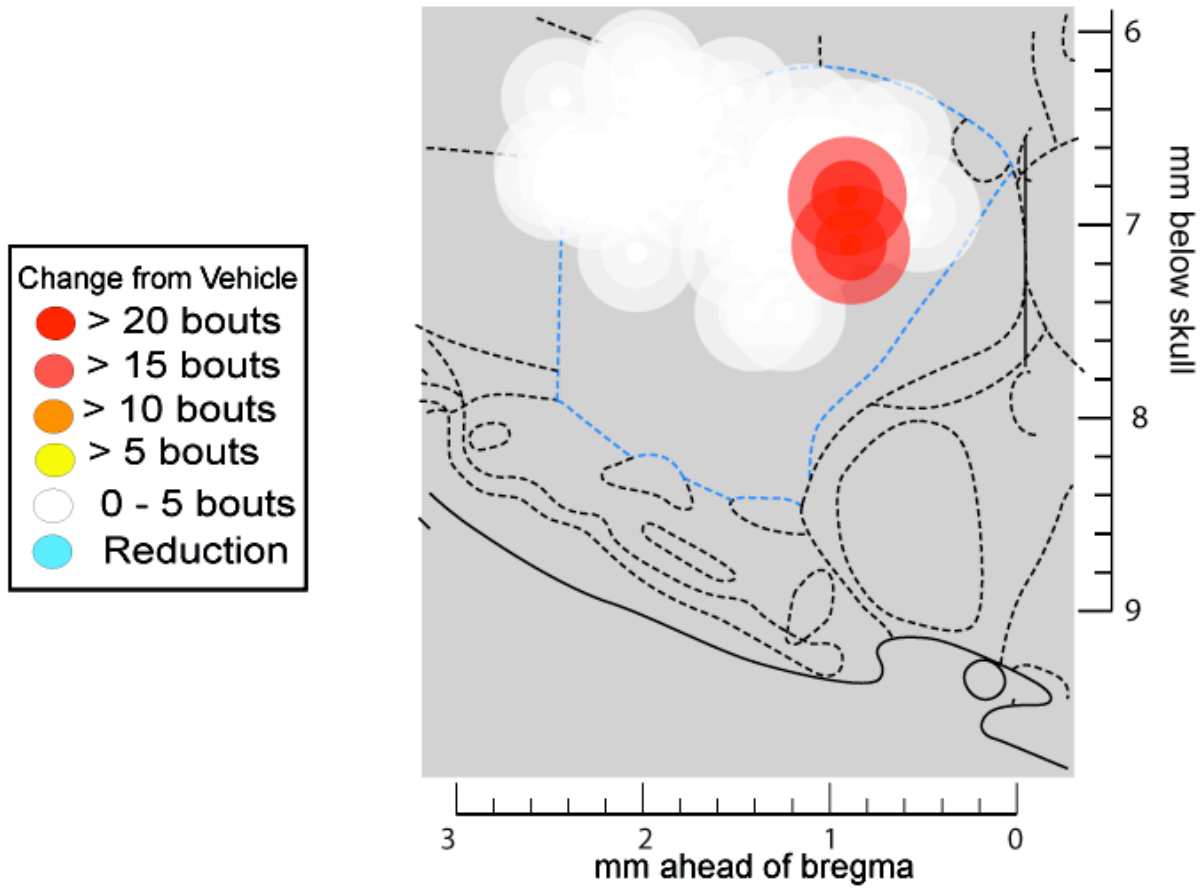




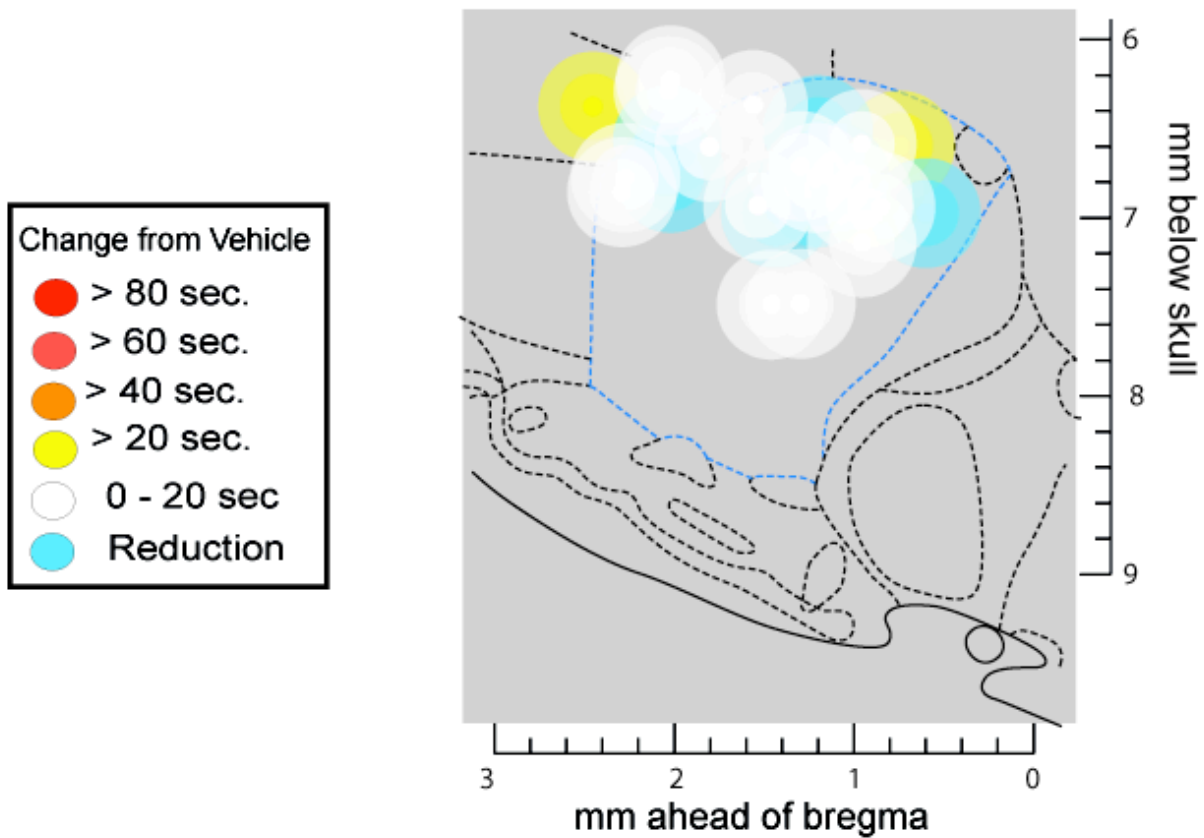
*Figure 7.* (A) The grams of food eaten under a DMSO/saline vehicle or CNQX in rostral and caudal shell. The error bars represent SEM. (B) The time spent eating in seconds under a DMSO/saline vehicle or CNQX in rostral and caudal shell. The error bars represent SEM. (C) The number of treading bouts under a DMSO/saline vehicle or CNQX in rostral and caudal shell. The error bars represent SEM.



*Figure 8.* Fos plume map indicating the amount of eating induced by CNQX. The colors represent the amount of eating induced by CNQX as a percentage of eating under a DMSO/saline vehicle. The inner circle represents 5 times Fos elevation over vehicle (0.025 mm radius). The intermediate band represents 2 times Fos elevation over vehicle (0.19375 mm radius). The outer ring represents 1.5 times Fos elevation over vehicle (0.30625 mm radius). The map shows a sagittal view of the nucleus accumbens shell (dotted blue outline).



*Figure 9.* Fos plume map indicating the amount of defensive treading induced by CNQX. The colors represent the number of treading bouts induced by CNQX in comparison to a DMSO/saline vehicle. The inner circle represents 5 times Fos elevation over vehicle (0.025 mm radius). The intermediate band represents 2 times Fos elevation over vehicle (0.19375 mm radius). The outer ring represents 1.5 times Fos elevation over vehicle (0.30625 mm radius). The map shows a sagittal view of the nucleus accumbens shell (dotted blue outline).



*Figure 10.* Fos plume map indicating the amount of freezing induced by CNQX. The colors represent the time spent freezing induced by CNQX in comparison to a DMSO/saline vehicle. The inner circle represents 5 times Fos elevation over vehicle (0.025 mm radius). The intermediate band represents 2 times Fos elevation over vehicle (0.19375 mm radius). The outer ring represents 1.5 times Fos elevation over vehicle (0.30625 mm radius). The map shows a sagittal view of the nucleus accumbens shell (dotted blue outline).