Title: Dorsal striatal dopamine induces fronto-cortical hypoactivity and attenuates anxiety and compulsive behaviors in rats

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Supplementary Materials and Methods

Study Design

In the first cohort (cohort 1) of animals, we confirmed the established targeted transduction model, with random assignment of rats to either the chemogenetic or the control group (Fig. S1). We then tested the effects of chemogenetic activation on open field locomotor behavior as well as CSTC-specific behavioral phenotypes, i.e. self-grooming and sensory gating. In the second cohort (cohort 2), we repeated the locomotor assessment and measured metabolic activity and functional connectivity in the CSTC circuit using [^{18}F]FDG PET, while also measuring local concentrations of energy metabolites with MRS. We excluded from consideration rats not showing bilateral expression of hM3Dq-DREADD to immunohistochemistry post hoc, along with statistically significant outliers in other experiments. We conclude by discussing the various results in the context of how altering dopamine transmission in the mDS may be relevant to the symptoms of OCD.

Chemicals

Clozapine N-oxide (CNO) purchased from Cayman Chemical Company (Cat. #16882) was diluted to 10 mg/ml in dimethyl sulfoxide (DMSO, 99% Merck) and stored at -20 ºC. Before use, it was further diluted 20-fold in normal saline and administered intraperitoneally at a dose of 0.5 mg/kg.

Viral vectors

The DREADD (hM3Dq-containing) adeno-associated virus (AAV) – rAAV6-hSYN-DIO-hM3Dq-mCherry *4.3x10E12 vg/mL* – was produced by the Vector Core at the University of North Carolina at Chapel Hill (UNC Vector Core). The control (hM3Dq-negative) virus, – rAAV6-hSYN-DIO-mCherry – was produced by the Viral Vector Facility at the University of Zürich (UZH) *8.5 x 10E12 vg/mL*.

*Software*We used the following commercial programs for data analysis and plotting: Matlab vR2018b (Mathworks), PMOD v3.7 (PMOD Technologies LLC), RStudio v1.2.1335 *(95)* (RStudio), FSL 5.0.11 (FMRIB Software Library), Prism v8 (GraphPad). EthoVision v.11.5 (Noldus Information Technologies), LC Model (Provencher).

Breeding and Phenotypic Assessment in Animals

TH:Cre positive males were crossed with wildtype females and outbred, resulting in hemizygous TH:Cre positive or wild-type pups. The breeding was conducted at the Department of Experimental Medicine (AEM, University of Copenhagen). The rats were ear-marked and

transferred to the rodent facility at Rigshospitalet to habituate for at least one week before surgery. Animals were genotyped using the REDExtract-N-Amp™ Tissue PCR Kit (Cat. #XNAT, Sigma) and Cre recombinase oligonucleotides (Eurofins): forward 5'-GCG GTC TGG CAG TAA AAA CTA TC-3' and reverse: 5'-GTG AAA CAG CAT TGC TGT CAC TT-3'. The rats were housed in same sex groups of 2-3 animals per cage with 12 h light cycle beginning at 0600 and ending at 1800. All experiments were conducted during the light phase. Females were fed *ad libitum* (1319 Avlsfoder, Rotte/Mus, Brogaarden), but males were diet-restricted after their eighth week of age to 20 g of food pellets per day to keep their body weight below 500 g, as required for scanning. We conducted all procedures in accordance with the FELASA guidelines and with approval from The Danish Animal Experiments Inspectorate (license number: 2016-15-0201-01031). GMO procedures were approved by the Danish Working Environment Authority (Lab ID 229 806).

Immunohistochemistry details

In the ABC-P method, slices were washed in 10 mM PBS, and then in 1% H_2O_2 in PBS. Non-specific binding was blocked with 5% normal sheep serum (NSS), 1% BSA, and 0.3% Triton X-100 (TX) in PBS. Sections were incubated at 4 ºC in primary antibody (1:1000 dilution in 1% BSA, 0.3% TX-100 in PBS) overnight and later washed in 0.1% TX in PBS followed by one hour incubation with secondary antibody at room temperature (1:1000 dilution in 1% BSA, 0.3% TX in PBS). After washing again, the samples were incubated another hour in ABC-P (Cat. #PK-6100, Vector laboratories) and rinsed in Tris-HCl before being developed in 0.1% diaminobenzidine (DAB). In the fluorescent method, nonspecific binding was blocked with 0.2% BSA, 0.2% TX, and normal donkey serum (NmDS) in PBS at room temperature, before incubation with primary antibody (diluted 1:500 in PBS) overnight at 4 ºC. Sections were then washed and blocked with 2% NmDS before a 2-hour incubation with the fluorescent secondary antibody (1:200 in PBS) at room temperature in the dark. Stained sections were mounted on slides, covered with Pertex or antifade mounting medium, and imaged with a Zeiss Axio Imager Z.1 imager microscope. Digital images of ABC-P stained brain slices were analyzed to assemble a depiction of the extent and pattern of transduction in the target regions.

Acoustic startle response

Startle tests were conducted in a sound-attenuated, lighted and ventilated chamber (StartFear Combined system, Panlab Harvard Apparatus, Barcelona, Spain). The chamber (67 x 53 x 55 cm) contains a behavioral box (25 x 25 x 25 cm) mounted on a load cell platform, a loudspeaker mounted above the animal, and a small LED light for illumination during testing. Animals were held in a Plexiglas cylinder (225 mm length x 74 mm diameter) mounted on a grid floor by plastic screws. Startle responses were detected using weight transducers placed under each leg of the load cell platform, and the response was amplified and digitized over a range of 0–100 arbitrary units. The amplitude of the startle response is defined as the largest peak value within 1 s after the onset of the startle stimulus. Data collection and sequencing of all stimuli are controlled by the supplied hardware and software (Packwin V2.0). The startle response of the rats was assessed before the PPI tests. After an exploration period of 10 min with a constant background white noise of 65 dB for acclimation, 55 startle stimuli of 20 ms duration. (70-120 dB white noise (w.n.), in 5 dB intervals, 5 of each) were presented in a pseudo-randomized order, with variable pseudo-randomized inter-trial intervals (ITI; range 6-24s, mean 15 s) to avoid predictability of the next

stimulation. The startle response corresponding to each pulse was plotted for each rat and the maximum pulse intensity was established.

Prepulse inhibition of the acoustic startle response

The session consisted of four blocks. Block I was a 5 min exploration period with only background noise (65 dB w.n.). Block II was a short-term habituation (STH) test with 10 pulses (110 dB w.n., 30 ms each at ITI of 10 s). Block III was the proper startle and pre-pulse inhibition (PPI) test, consisting of seven different trials; a) no stimuli (65 dB w.n.); b) pulse alone (110 dB w.n., 30 ms); c) pre-pulse alone (81 dB w.n., 20 ms); d-g) pulse (110 dB w.n., 30 ms) preceded 100 ms by a 20 ms w.n. pre-pulse at 69 dB (d), 73 dB (e), 77 dB (f) or 81 dB (g). Each trial type was presented 15 times in a pseudo-randomized order with a variable ITI ranging from 6-24 s (15 s mean). Block IV was another STH test, conducted just as Block II. The percentage of PPI for each pre-pulse intensity was expressed as: % PPI = [(mean startle amplitude on pulse alone trial – mean startle amplitude on pre-pulse trial)/mean startle amplitude on pulse alone trial] *100.

[^{18}F]FDG PET scanning

The rats were fasted overnight and brought to the scanning room at least two hours before the scan. Here they received an s.c. injection of 0.5 mg/kg CNO (n=14, (7 mDS-DREADD, 4F/3M), (7 mDS-mCherry, 4F/3M)) or saline followed 20 min later by an i.p. injection of 14.1 ± 1.6 MBq FDG (from the clinical in-house production of he Department of Clinical Physiology, Nuclear Medicine and PET, Righospitalet, Denmark, where the PET scans were also conducted). The rats remained in their home cage for 45 minutes following the [^{18}F]FDG injection. At the end of this tracer uptake period, the rats were rapidly sedated with isoflurane (2–2.5% in oxygen) and placed in a homemade four-rat insert at the center of the aperture of a Siemens HRRT (High Resolution Research Tomograph) scanner (20) for a 45-min list-mode emission recording followed by a 6-min rotating point source ^{137}Cs transmission scan. The rats were kept warm using an infrared lamp and monitored for respiration throughout the scan. The list-mode emission data were dynamically reconstructed with the standard 3D-OP-OSEM- algorithm with point-spread function modelling[1] (16 subsets, 10 iterations and no postreconstruction filtering) using a MAP-TR attenuation map with human head prior (20) and scatter correction . PET images were cropped to brain only images for each rat in PMOD and manually co-registration to an-FDG-specific rat brain template. Each individual scan was normalized using a whole-brain normalization factor (NF = Average whole-brain uptake of all animals/individual whole-brain uptake), this was done to preserve the scans in SUV (SUV = kBq·cc^{-1} in region/ kBq·g^{-1} injected) and not a ratio. A VOI-atlas with select regions from the CSTC circuit was used for VOI-extraction.

PET data analysis

The images were averaged per group/condition and the difference, (higher or lower tracer uptake in CNO condition compared with baseline) was calculated within each group (DS-DQ and DS-

[1] Ref: Sureau FC, Reader AJ, Comtat C, et al. Impact of image-space resolution modeling for studies with the high-resolution research tomograph. J Nucl Med. 2008;49: 1000–8, and Hong IK, Chung ST, Kim HK, et al. Ultra fast symmetry and SIMD-based projection-backprojection (SSP) algorithm for 3-D PET image reconstruction. IEEE Trans Med Imaging. 2007;26: 789–803.

VEH) in PMOD. A regional analysis within the CSTC circuit (NAc, ACC, DMS, DLS, oFC, mPFC, Thalamus) was done for each animal. The mean difference in FDG uptake between conditions (baseline & CNO) in the two groups (DS-DQ, DS-VEH) was calculated in Prism using a mixed-effect model with Sidak's multiple comparisons test. Subsequently, we made a post hoc test of FDG uptake correlations between regions in the CSTC circuit (Fig. 4D and E) for the mDS-DREADD animals at baseline and after CNO. For a specific group/condition, a Pearson's correlation coefficient was calculated between each region pair and transformed using Fisher's r-to-z transformation, to allow for comparison of data with heterogeneous distributions (92). Circuit-wide FDG correlations for each condition/group were estimated as the sum of the absolute value of z-transformed values across all unique region pairs (i.e., lower or upper triangles of the correlation matrix). Permutation testing (10,000 permutations) was used to derive an empirical null distribution and estimate statistical significance. For each permutation, respective condition/group status was scrambled prior to computing the CSTC circuit correlation matrix and sum of absolute z-transformed values. Condition/group differences were considered statistically significant if the permutation derived p-value was less than 0.05. in DS-DQ animals was performed for each region-to-region correlation. For each region pair, a null distribution was derived by scrambling regional FDG values with respect to each other and estimating the correlation. Observed p-values for the post hoc analysis were family-wise error-corrected across all the region-to-region pairs using Bonferroni-Holm (93).

Supplementary Figures

Fig. S1. Experimental setup

Fig. S2. Quantification of transduction in rats

Fig. S3: Second batch of locomotor experiments in animals for imaging experiments

Fig. S4. Locomotor data per time from manuscript figure 2 for each sex

Fig. S5: Additional acoustic startle data.

Fig. S6: Representative [^{18}F]FDG-PET and MR rat brain image with all atlas VOIs

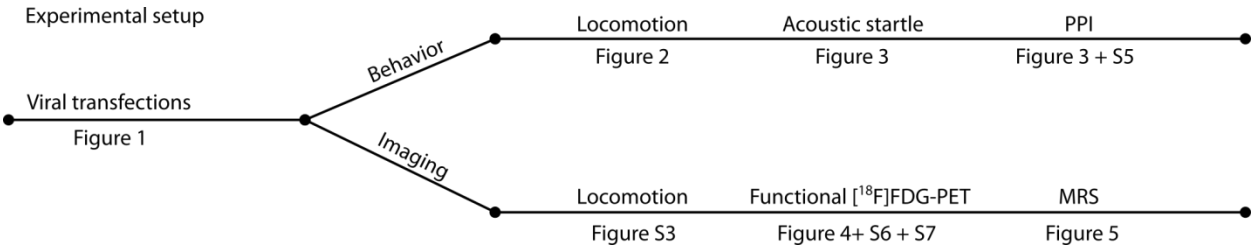

Figure S1: Experimental setup. The experiment was designed in three phases: (1) validation of the novel transduction approach, which selectively targets the nigrostriatal dopamine neurons innervating the mDS, (2) behavioral analyses following chemogenetic activation of these neurons, and (3) in vivo imaging of its effects on metabolic activity, functional connectivity and neurochemical markers in mDS and frontal cortex. We use immunohistochemical methods to confirm the targeted transduction, and behavioral testing to confirm locomotor activation in the mDS-DREADD

and mDS-mCherry rats upon chemogenetic dopamine activation. In the first cohort of rats, we tested the behavioral effects of chemogenetic activation on self-grooming and sensory gating. In a second cohort of rats, we performed molecular imaging with [^{18}F]FDG-PET to map the brain-wide metabolic effects of chemogenetic activation, and MRS to map effects on neurochemical levels, specifically glutamate, glutamine and NAA and NAAG.

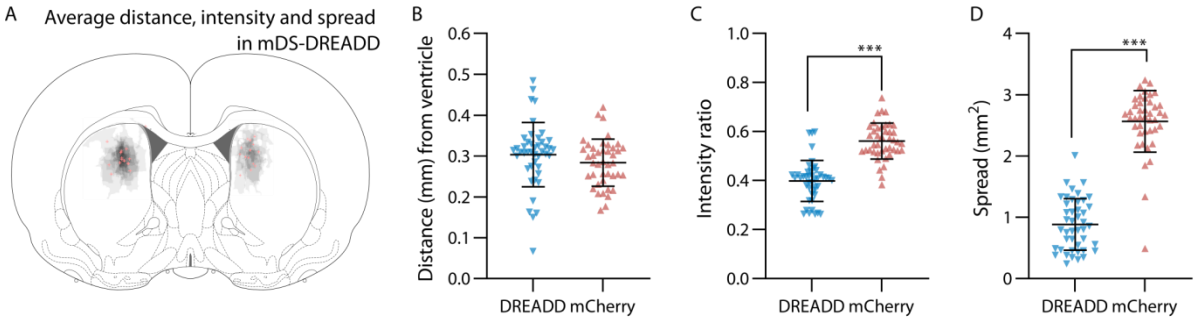

Figure S2: Quantification of transduction in rats, A) Visual representation of the average distance, intensity and spread of mDS-DREADD viral transductions. B,C,D) Quantified average distance, intensity and spread of mDS-DREADD viral transductions in relation to the smaller vector used in -mCherry.

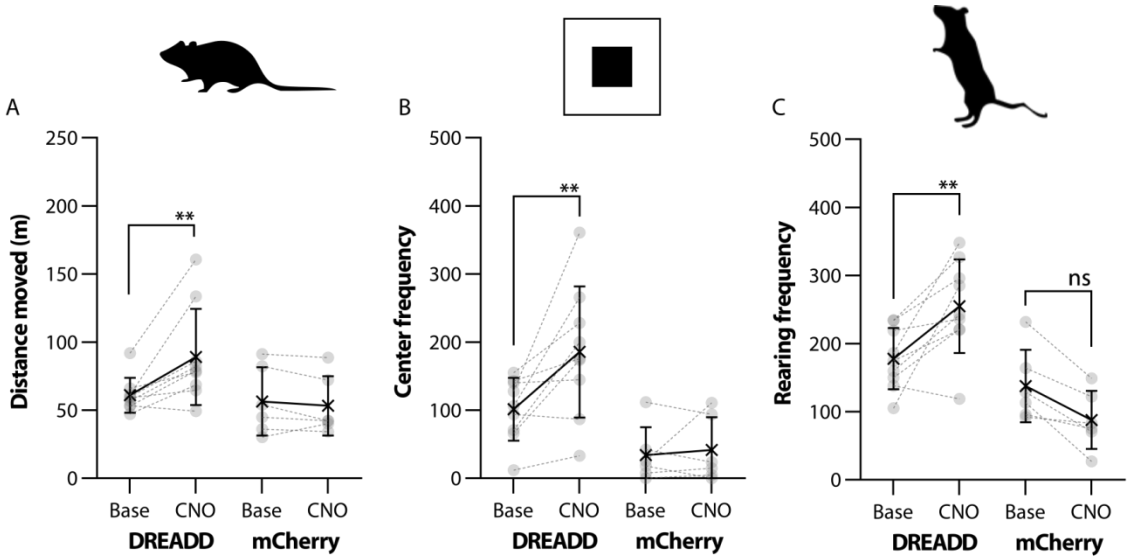

Figure S3: Second batch of locomotor experiments in animals for imaging experiments. A: 46% increase, from 610 cm to 890 cm, P = 0.0031. B: 82% increase, from 102 cm to 186 cm, P = 0.0053. C: 43% increase, from 178 cm to 255 cm, P = 0.0017.

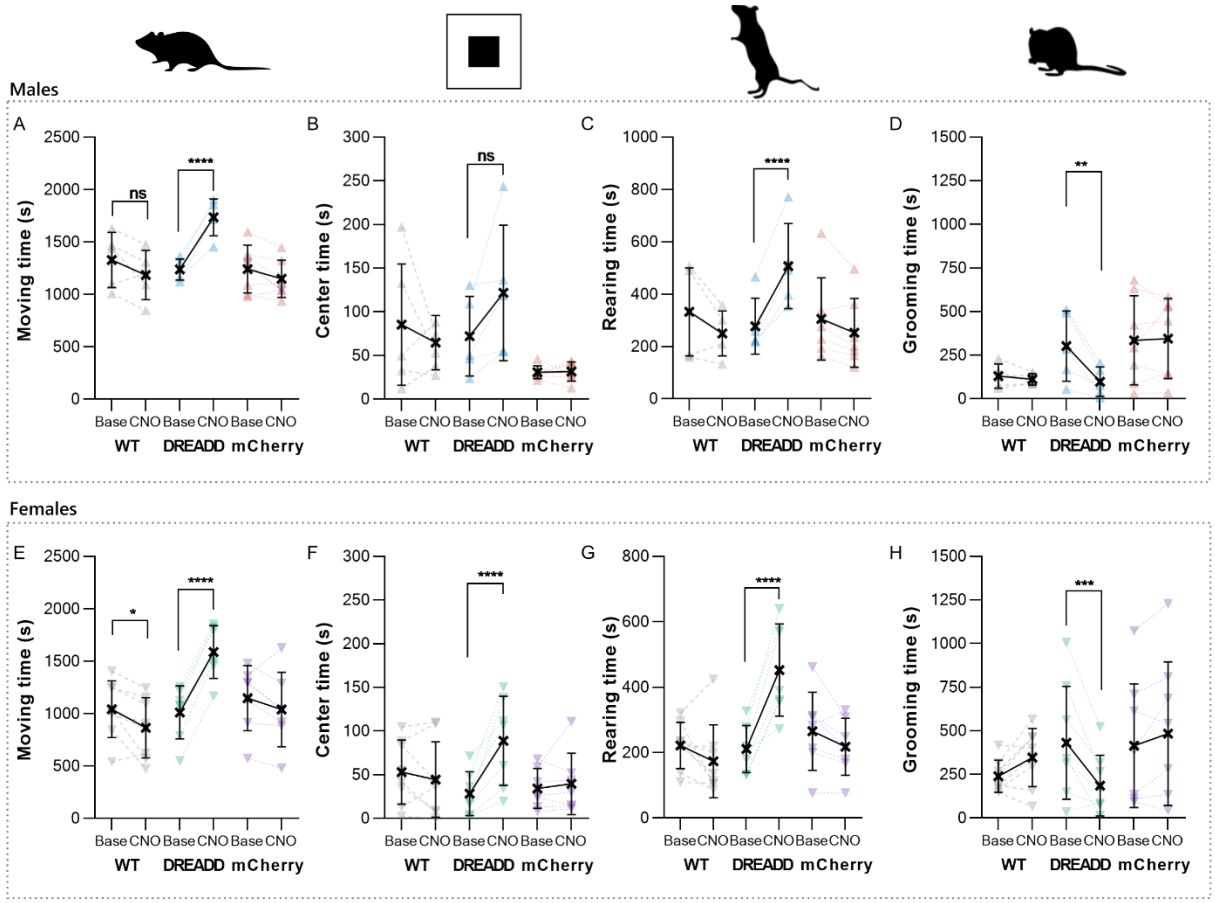

Figure S4: Locomotor data per time from manuscript figure 2 shown for each sex in individual graphs.

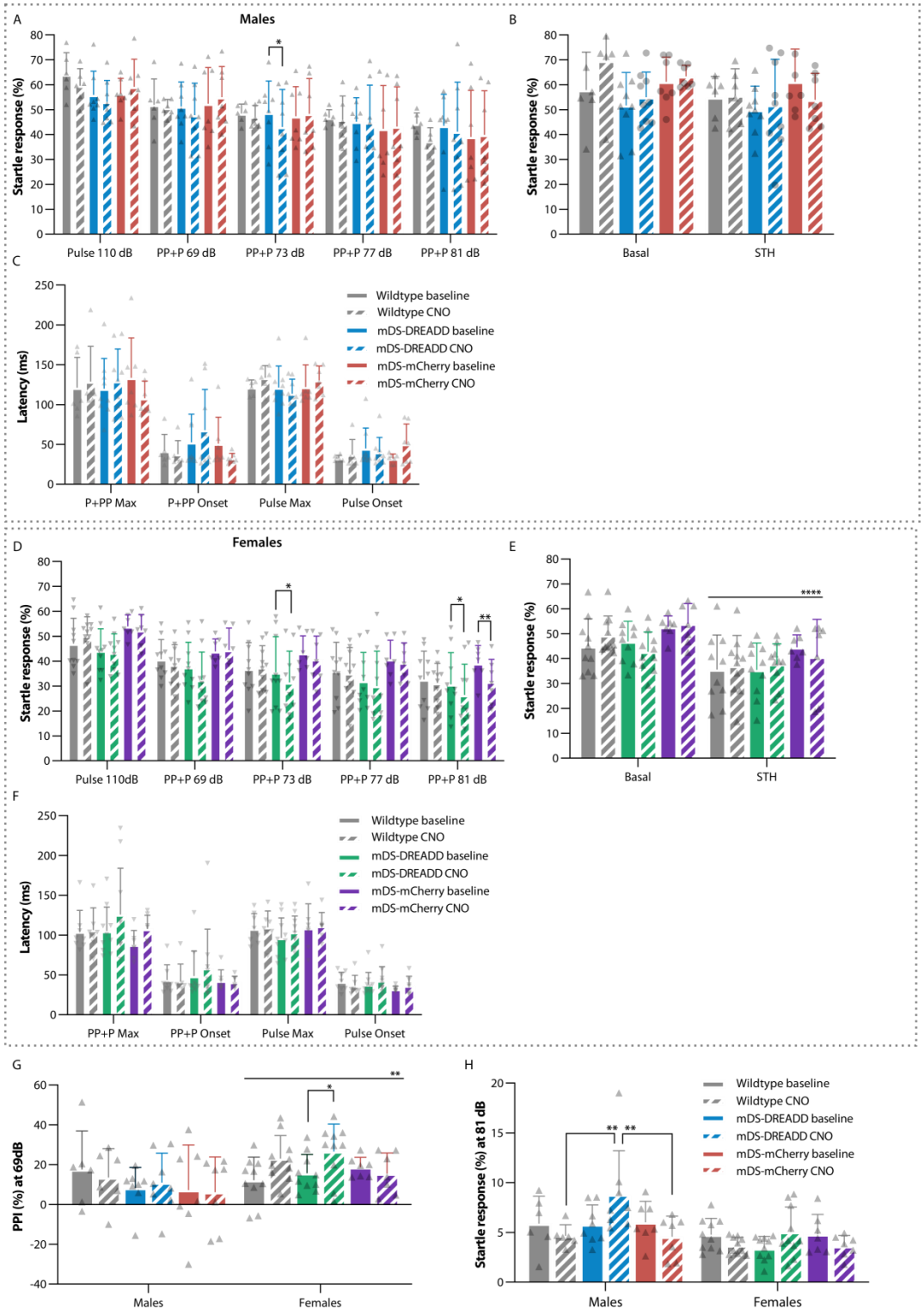

Figure S5: Additional acoustic startle data in males (top) and females (middle). A,D) Startle response following pulse and pulse + prepulse (PP+P) at each dB. B,E) Short term habituation within a startle sessions. C,F) Latency from pulse to startle. G) Sex comparison of PPI (%) at 69dB. H) Startle responses (or excessive movement) when no stimuli was present.

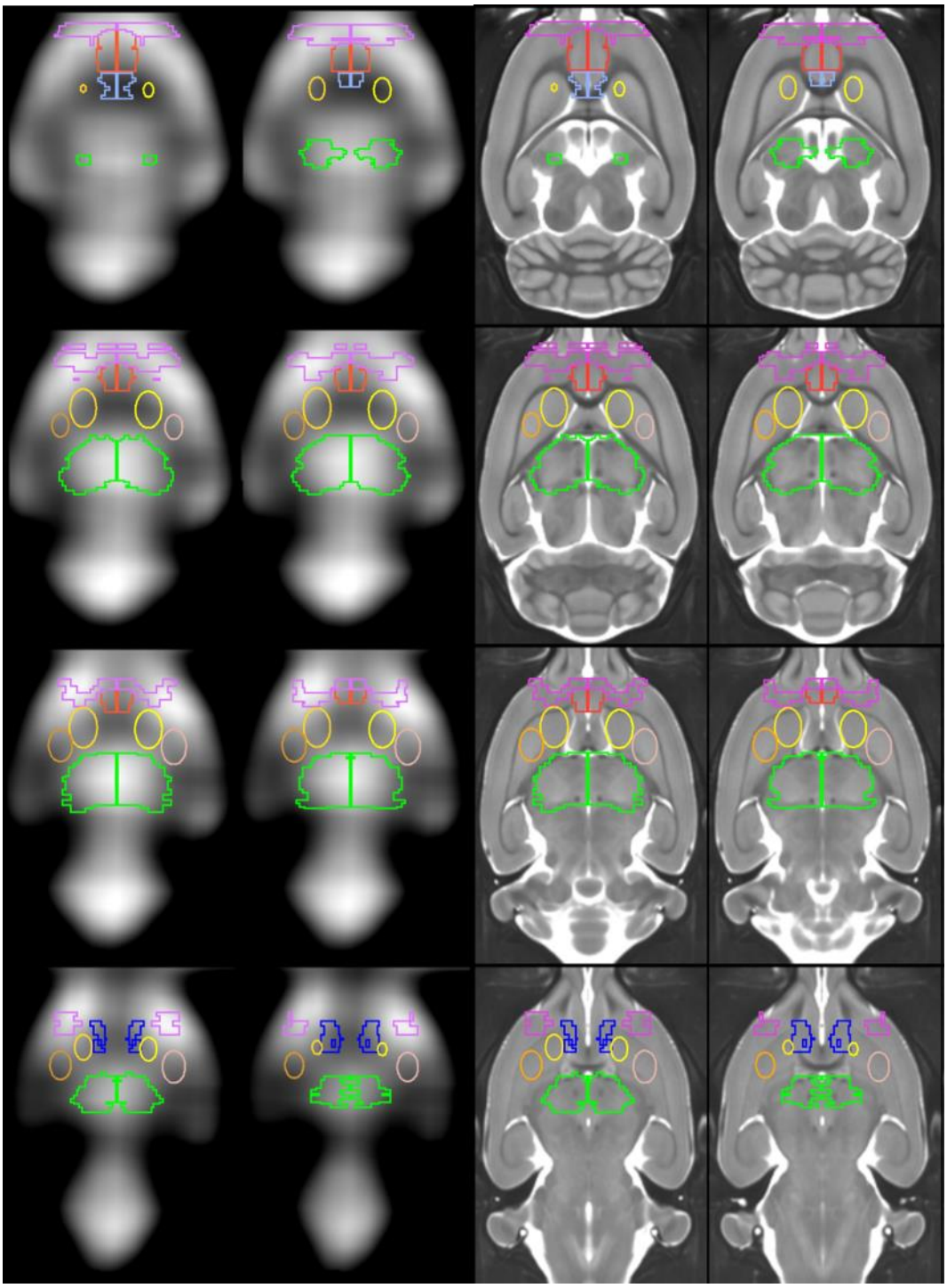

Figure S6: A representative [^{18}F]FDG-PET rat brain image (left side) and a reference MR (right side) with all VOIs, nucleus accumbens (NAc, dark blue), anterior cingulate cortex (ACC, light blue), medial prefrontal cortex (mPFC, red), orbitofrontal cortex (OFC, purpul), thalamus (Thal, green), medial dorsal striatum (mDS, yellow), ventral dorsal striatum (lDS, brown).