Prefrontal Cortex Modulates Desire and Dread Generated by Nucleus Accumbens Glutamate Disruption

Supplemental Information

Supplemental Methods

Cranial Cannulation Surgery

Under surgical anesthesia, all rats received bilateral implantation of permanent cranial cannulae aimed at points throughout the rostrocaudal extent of medial shell of nucleus accumbens (NAc) (19 mm, 23 gauge) and either the infralimbic, prelimbic, or medial orbitofrontal cortex regions of prefrontal cortex (PFC) (15 mm, 23 gauge). Coordinates for each rat were bilaterally symmetrical, and aimed 2 mm above target sites. Following induction of anesthesia with ketamine hydrochloride (80 mg/kg) and xylazine (5 mg/kg), and treatment with atropine (0.05 mg/kg) to prevent respiratory distress, rats were placed in a stereotaxic apparatus (David Kopf Instruments) with a flat-skull angle (incisor bar set at -3.3 mm). Cannulae aimed at prefrontal cortex were angled laterally 5° so that injection sites were sufficiently medial following implantation centered on the following coordinates: medial orbitofrontal cortex (*n* = 45): anteroposterior (AP) +4.5 mm, mediolateral (ML) +/-.8 mm, dorsoventral (DV) -3.0 mm; infralimbic (n = 34): AP +3.0 mm, ML +/-.8 mm, DV -3.0 mm; prelimbic (*n* = 14): AP +3.4 mm, ML +/-.8 mm, and DV -2.0 mm. To avoid the lateral ventricles, and to allow sufficient space between cannulae aimed at NAc and prefrontal cortex, cannulae aimed at NAc were angled laterally 15º and aimed at coordinates between AP +.8 to +2.5 mm, ML +/- 3.0 to +/- 3.2 mm, and DV, -6.0 to -6.5 mm. Cannulae were anchored to the skull using four surgical screws and dental acrylic, and stainless steel obturators (28 gauge) were inserted to avoid cannulae occlusion. Post-surgery, rats received injections of chloramphenical sodium succinate (60 mg/kg)

to prevent infection and carprofen (5 mg/kg) for pain relief, and were allowed to recovery for at least 7 days before testing began.

Bicuculline Dose

Many precedents exist for the use bicuculline or other gamma-aminobutyric acid antagonists in cortical microinjections for the activation of prefrontal cortex (1-10). Doses of bicuculline similar or larger than here have been shown to depress heat-induced tail flick (100, 200 or 300 ng; 5, 9), block acquisition of conditioned defeat (300 ng; 6), block increases in impulsivity produced by an *N*-methyl-D-aspartate antagonist (500 ng; 7), impair attention (25 ng; 8) and increase response latencies in a working memory task (50 ng; 4). No generalized behavioral deficits or seizure-like activity were reported by those studies, even at a dose 3-times that used in our current study (300 ng; 6). Our specific dose (100 ng in .2 µl per side) was chosen based on pilot studies (11). All of our testing was video recorded and watched carefully by trained observers, who quantified all of the behaviors of interest and looked for any unusual activity, including seizures. We observed no seizure-like behaviors at this dose in either the pilot experiment or in the experiments reported here.

Microinjection Procedure

All drug conditions were received in counterbalanced order, on testing days spaced at least 48 hours apart. Rats used for Fos analysis received only one set of microinjections. On testing days, all solutions were brought to room temperature (~21ºC) and bilaterally infused, at a speed of 0.2 µl / minute, through stainless steel injectors (16 mm to prefrontal cortex, 21 mm to NAc, 29 gauge), which extended 2 mm beyond the tip of the cannulae into the target region.

Following infusion, injectors were left in place for 1 minute to allow drug diffusion, after which obturators were replaced and rats were immediately placed in the testing chamber.

Behavioral Tests of Spontaneous Motivated Behaviors

All behavioral testing rats (n = 98) were habituated to the testing procedure and apparatus on 4 days. On each testing day, rats received one of the drug conditions described previously, and were placed immediately in the transparent testing chamber (23 x 20 x 45 cm) which contained preweighed food (~20g of rat chow) and *ad libitum* water, to allow the expression of appetitive behavior, and ~3 cm deep of granular corn cob bedding, to allow the expression of defensive treading behavior. Behavior in the chamber was video recorded for 60 minutes and scored later offline for analysis. Rats were removed by the experimenter at the end of the session using a standardized slow-approach hand motion. The experimenter recorded whether rats made any audible distress vocalizations, attempts to escape, or attempts to bite the experimenter.

Fos-like Protein Immunohistochemistry

Following transcardial perfusions, brains used for Fos analysis were removed and placed in 4% paraformaldehyde for 4–24 hours, and then transferred to 25% sucrose (in 0.1 M NaPB) for at least 3 days. Brains were sliced at 40 microns on a freezing microtome, and processed for Fos-like immunoreactivity using NDS, goat anti-cfos (Santa Cruz Biotechnology, Santa Cruz, CA) and donkey anti-goat Alexa Fluor 488 (Invitrogen, Carlsbad, CA) as described previously (12-14). Sections were mounted, air-dried and coverslipped with ProLong Gold antifade reagent (Invitrogen).

Fos Identification and Assessment: Local Fos Plumes and PFC-NAc Interactions

Immunoreactivity for Fos-like proteins was visualized using a Leica microscope equipped for fluorescent microscopy, using a filter with an excitation band at 480-505 nm for Fos-positive cells and images were taken using MCID Core software. For analysis of drug spread, Fos plumes images were taken in the areas surrounding the microinjection with the most intense areas of Fos expression, just medial to the end of the injector tip, surrounding a small focal point of necrosis. Fos labeled cells were individually counted within successive blocks (50 µm x 50 µm), along 8 radial arms emanating from the center of the necrosis, with 10x magnification (Figure 1). Zones of Fos elevation (or "plumes") were assessed as described previously (12). Additionally, to assess whether prefrontal cortex microinjections had direct neurobiological effects on NAc shell, we assessed immunoreactivity for Fos-like protein in NAc shell of rats who received no microinjections in NAc, following either vehicle or bicuculline prefrontal cortex microinjections. We counted Fos densities in NAc shell at three coronal sections (rostral, middle and caudal) in boxes (200 µm x 200 µm) centered over ventral and dorsal regions, spaced 200 µm apart dorsoventrally.

Histology

Following all testing, behavioral testing rats were deeply anesthetized with an overdose of sodium pentobarbital. Brains were removed and fixed in 10% paraformaldehyde for 2 days and in 25% sucrose solution for 3 days. Brains were sliced at 60 microns on a freezing microtome, and stained with cresyl violet for verification of microinjection sites. Maps of bilateral Fos plume-sized placements in the sagittal plane were then color-coded to express changes in behavior for individual rats in the figures, as either a percent of vehicle control (for

DNQX effects) or percent of DNQX in NAc and vehicle in prefrontal cortex (for effects of prefrontal activation or inhibition). NAc placements were classified as rostral if their placements were located +1.8 to +2.4 mm ahead of bregma, middle if their placements were located +1.0 to +1.8 ahead of bregma, and caudal if their placements were located +0.4 to +1.0 mm ahead of bregma.

Statistical Analysis

The effects of DNQX on each continuous behavior were assessed using a three-factor mixed within- and between-subject analysis of variance (ANOVA) (drug [DNQX versus vehicle] x group [activation versus inactivation] x NAc placement [rostral versus caudal]) to verify elicitation of eating and defensive behavior along a rostrocaudal gradient, equally within both groups. The effects of prefrontal activation and inactivation on DNQX-induced behavior were assessed using an additional four-factor mixed within- and between-subject ANOVA, in comparison to behavior on DNQX-alone (prefrontal drug x DNQX x prefrontal placement x NAc placement). When significant interactions were found, rats were split by anatomical location and additional analysis was done using a two-way ANOVA (prefrontal drug x NAc drug) and additional one-way ANOVAs with pairwise comparisons using Sidak corrections for multiple comparisons. For the purposes of visualizing data, NAc placements were further divided into six rostrocaudal bins and dorsal and ventral prefrontal placements were divided into eight rostrocaudal bins. Bar graphs of these bins were placed next to maps of behavioral effects produced at individual NAc or prefrontal sites.

Supplemental References

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