Supplemental Material

The Drosophila brain on cocaine at single cell resolution

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Supplemental Methods

Drosophila stock

Canton S (B) flies (Norga et al. 2003) were maintained on standard cornmeal/yeast/molasses-agar culture medium at 25°C on a 12:12 hour light:dark cycle with 50% humidity in controlled adult density vials to prevent overcrowding. Briefly, 5 males and 5 females were placed into a vial and allowed to mate for two days before being cleared. Progeny from these vials were collected after eclosion and aged for 3-5 days before experimentation.

Cocaine exposure

Cocaine.HCl was obtained from the National Institute on Drug Abuse under Drug Enforcement Administration license RA0443159. To expose flies to cocaine, we performed a modified version of the capillary feeder (CAFÉ) assay (Ja et al. 2007). We collected 200 Canton S (B) flies between 3 and 5 days old using CO₂ anesthesia, sexes separately. We placed them individually in culture vials containing cornmeal/yeast/molasses/agar culture medium (Genesee Scientific, Inc., San Diego, CA) and allowed them to recover for 24 hours before experimentation. Between 3:00-5:00 PM on the day before the assay, we transferred the flies to vials containing 1.5% agar (Sigma Aldrich, St. Louis, MO) in which a capillary (VWR International, Radnor, PA: 12.7 cm long, 5 μl total volume) filled completely with a solution of 4% sucrose (Sigma Aldrich) and 1% yeast (Fisher Scientific, Hampton, NH) was inserted. The next morning, we replaced the sucrose capillaries for 100 flies with capillaries containing 4% sucrose supplemented with 1 μg/μL of cocaine and 1% yeast; for the other 100 flies, we replaced the sucrose capillaries with fresh 4% sucrose and 1% yeast with no drug. A droplet of mineral oil (Sigma Aldrich) was added to the top of each capillary to minimize evaporation. We collected the first 40 flies that consumed 0.53 μL of cocaine and the first 40 flies that consumed 0.53 μL of sucrose, corresponding to an 8 mm reduction in the height
of the solution in the capillary. All experiments were carried out between 8 AM and 11 AM. Flies were allowed to feed for no more than 2 hours.

**Behaviors**

We measured negative geotaxis and startle response of individual flies within a 10-minute time-frame immediately following acute exposure to cocaine in the CAFÉ assay. We quantified grooming and seizures in addition to measuring the behavioral response in each assay. Excessive grooming was defined as more than 10 seconds of constant grooming (Video S2). Seizure activity was defined as severe muscle tremors that prevented the fly from moving normally (Video S3).

**Negative geotaxis:** Following acute cocaine consumption, we placed each fly in a 14.8 cm-tall clear glass vial with its circumference marked 7.5 cm up the vial. Flies were given 30 seconds to acclimate to the vials. We then tapped the flies to the bottom of the vial and recorded the time taken for each fly to cross the 7.5 cm mark, with a maximum allowed climb time of 30 seconds. Flies that did not pass the mark within 30 seconds were designated as “did not finish”. The numbers of flies tested are indicated in the legend to Figure 1. Significant differences from control were assessed using one-tailed Student’s t-test. Grooming and seizure activity were also scored at this time and differences between control flies and flies exposed to cocaine were assessed using Fisher’s exact test.

**Startle response:** Following acute cocaine consumption, we tested single flies in their vials for acute startle response. To ensure the same amount of mechanical stimulation for all trials, we constructed a ‘fly drop tower’ in which all vials were dropped 42 cm and then secured in a horizontal position. As soon as the vials attained a horizontal position the flies were observed for 45 seconds and the total time each fly spent moving was recorded (Video S1). The numbers of flies tested are indicated in the legend to Figure 1. Significant differences from control were
assessed using one-tailed Student’s t-test. Grooming and seizure activity were also scored at this
time and differences between control flies and flies exposed to cocaine were assessed using
Fisher’s exact test. While grooming, flies were stationary.

**Brain dissection and dissociation**

Brains were dissected from each fly immediately after it consumed the designated amount of
sucrose or cocaine solution. We used a dissociation protocol modified from Croset et al. (2018)
and Davie et al. (2018). We dissected brains in cold D-PBS (Gibco, Thermo Fisher Scientific,
Waltham, MA) and collected them into 1.7 ml tubes in cold Schneider’s medium (Gibco). We
collected 20 brains per sample within one hour. We collected eight samples of 20 brains from
males and females exposed to cocaine or sucrose, with two biological replicates per treatment
and sex. We replaced the D-PBS in the dissection dish after dissecting 2 brains to ensure that it
stayed cold and we used separate drops of buffer for decapitation and brain dissection to avoid
contaminating the brain samples. We centrifuged the samplesn at 300xg at 4°C for 5 min and
removed the supernatant. We then added 450µl of collagenase solution (50 ul of fresh 25mg/ml
collagenase (Gibco) in sterile water + 400µl of Schneider’s medium), flicked the tube gently and
allowed the brains to incubate at room temperature for 30 min. We replaced the collagenase
solution after centrifugation with PBS + 0.04% BSA (NEB, Ipswich, MA). We mechanically
dissociated the brains slowly and gently, using stepwise trituration - P200 pipette 5 times, 23G
needle pre-wetted with PBS + BSA 5 times, and 27G pre-wetted needle 5 times. We passed the
suspension through a pre-wetted 10µm strainer (Celltrics, Görlitz, Germany) aided by gentle
tapping. We added 50ul of PBS+BSA to aid flow of the suspension through the strainer. We
counted live cells using a hemocytometer with trypan blue exclusion. We proceeded with GEM
generation using the Chromium controller (10X Genomics, Pleasanton, CA) if we had a live cell
count of > 500 live cells/µl.
Library preparation and sequencing

We made libraries after GEM generation in accordance with 10X Genomics v3.1 protocols. We determined fragment sizes using Agilent Tapestation kits (Agilent, Santa Clara, CA) - d5000 for amplified cDNA and d1000 for libraries. However, the samples were not diluted at either step since these were not high concentration libraries. We measured the concentrations of amplified cDNA and the final libraries using a Qubit 1X dsDNA HS kit, also without dilution. In addition to Qubit, we quantified the final library concentrations using a qPCR based library quantification kit (KAPA Biosystems, Roche, Basel, Switzerland) in order to measure the concentration of fragment sizes of interest in accordance with the manufacturer’s recommendations. We used 12 cycles for the cDNA amplification and 14 cycles for indexing PCR. We sequenced the final libraries on an S1 flow cell using a Novaseq (Illumina, Inc., San Diego, CA) according to the manufacturer’s instructions.

FASTQ generation, demultiplexing and alignment

The mkfastq pipeline within Cell Ranger v3.1 (10X Genomics, Pleasanton, CA) was used to convert BCL files from the sequence run folder to demultiplexed FASTQ files. Release 6 version of the Drosophila melanogaster reference GCA_000001215.4 from NCBI Genbank was indexed using the mkref pipeline and used for alignment using the count pipeline within Cell Ranger v3.1 with the expected cell count parameter set to 5,000 cells. The sequencing and alignment summary is given in Supplemental Table S2.

Preprocessing, integration and cell-type clustering

Raw expression counts output for each sample from the Cell Ranger pipeline was imported and analyzed using the Seurat v3 package in R (Butler et al. 2018). Genes expressed in less than 5 cells and cells with less than 300 or greater than 2500 RNA features were filtered out. Normalization and subsequent integration were performed using scTransform pipeline
To identify the cell-type clusters within the dataset, unsupervised clustering using the `FindClusters` function and a resolution of 0.8 was used. Cluster marker genes were identified using `FindAllMarker` function (min.pct=0.25, logfc.threshold = 0.5, only.pos = TRUE). The top three genes with positive expression for each cluster were extracted and used for cell-type characterization.

**Differential expression**

Differential expression was performed for each cluster in two ways: (i) after combining male and female samples together to test for effects of cocaine that are common to both sexes; and (ii) testing for effects of cocaine in males and females separately to identify sexually-dimorphic responses. The Pearson residuals output from `scTransform` pipeline was used as input for differential expression (DE) calculation (Hafemeister and Satija, 2019). The `MAST` algorithm was used as the testing methodology in the `FindMarkers` function (test.use = "MAST", assay = "SCT", slot = "scale.data") for each cluster to calculate DE. Clusters with sufficient number of DEGs were subjected to pathway enrichment analysis using the statistical overrepresentation test using the PantherDB (Thomas et al. 2003) and Reactome databases (Fabregat et al. 2016). Pathways with BH-FDR adjusted $P$ values < 0.05 were considered statistically enriched.

**Simulation of bulk RNAseq response**

The results from DE calculation from the combined dataset were used to determine which genes were consistently upregulated and downregulated, respectively, across all clusters as a result of exposure to cocaine. The top 50 ranked differentially upregulated genes for each cluster and the top 20 ranked differentially downregulated genes for each cluster were input into `TopKLists` R package (Schimek et al. 2015).
**Cluster-specific co-expression networks**

The scaled data from the *scTransform* pipeline for differentially expressed genes from clusters 16 and 22 were extracted for the male samples. These scaled data were used as input for filtering through Random Matrix Theory (RMT; Gibson et al. 2013). The correlations that passed the filtering process were visualized using Cytoscape version 3.7.2. The MCODE algorithm (Bader et al. 2003) was utilized to identify highly interconnected modules within the larger cluster network. Genetic interaction networks were constructed by converting the gene IDs to gene names/symbols using the FlyBase Consortium’s ‘Query-by-symbols/ID’ tool and calculating interactions between gene products using the stringApp plugin within Cytoscape (Doncheva et al. 2019). To identify specific pathways that are enriched in genes within each of the circular groups, we performed statistical overrepresentation tests on the gene IDs from each group using the PantherDB (Thomas et al. 2003) and Reactome (Fabregat et al. 2018) databases. Pathways with BH-FDR adjusted *P* values < 0.05 were considered statistically enriched.
Supplemental Tables

Table S1: Raw behavioral data of flies exposed to cocaine. Refer to Supplemental_Table_S1.xlsx
| Sequencing Sample ID | Number of Reads | Estimated Number of Cells | Mean Reads per Cell | Median Genes per Cell | Sequencing Saturation | Fraction Reads in Cells | Total Genes Detected | Median UMI Counts per Cell |
|----------------------|----------------|--------------------------|---------------------|-----------------------|-----------------------|-----------------------|------------------------|--------------------------|
| ♀ Sucrose Rep 1      | 87,765,954     | 9,072                    | 9,674               | 772                   | 41.60%                | 71.50%                | 11,958                 | 1,347                    |
| ♀ Sucrose Rep 2      | 72,264,746     | 11,693                   | 6,180               | 807                   | 29.70%                | 80.90%                | 12,049                 | 1,476                    |
| ♂ Sucrose Rep 1      | 123,911,975    | 13,193                   | 9,392               | 769                   | 29.20%                | 79.50%                | 12,523                 | 1,378                    |
| ♀ Sucrose Rep 1      | 114,946,639    | 11,033                   | 10,418              | 799                   | 31.10%                | 72.00%                | 12,354                 | 1,440                    |
| ♀ Cocaine Rep 1      | 120,400,905    | 13,072                   | 9,211               | 733                   | 22.40%                | 69.70%                | 12,203                 | 1,304                    |
| ♀ Cocaine Rep 2      | 142,640,818    | 9,367                    | 15,228              | 1,080                 | 52.80%                | 83.30%                | 12,618                 | 2,286                    |
| ♂ Cocaine Rep 1      | 78,416,962     | 10,437                   | 7,513               | 680                   | 22.00%                | 68.40%                | 11,631                 | 1,190                    |
| ♂ Cocaine Rep 2      | 162,182,108    | 11,124                   | 14,579              | 1,207                 | 21.70%                | 58.70%                | 12,464                 | 2,868                    |
| Undetermined         | 49,596,498     |                          |                     |                       |                       |                       |                        |                          |
| **Total**            | 967,684,238    | 88,991                   |                     |                       |                       |                       |                        |                          |
| **Minimum**          | 72,264,746     | 9,072                    | 6,180               | 680                   | 21.70%                | 58.70%                | 11,631                 | 1,190                    |
| **Maximum**          | 162,182,108    | 13,193                   | 15,228              | 1,207                 | 52.80%                | 83.30%                | 12,618                 | 2,868                    |
| **Ratio**            | 2.24           | 1.45                     | 2.46                | 1.78                  | 2.43                  | 1.42                  | 1.08                   | 2.41                     |
| **Mean**             | 112,816,263    | 11,124                   | 10,274              | 856                   | 31.31%                | 73.00%                | 12,225                 | 1,661                    |
| **Standard deviation**| 31,547,982     | 1520.21                   | 3156.57             | 185.07               | 10.91%                | 8.02%                 | 332.22                 | 592.64                    |

Table S2: Summary of sequencing and alignment statistics.
Table S3: Differentially expressed genes after cocaine exposure of males and females
and their top human orthologs. Refer to Supplemental_Table_S3.xlsx
| Cluster | Cluster defining genes | Cluster annotation | References |
|---------|------------------------|--------------------|------------|
| 0       | VGlut/CG2269/CG32017   | Glutamatergic neurons | Croset et al., 2018 |
| 1       | CG14989/Gad1/CG32017  | GABAergic neurons   | Croset et al., 2018 |
| 2       | spab/jeb/CG31221      | neuropeptides/cholinergic neurons | Croset et al., 2018 |
| 3       | VGlut/CG34355/CG9650  | Glutamatergic neurons | Croset et al., 2018 |
| 4       | SoxN/CG9650/klg/scro  | optic lobe         | Davie et al., 2018 |
| 5       | pros/dati/spab        | Central brain neurons type A | Davie et al., 2018 |
| 6       | toy/bi                | undetermined       | Davie et al., 2018 |
| 7       | pros/br/beat-llc/dati | Central brain neurons type A | Davie et al., 2018 |
| 8       | sosie/acj6/nAChRalpha6/ct | olfactory projection neurons | Croset et al., 2018 |
| 9       | CG14989/Gad1/Lim3     | GABAergic neurons   | Davie et al., 2018 |
| 10      | mbl/CG31345/Imp/ChaT  | Central brain type B cholinergic neurons | Davie et al., 2018 |
| 11      | jdp/Pka-R2/Rgk1/rut  | Kenyon cells       | Murakami et al., 2017 |
| 12      | CG14989/ct/Gad1       | GABAergic neurons   | Croset et al., 2018 |
| 13      | Obp44a/CG8369/CG15201/MtnA | Gila | DeSalvo et al., 2014 |
| 14      | CG18598/pdm3/vvl/scro/jus | optic lobe neurons | Davie et al., 2018 |
| 15      | Pka-C3/CG2016/sosie   | undetermined       | Croset et al., 2018 |
| 16      | CNMaR/SoxN/Sox102F/acj6 | optic lobe and antennal lobe neurons | Croset et al., 2018 |
| 17      | Eaat1/CG2016/CG15522 | Astrocytes         | MacNamee et al., 2016 |
| 18      | DIP-theta/CG42458/aop/scro | optic lobe neurons | Davie et al., 2018 |
| 19      | CG8369/CG1552/Gs2    | Gila               | Huang et al., 2015 |
| 20      | Pka-R2/crb/Rgk1      | Kenyon cells       | Murakami et al., 2017 |
| 21      | VGlut/mbl/Proc/Imp   | Central Brain type 2 glutamatergic neurons | Croset et al., 2018 |
| 22      | CG3168/Cys/SPARC     | Surface glia and fat body | DeSalvo et al., 2014 |
| 23      | Nos/Octbeta1R/Gad1/vMat | GABAergic and monoaminergic neurons | Croset et al., 2018 |
| Cluster | Cluster defining genes | Cluster annotation | References |
|---------|------------------------|--------------------|------------|
| 24      | Vmat/Hsp23/CG457784//   | Central brain type 2 | Croset et al., 2018 |
|         | Imp/Nep1               | monoaminergic neurons | Turrel et al., 2016 |
|         |                        |                    | Davie et al., 2018 |
| 25      | bi/shakB/Octbeta1R     | undetermined       | Ye et al., 2004 |
| 26      | CG31345/CG18598/shakB  | undetermined       | Turrel et al., 2016 |
| 27      | CG14687/Dh31/Nep1/jdp  | mushroom body and ellipsoid body | Croset et al., 2018 |
|         |                        |                    | Nixon et al., 2019 |
| 28      | CG43795/CG14274/Frq1   | undetermined       | Croset et al., 2018 |
| 29      | DAT/Vmat/ple           | Dopaminergic neurons | Croset et al., 2018 |
| 30      | Arr2/Arr1/ninaE        | Photoreceptor cells | FlyBase, Thurmond et al., 2019 |
| 31      | CG2016/CG6044/CG42540  | undetermined       | Croset et al., 2018 |
| 32      | IncRNA:CR45223/kn/sNPF | undetermined       | Croset et al., 2018 |
| 33      | CG8034/VGlut/Ca-alpha1T| Glutamatergic neurons | Ignell et al., 2009 |
| 34      | Tk/Nplp1/nAChRalpha6  | Tachykinin/neuropeptidergic neurons | Asahina et al., 2014 |
| 35      | IncRNA:CR45223/sNPF/Gad1| Central brain GABAergic neurons | Croset et al., 2018 |

Table S4: Characterization of cell clusters through combinations of biomarkers. Genes indicated in bold font were used to annotate the clusters.
Table S5: Genes that were consistently upregulated and downregulated across multiple clusters due to acute cocaine exposure. Refer to Supplemental_Table_S5.xlsx

Table S6: Results from differential expression analysis for each cluster from the male and female datasets combined. Refer to Supplemental_Table_S6.xlsx

Table S7: Results from differential expression analysis for each cluster from the male dataset. Refer to Supplemental_Table_S7.xlsx

Table S8: Results from differential expression analysis for each cluster from the female dataset. Refer to Supplemental_Table_S8.xlsx

Table S9: Quantification of shared and unique differentially expressed genes due to exposure to cocaine between the males and females within each cluster. Refer to Supplemental_Table_S9.xlsx

Table S10: Functional enrichment analyses of differentially expressed genes in selected clusters due to cocaine exposure. Refer to Supplemental_Table_S10.xlsx
Figure S1: Visualization of gene expression patterns using UMAP projections. Gene expression patterns of single cells (n = 86,224) from all 8 (2 ♀ cocaine, 2 ♀ sucrose, 2 ♂ cocaine, 2 ♂ sucrose) samples were visualized in low dimensional space using a graph-based, non-linear dimensionality reduction method (UMAP). Individual dots represent each cell and the colors of the dots represent the samples to which the cells belong.
Figure S2: UMAP visualization of expression patterns for genes that respond globally to cocaine exposure. Visualization of gene expression patterns was performed using UMAP projections. Each dot represents a cell within the integrated dataset from all 8 samples. The color gradients of the dots represent the normalized and scaled expression value of the genes that respond globally due to acute cocaine exposure in each sample group (separated based on sex and condition). Identities of specific clusters and groups of cell types are indicated in the Rpl41 panel A: GL - Glia, OL - Optic Lobe, AC - astrocytes, GLMN - Glutamatergic neurons, MB - Mushroom Body, GBN - GABAergic neurons.
Figure S3: Full co-expression network analysis of DEGs from the male C16 cluster. Network constructed from Pearson Coefficient based co-expression values calculated from scaled data of genes that were differentially expressed (filtered for $|\log_{10} FC| > 0.5$, Bonferroni adjusted P value < 0.05) due to cocaine exposure in C16 of the male dataset. Co-expressions have been filtered using Random Matrix Theory. The colors of the dots represent the connectivity index derived from MCODE scores. The colors of edges represent the positive (red) and negative (green) correlations.
Figure S4: MCODE Subnetworks from co-expression network analysis of DEGs from the male C22 cluster. Network constructed from Pearson Coefficient based co-expression values calculated from scaled data of genes that were differentially expressed (filtered for $|\log_{10} FC| > 0.5$, Bonferroni adjusted P value < 0.05) due to cocaine exposure in C22 of the male dataset. Co-expression values have been filtered using Random Matrix Theory. (a-c) MCODE subnetworks derived from the full network. The colors of the dots represent the connectivity index derived from MCODE scores. The colors of edges represent the positive (red) and negative (green) correlations.
Supplemental Movies

Movie S1: Startle response assay. Refer to Supplemental_Movie_S1.mp4

Movie S2: Grooming behavior of a male exposed to cocaine. Refer to Supplemental_Movie_S2.mp4

Movie S3: Seizure of a male exposed to cocaine. Refer to Supplemental_Movie_S3.mp4
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