Connections of the Mouse Orbitofrontal Cortex and Regulation of Goal-Directed Action Selection by BDNF-TrkB

Supplemental Information

Supplemental Methods & Materials

Surgery

Mice were anaesthetized with ketamine/xylazine, ketamine/dormitor, or 1:1 2-methyl-2-butanol and tribromoethanol (Sigma) diluted 40-fold with saline in the case of lesions. With needles centered at bregma, stereotaxic coordinates were located on the leveled skull using a digitized stereotaxic frame (Stoelting). For tracing experiments, mice received 0.15 ml unilateral infusions of BDA 10,000 in either VLO or DLO/AI. Coordinates from bregma, based on the mouse brain atlas of Paxinos and Franklin (1), were as follows: AP +2.22 to 2.8, ML $\pm$1.2 to $\pm$2.2, DV -2.8 to -3.0. The microsyringe remained in place for 10 min following infusions. One week following surgery, mice were deeply anesthetized and transcardially perfused with 4% paraformaldehyde. Brains were collected, and BDA expression was analyzed as described below under “Histology.”

For viral vector surgeries, lentiviral vectors expressing GFP or Cre recombinase were generated by the Emory University Viral Vector Core. AAV5-CaMKII-HA-hM$_4$D(Gi)-IRES-mCitrine or AAV5-CaMKII-GFP were purchased from the UNC Viral Vector Core. Infusion coordinates for the VLO were AP +2.6, DV -2.8, ML ±1.2. Amygdala coordinates were AP -1.4, DV -4.9, ML ±3.0. Viral vector volumes were 0.25 ml; 0.1 ml was used for NMDA (20 mg/ml in sterile saline; Sigma). Infusions were delivered over 5 min with needles left in place for 4 additional min. Mice were sutured and allowed to recover for at least 3 weeks before behavioral testing. After testing, mice were deeply anaesthetized and transcardially perfused with 4% paraformaldehyde, then brains were processed as described under “Histology.”

Vectors were infused bilaterally, or in the case of “disconnection” experiments, lenti-Cre was infused *unilaterally* in the VLO and NMDA was infused either ipsilaterally or contralaterally in the amygdala. Additional mice were infused ipsilaterally or contralaterally with lenti-GFP and saline, respectively. Throughout, no differences were observed between these two control groups, which were combined for statistical and graphical purposes.

Extinction Conditioning

After instrumental conditioning as described in the main text, mice in one experiment were placed in the conditioning chambers for an additional 15 min/day for 7 days. Responding was not reinforced, and mice were injected with saline or 7,8-DHF immediately after each session. This protocol was chosen because it is sensitive to between-group differences, if any, in appetitive response extinction (2). Graphically, response rates on both apertures are represented and were compared by ANOVA with repeated measures.

Dendritic Spine Imaging and Enumeration

Dendritic spine imaging was accomplished as described (3,4) immediately following the final extinction training session. Briefly, fresh YFP-expressing brains were submerged in 4% paraformaldehyde for 48 hours, then transferred to 30% w/v sucrose, followed by sectioning into 40 µm-thick sections on a microtome held at -15°C. Unobstructed dendritic segments running parallel to the surface of the section were imaged on a spinning disk confocal (VisiTech International) on a Leica microscope. Z-stacks were collected with a 100X 1.4NA objective using a 0.1 mm step size, sampling above and below the dendrite. After imaging, we confirmed at 10X that the image was collected from the VLO.

Collapsed z-stacks were analyzed using NIH ImageJ. Each protrusion $\leq$4 mm was considered a spine and counted (5). Individual planes were evaluated to detect protrusions

perpendicular to the z-stack. Bifurcated spines were considered singular units. To generate density values, spine number for each segment was normalized to the length of the segment. 4-6 independent segments from secondary and tertiary dendritic branches within 50-150 mm of the soma were collected. Each animal contributed a single density value (its average) to statistical analyses. Due to the relatively stellate appearance of VLO neurons, apical *vs*. basal branches were not distinguished (e.g., see 6,7). A single blinded rater scored all spines.

BDA Histology

Brains were sectioned into 55 mm-thick sections on a microtome held at -15°C. For BDA tracing studies, BDA signal was amplified with a standard Vectastain Elite ABC kit and revealed by nickel-enhanced-diaminobenzidine staining. Sections were then mounted and lightly counterstained with Cresyl violet before being coverslipped. BDA signal was examined in both brightfield and darkfield. Maximum diffusion around the infusion site was mapped, and labeling patterns of axon terminals from each infusion site were transposed onto representative coronal sections from *The Mouse Brain in Stereotaxic Coordinates, Second Edition* (1). Labeling from 2-3 animals was analyzed for each site.

BDNF Quantification

Mice were rapidly decapitated, and brains were extracted and immediately frozen on dry ice for BDNF quantification by enzyme-linked immunosorbent assay (ELISA). Frozen tissue was sectioned into 1 mm-thick coronal sections, and the VLO and amygdala were extracted with bilateral tissue punches (1 mm core) likely containing both infected and uninfected tissues. Tissue samples were homogenized in lysis buffer [200 ml: 137 mM NaCl, 20 mM tris-Hcl (pH = 8), 1% igepal, 10% glycerol, 1:100 Phosphatase Inhibitor Cocktails 2 and 3 (Sigma)] by sonication.

BDNF quantification was accomplished using a 2-site BDNF ELISA kit in accordance with the manufacturer's instructions (Promega) except that 75 µl of sample/well was loaded, and the extraction procedure was excluded. BDNF concentrations were normalized to the total protein content in each sample as determined by Bradford colorimetric assay; covariance with behavioral measures was tested using a linear regression analysis.

In a replication study, an independent group of mice was infused with lenti-Cre or lenti-GFP into the VLO, then euthanized in the absence of behavioral testing 4 weeks later for BDNF quantification. The values in the histograms in figure 4 of the main text represent both behaviorally-characterized and behaviorally-naive cohorts. Amygdala BDNF was compared by t-test. VLO BDNF concentrations were non-normally distributed and thus compared by Mann-Whitney comparisons.

Statistical Analyses

Two-tailed statistical analyses with $\alpha \leq 0.05$ were performed using SigmaStat v.3.1 or SPSS v.21. Tukey's post-hoc tests were utilized in the event of significant interaction effects; significant posthoc comparisons are indicated graphically.

In the case of values lying >2 standard deviations outside of the mean, these values were considered outliers and excluded. As a result, three outlying values were eliminated from the BDNF ELISA experiments (Fig. 4). Two mice were removed from the "disconnection" study (Fig. 5) – one from the GFP/saline control group, and one from the ipsilateral control group. Of note, both generated exceptionally high response rates associated with the intact response-outcome contingency; therefore, we biased against the reported finding by removing them. Last, in the *Bdnf* knockdown/7,8-DHF experiment (Fig. 6), one mouse was removed from each of the four groups because they met the statistical criterion for being considered an outlier.

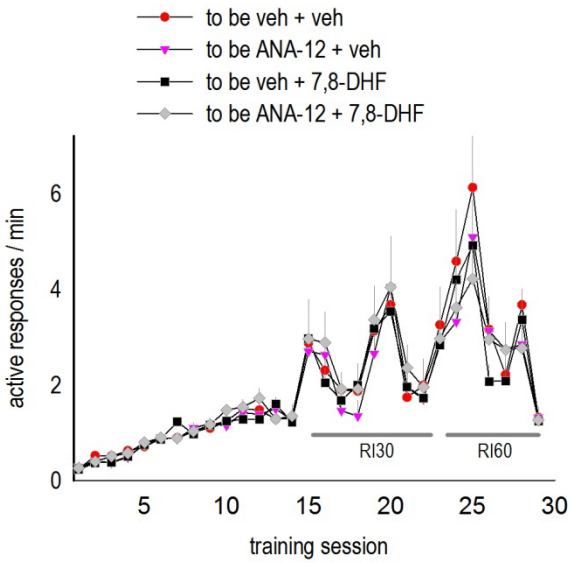

Figure S1. Response acquisition curve associated with main text Figure 6C. Mice acquired the nose poke responses without differences between groups (*F*s < 1). “RI” refers to random interval schedules of reinforcement. Symbols represent means + SEMs.

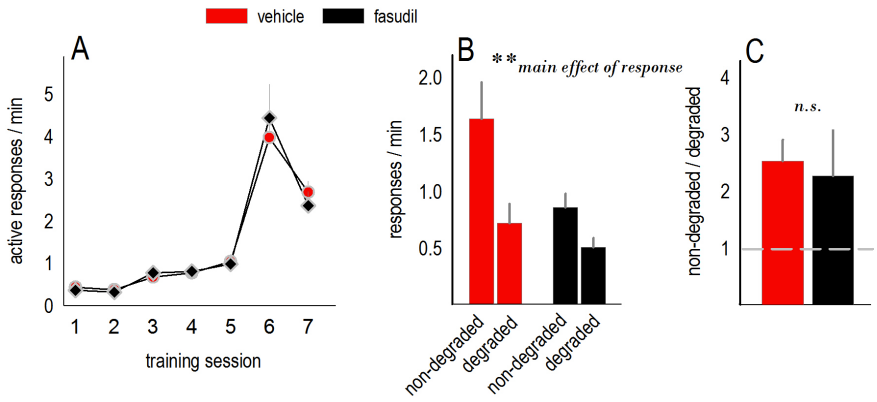

Figure S2. Acute fasudil treatment does not interfere with goal-directed action selection in intact mice. (**A**) Mice acquired the nose poke responses without differences between groups ($F < 1$). (**B**) Following the degradation of one action-outcome contingency, all mice preferentially performed the remaining response, that most likely to be reinforced [main effect of response $F_{(1,9)} = 21.5$, $p < .001$]. We also detected a trend for a main effect of fasudil [$F_{(1,9)} = 4.6$, $p = .06$]. (**C**) Nonetheless, the ratio of responses directed towards the "non-degraded" action-outcome contingency, relative to the degraded contingency, did not differ between groups. In other words, both groups generated the response that was more likely to be reinforced ~2.5-fold more than the alternative response. A ratio of 1 reflects chance-level responding. Symbols and bars represent means + SEMs. $^{**}p < .001$.

Supplemental References

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