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Supplemental Information

Abnormal Behavior of Zebrafish Mutant in Dopamine Transporter Is Rescued by Clozapine

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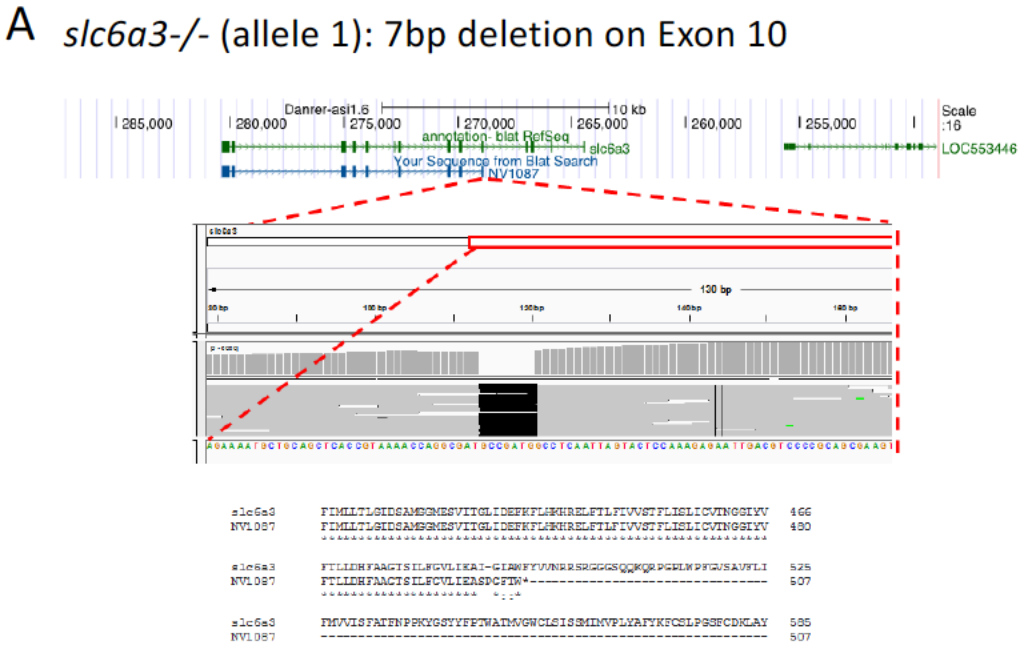
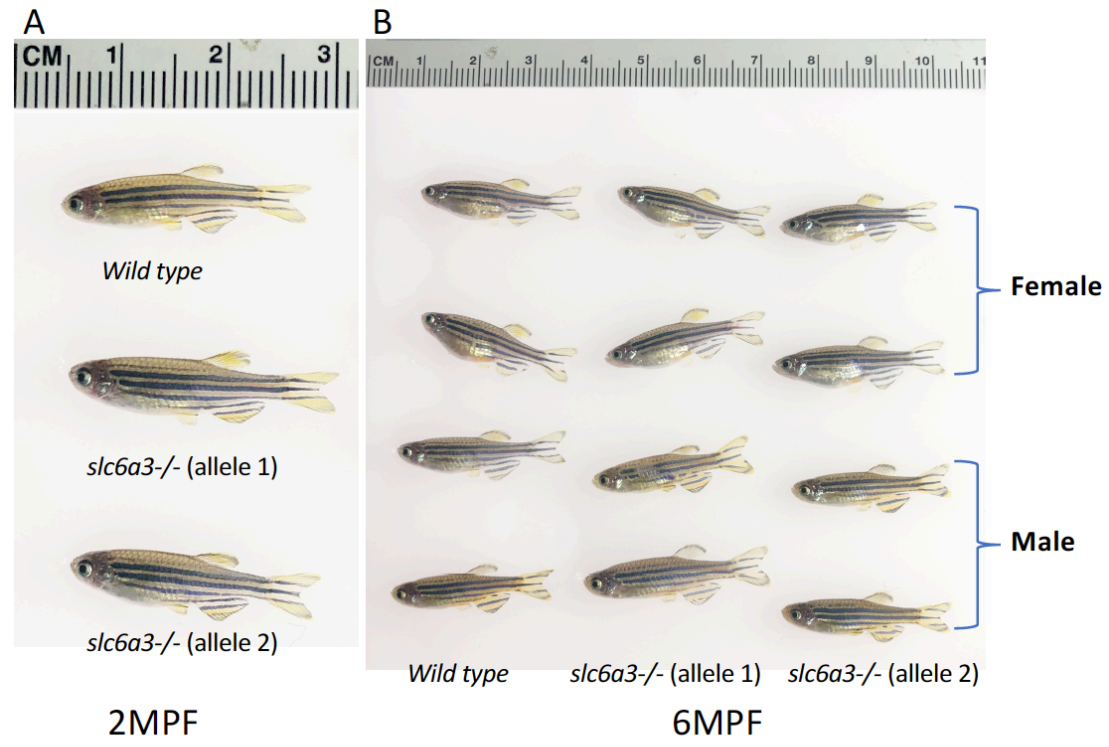
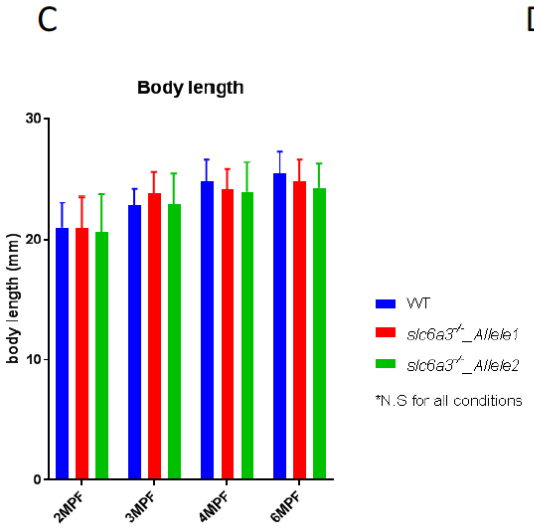
Supplemental Information

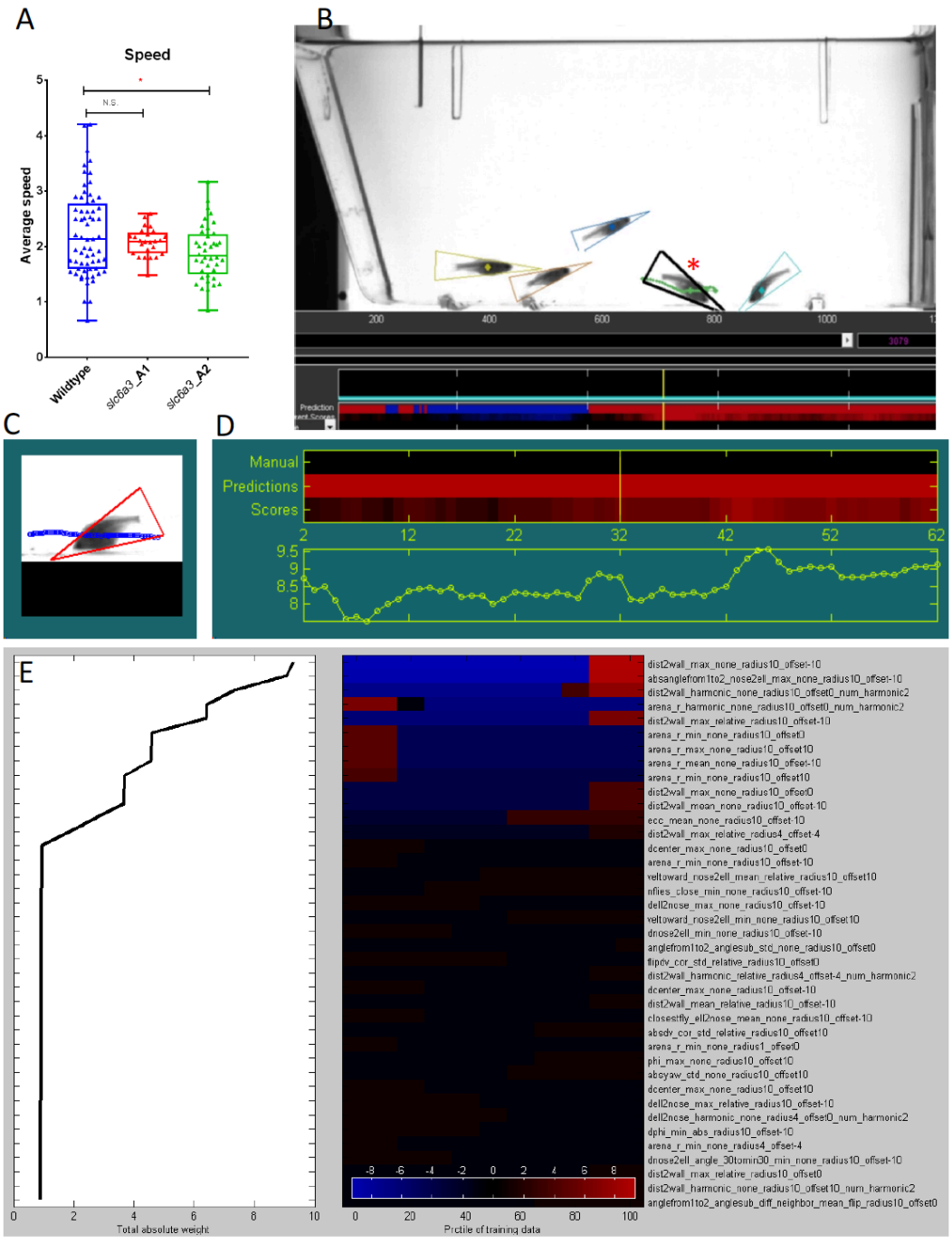


Supplementary Figure 1. Sequence confirmation of CRISPR deletions in the two *slc6a3-/-* alleles, related to Figure 1. (**A**) Mutation in *slc6a3-/-* allele1 is a 7bp deletion in Exon10. (**B**) Mutation of *slc6a3-/-* allele2 is a 4 bp deletion in Exon 3.



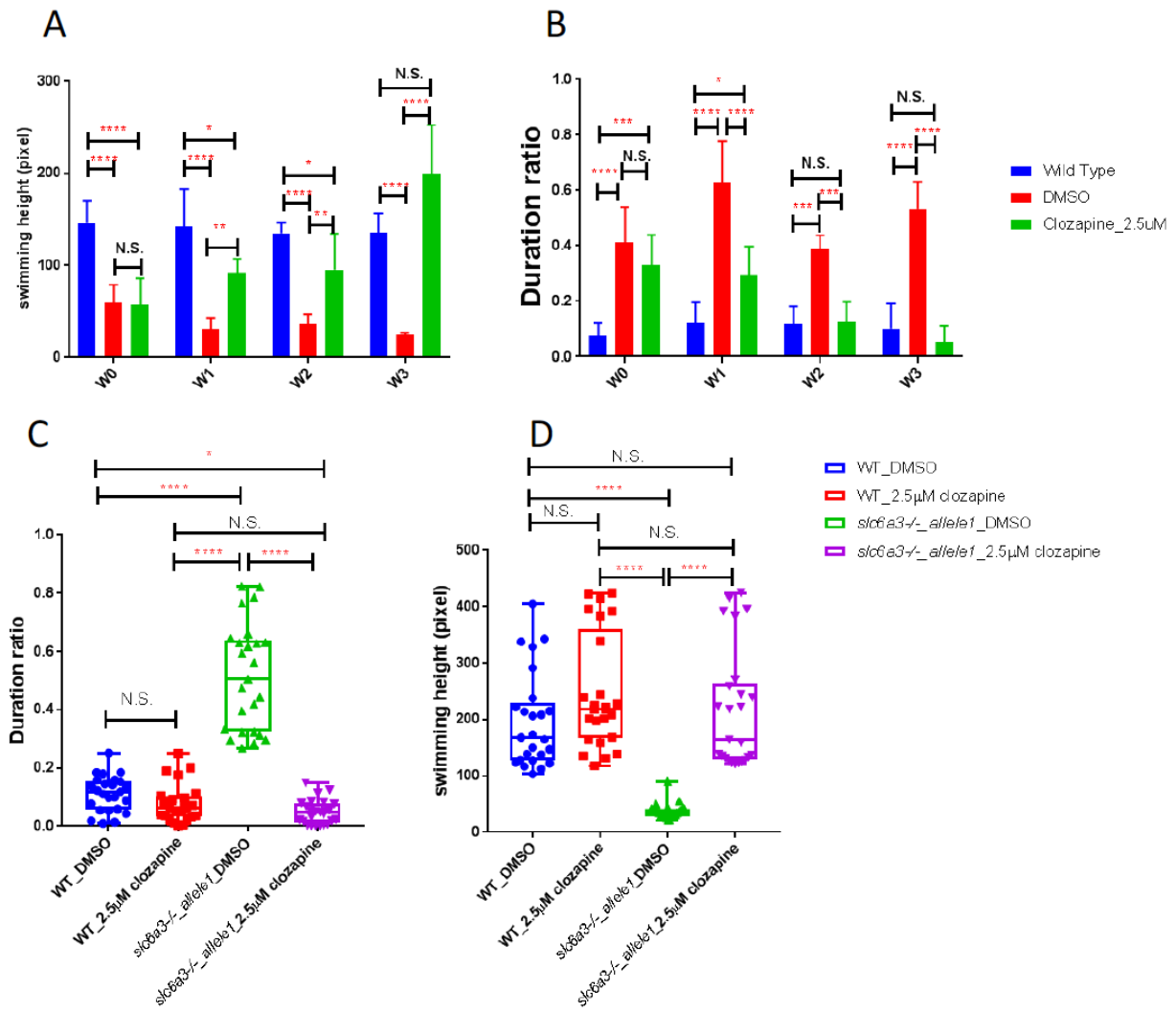


Supplementary Figure 2. *slc6a3* knockout does not affect overall morphology and development, related to Figure 1. (**A**) Zebrafish images of WT, *slc6a3-/- allele1*, and *slc6a3-/- allele2* at 2 months post-fertilization (2mpf). It should be noted that fish do not show gender-related morphology yet at this stage. (**B**) Zebrafish images of wild-type (WT), *slc6a3-/- allele1*, and *slc6a3-/- allele2* at 6 months post-fertilization (6mpf). (**C**) Fish body length (head to tail) measurement of WT, *slc6a3-/-* allele1, and *slc6a3-/- allele2* at 2mpf, 3mpf, 4mpf, and 6mpf. For all n>=16, significance test: one-way ANOVA Kruskal-Wallis test.

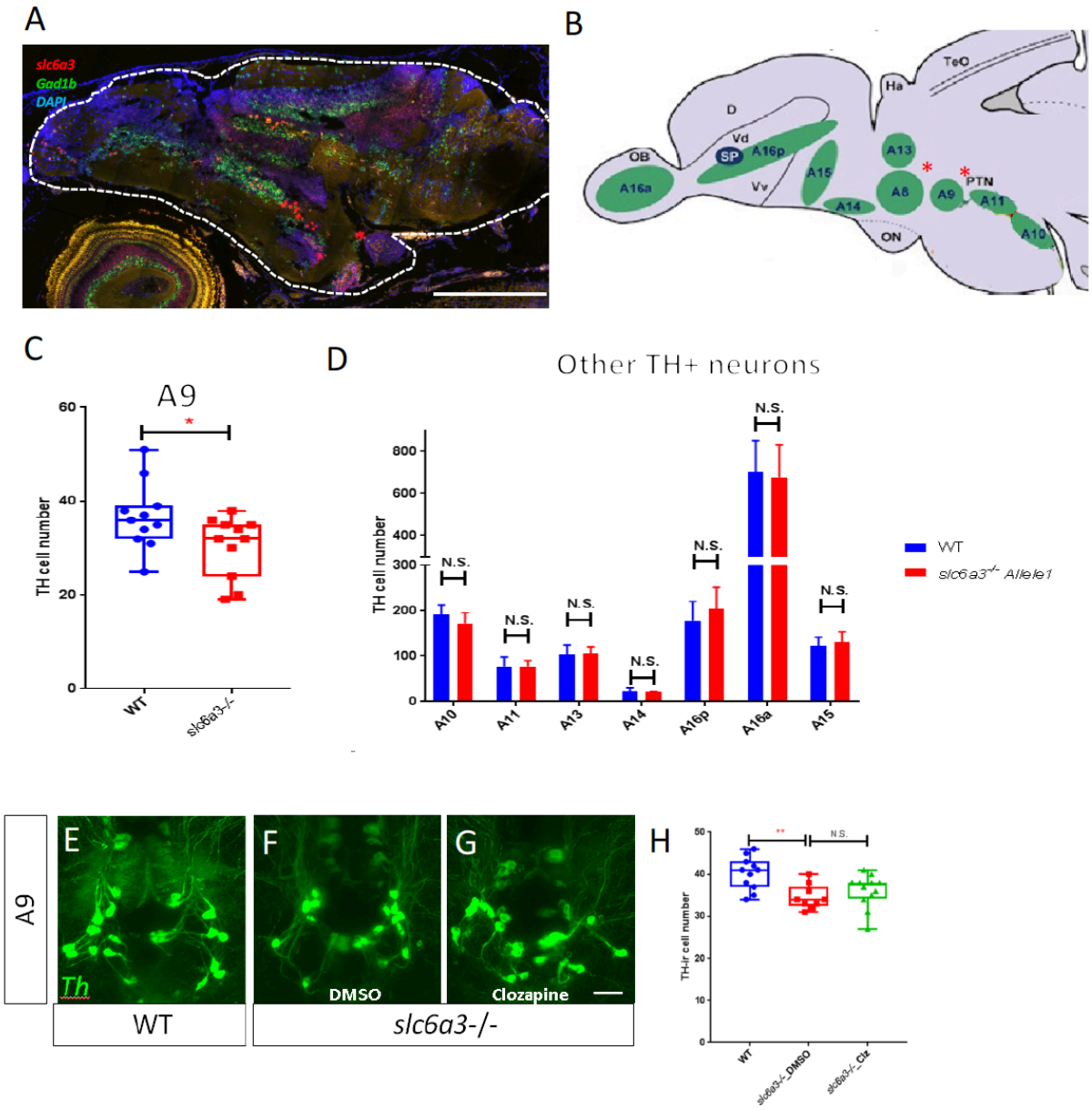


Supplementary Figure 3. Training the "digging" classifier in JABBA, related to Figure 1. (**A**) the average swimming speed of WT, *slc6a3-/- allele1*, and *slc6a3-/- allele2* (n_{WT}=64, $n_{slc6a3\text{-}/\text{-}\_A1}$=25, $n_{slc6a3\text{-}/\text{-}\_A2}$=40, *$p<0.05$, N.S.= no significance. Significance test: one-way ANOVA Kruskal-Wallis test). (**B**) The interphase of training "digging" classifier in JABBA. The fish posture is highlighted by a triangle (asterisk) with a centroid in the center of the fish. The triangle indicates the angles of fish toward to the bottom. (**C**) The view of individual fish during the

training. The blue line indicates the fish trajectory ±30 seconds of the timepoint. (**D**) The view of the "digging" label in a 60 second timeframe. Manual=manually labelled "digging" behavior (left blank here as the timeframe is randomly selected and not manually labelled). Prediction=predicted probability after training. Score=the probability score based on on all weighted features. A score of 0.75 was used for as the threshold for filtering the "digging" motif in video. (**E**) The rank of total absolute weights of all features for training the "digging" classifier.



Supplementary Figure 4. Statistical analysis for 2.5uM clozapine treatment. (**A-B**), **related to Figure 3.** The bar-graphs of swimming height (**A**) and the "digging" behavior (**B)** from week zero (W0) to week 3 (W3) in WT, *slc6a3-/- allele1* treated by DMSO and 2.5uM clozapine in drug screen. n=5 for all. Error bar=standard deviation. *****p<0.0001; ***p<0.001; **p<0.01; *p<0.05,* N.S.= no significance. Significance test: one-way ANOVA Kruskal-Wallis test. (**C-D**) The box plots of swimming height (**C**) and the "digging" behavior (**D)** at week 3 in WT and *slc6a3-/- allele1* treated by DMSO and 2.5uM clozapine in a follow-up experiment. n=25 for all conditions. *****p<0.0001; ***p<0.001; *p<0.05,* N.S.= no significance. Significance test: one-way ANOVA Kruskal-Wallis test.



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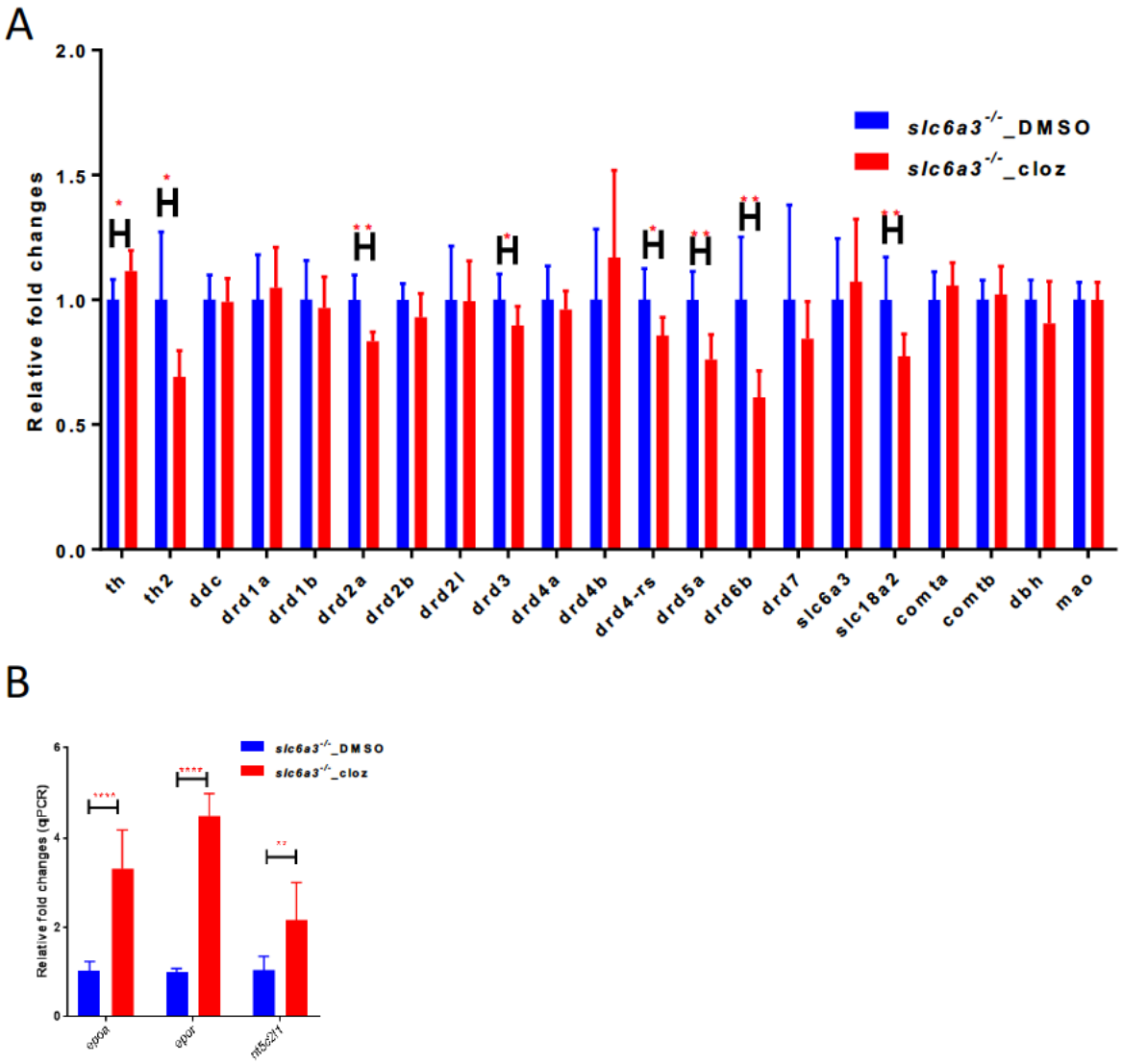
72 **Supplementary Figure 5. Whole brain CUBIC clearing to visualize TH+ neurons in the**
73 **adult brain, related to Figure 2 and Figure 3.** (**A**) RNAscope confirms expression of *slc6a3*
74 expression in the zebrafish brain. gad1b is a marker of brain glutamatergic neurons and used as
75 a reference of brain areas. Scale bar=250μm (**B**) Diagram of location of TH+ neuron groups
76 (dark green) in the zebrafish brain, in a sagittal view. D, dorsal telencephalic area; Ha,
77 habenula; OB, olfactory bulb; ON, optic nerve; PTN, posterior tuberculum; Vd, dorsal
78 telencephalic area; Vv, ventral telencephalic area; TeO, optic tectum; SP, Subpalium. **(Parker**
79 **et al., 2013) (C)** *slc6a3-/- allele1* fish manifest slight reduction of A9 DA neuron numbers at
80 2mpf. Box plot of cell numbers of A9 neurons are shown. Error bar=standard deviation. n=11 for

both WT and *slc6a3-/- allele1. *p<0.05.* Significance test: Wilcoxon–Mann–Whitney test. **(D)** TH+ neuronal number is unaffected in other regions of the *slc6a3-/- allele1* brain. Error bar=standard deviation. N>=8 for both WT and *slc6a3-/-* allele1 conditions. N.S.=no significance. Significance test: Wilcoxon–Mann–Whitney test. (**E-H**) 2.5uM clozapine treatment does not significantly rescue the A9 cell number in *slc6a3-/-* allele1. (**E-G**) Representative images of A9 neurons by z-projection of A9 neuron images. Scale bar=10μm. (**H**) Box plot of A9 DA cell number in wild-type, DMSO and 2.5uM clozapine treated *slc6a3-/- allele1* at 3mpf. For all n>=12. Error bar=standard deviation. **p<0.01.* N.S.=no significance. Significance test: one-way ANOVA Kruskal-Wallis test.



Supplementary Figure 6. Erythropoietin and 5'-nucleotidase pathway genes are upregulated by clozapine treatment in *slc6a3-/-* allele1, related to Figure 4. (**A**) The relative fold changes of all dopamine pathway genes by RNA-seq analysis, show minimal changes in gene expression levels. n=8 for all. Error bar=standard deviation. **p<0.05, *p<0.01.* N.S.=no significance. Significance test: Wilcoxon–Mann–Whitney test. (**B**) qRT-PCR confirms the up-regulations of *epoa, epor*, and *nt5c2l1* in slc6a3-/- allelel1 by 2.5uM clozapine treatment. n=6 for all. ***p<0.01. ****p<0.0001.* Significance test: Wilcoxon–Mann–Whitney test.

Supplementary Video 1. Wild-type behavior, related to Figure 1.

Supplementary Video 2. *slc6a3-/-* allele1 behavior, related to Figure 1.

Supplementary Video 3. *slc6a3-/-* allele2 behavior, related to Figure 1.

Supplementary Video 4. *slc6a3-/-* allele1 behavior with 3-week DMSO treatment, related to Figure 3.

Supplementary Video 5. *slc6a3-/-* allele1 behavior with 3-week 2.5uM clozapine treatment, related to Figure 3.

Supplementary Video 6. *slc6a3-/-* allele1*;Tg(aTub:iTTA; TetO:EpoA-2a-GFP)* behavior at 2 months post fertilization, related to Figure 4.

Supplementary Excel table. Normalized RNA-seq counts of all genes in *slc6a3-/-* allele1 treated by DMSO and 2.5uM clozapine, related to Figure 4.

Transparent Methods

Animal husbandry

Zebrafish (*Danio rerio*) were housed in 3L tanks in a recirculating Aquatic Habitats facility (Pentair, USA) at 28±0.5°C water temperature. Fish were maintained on a 14-hour light/10-hour dark cycle with light turning on at 07:00 AM. Fish were fed a diet of brine shrimp (Catalog #BSEPCA-Brine Shrimp Direct, USA) twice daily and supplemented with flake fish food (Tetramin, Catalog# 98525-Pentair, USA) daily. All animals were maintained and procedures were performed in accordance with the Institutional Animal Care and Use Committees (IACUC) of NIBR.

Generation of the CRISPR mutants

The *slc6a3* CRISPR sgRNAs were designed based on an in-house genome assembly of the AB strain (unpublished, sequences available upon request). Two sgRNAs targeting different loci were selected (Table 1). sgRNAs were synthesized using T7 in vitro transcription using the MEGAshortscript™ T7 Transcription Kit (ThermoFisher, AM1354). Then sgRNAs were purified with MEGAclear™-96 Transcription Clean-Up Kit (ThermoFisher, 325 AM1909). The purified sgRNAs (125 ng/µL) were co-injected with Cas9 protein (500 ng/µL PNA bio, CP01) into 1-cell stage fertilized zebrafish embryos. CRISPR injected embryos were raised to adulthood and crossed with wild-type AB fish to get F1 generation. Mutants were identified in the F1 and maintained in heterozygous.

Behavioral assay

For behavioral analysis, we used a side-mounted camera (acA2000-165u mNIR, Basler) on a standard 1.4 L fish tank (Pentair, USA). To make the background uniform for tracking, a 25cmX25cm IR illuminating board was placed behind the fish tank for illumination. An optical filter (LP780-72 filter, MidOPT) was placed on a lens (LM8XC 1.3" (4/3") 8.5mm, F2.8, KOWA) to permit only infrared light being recorded. Five fish were netted into 1L fish water in the fish tank. After 5min acclimation, a 30min fish swimming video was recorded. The video dimension was 1224X500 (pixels). The recording frequency was 60hz. The video was tracked and annotated using Janelia Automatic Animal Behavior Annotator (JAABA) following the tutorial (http://jaaba.sourceforge.net) (Kabra et al., 2013). In total, eight 30min videos (240 minutes) were used for the "digging" classifier training (4 wild-type, 2 slc6a3-/- allele1, and 2 slc6a3-/- allele2). The parameters for training are: Iterations:100; Iterations for fast update: 10; Base Classifier: Decision Stumps; Sample points:2500; Bin:30; Cross Validation Fold: 7. All available features (appearance, social, locomotion, arena, position, identity, compatibility) were applied in the training. The weights of individual sub-features were ranked based on manual label (**Supplementary Figure 3B-E**). For the "digging" classifier, an accuracy of 94.1% was achieved in the positive frames (when fish show the "digging" behavior) and 98.2% in the negative frames (when fish do not show the "digging" behavior).

Whole brain immunofluorescence and TH+ cell counting

Whole brain immunofluorescence was adapted from the CUBIC clearing method (Susaki et al., 2015). Briefly, adult zebrafish were euthanized in ice-cold water and decapitated. The heads were fixed in 4% PFA for overnight at 4 °C. Then the brains were dissected and incubated in the Clearing reagent (250mg/mL urea; 250mg/mL N,N,N',N'-tetrakis (2-hydroxypropyl) ethylenediamine; 150mg/mL Triton X-100) at 37 °C with shaking for 4 days. The cleared brains were then incubated in blocking solution (1XPBS, 0.2% Triton X-100, 10% DMSO, 10% goat serum) at 37 °C for 1 day. The primary antibody (Anti-Tyrosine Hydroxylase (TH) , EMD Millipore, AB152) was added and incubated at 37 °C for 4 days. Following 1-day wash by wash solution (1XPBS, 0.1% Tween-20, 1% DMSO), the secondary antibodies (Alexa Fluor® 488 goat anti rabbit, ThermoFisher, A27034) were added an incubated at 37°C for 4 days. After 1 day wash, the samples were transferred into refractive index matching solution (RIMS) (30mL of 0.02 M phosphate buffer (Sigma P5244), 40g Histodenz (Sigma D2158), 0.1% Tween-20, 0.01% sodium azide, use NaOH adjust pH to 7.5) and imaged using a ZEISS lightsheet microscope with a 5X detection objective (Lightsheet Z.1, Zeiss). Images were processed using "lightsheet dualfusion" with default parameters in ZEN (Zeiss). Processed images were then

quantified using Arivis Vision4D software (Arivis Inc.) and FIJI (NIH). For counting of TH+ cells, the TH+ channel and the planes of interest were selected in ARIVIS Vision4D analysis panel. A filter of basic morphology (type: opening; radius:5) and a segmentation of blob finder (diameter 14um, threshold 17.32, high split sensitivity) were applied. After assessing the segmentation result we manually corrected the false positive segmentation. We used FIJI to generate all manuscript images.

RNA-seq and data process

Adult Zebrafish were euthanized by submersion in ice-cold water for 5min. The brains were dissected in RNAlater solution (ThermoFisher, AM7020). Total RNA from single zebrafish brains was prepared using the RNeasy Plus 96 Kit (Qiagen, 74192). mRNA libraries were generated using the TruSeq Stranded mRNA Library Prep Kit (Illumina, 20020595), and sequenced on Illumina HiSeq 2000 in paired-end mode. Four female and four male adult fish were sequenced for each condition at a mean depth of 34 million reads per sample. RNA sequencing reads were trimmed using Trimmomatic (version 0.32) to remove sequencing adaptors and low quality reads, mapped to internal zebrafish genome using STAR 2.5.3a with default parameters, and QCed using CollectRnaSeqMetrics from the picard-tools 1.113 package. An rRNA genome annotation generated using RepeatMasker prediction was downloaded from the UCSC genome browser and used for CollectRnaSeqMetrics; Uniquely mapped fragments were counted against a customized gtf file generated based on ENSEMBL zebrafish gene annotation (release 91). The featureCounts function (featureCounts -p -O -s 2) in the subread (version 1.5.0) package was used for counting. Differential brain gene expression analysis between DMSO and clozapine treatment was performed using DESeq2. The log2 fold changes were tested against 0 using the Wald test, and the p-values were adjusted using Benjamini-Hochberg multi-comparison correction.

Total RNA extraction and qRT-PCR

Adult Zebrafish were euthanized by submersion in ice-cold water for 5min. The brains were dissected in RNAlater solution (ThermoFisher, AM7020). Total RNA of zebrafish brain was prepared using RNeasy mini kit (Qiagen). The concentration of total RNA was measured and an equal amount of total RNA for all conditions was reverse transcribed using iScript™ Reverse Transcription Supermix (Bio-Rad, 1708840). The same amount of templates was used for qPCR with iTaq™ Universal SYBR® Green Supermix (Bio-Rad, 1725120) in QuantoStudio 7 Real-Time PCR (ThermoFisher Scientific). The sequences of all qPCR primers are listed in Table 2.

Cloning and overexpression of *epoa* in zebrafish brain

The *epoa* overexpression construct was cloned using a 3-way Gateway strategy based on the previously published Tol2kit cloning method (Kwan et al., 2007). A sequence of *epoa-2a-EGPF* flanked with a recombination sequence for the LR reaction were synthesized by Genewiz. A Gateway reaction was performed using LR Clonase™ II Plus enzyme (ThermoFisher, 12538120) to assemble three pieces of DNA fragments (5'-entry clone (TetO promoter), middle-entry clone (*epoa-2a-EGPF* cDNA), 3'- entry clone (PolyA tail)) into the destination vector (pDestTol2) at room temperature for overnight. The confirmed construct containing all DNA fragments (*TetO: epoa-2a-EGPF-PolyA*) was co-injected with a neuronal alpha-tubulin promoter driven itTA (*aTub:itTA*) and the Tol2 transposase mRNA in the *slc6a3*$^{-/-\_allele1}$ embryos at one-cell stage. The *epoa* expression level was verified by qRT-PCR of injected embryos at 5dpf. A stable transgenic line *slc6a3*$^{-/-\_allele1}$*; Tg(aTub:iTTA; TetO:EpoA-2a-GFP)* was screened by visualizing GFP fluorescence in the brain of progeny of injected fish.

Dopamine concentration measurement in whole zebrafish brains

Adult Zebrafish were euthanized using ice-cold water for 5min. The brains were dissected on an ice-cold stage and weighed using a Micro-balance (Mettler Toledo XPE56). The samples were randomized and transferred to Precellys tissue homogenizing CKmix tubes. Three hundred microliters of (70/30) acetonitrile/water containing 0.5% ascorbic acid and the dopamine internal standard 10 µM (Cambridge Isotopes d4) was added to each of the samples. The samples were homogenized at 2500 RPM for 3X30 seconds and then spun at 10,000 rpm for 10 minutes at 4°C. Fifteen microliters of the sample was transferred to a 384 well plate containing 30 µL of 100 mM borate buffer pH 9.0 and 15 µL of anhydrous acetonitrile containing 2% (v/v) benzoyl chloride (Sigma Aldrich) was added. The plate was sealed and shaken for 15 minutes. Calibration curves for dopamine starting at 125 µM were diluted 1:2 over 16 points. Calibration curves were treated the same as the samples. The samples and calibration curves were then transferred to an Agilent 1290 auto-sampler set a 4°C and 5 µL was injected onto a 2X50 mm ACE Excel 2 C18-Amide column connected to a Sciex 5500 operating in MRM mode. Solvent A was HPLC grade water containing 20 mM ammonium formate and 0.15% formic acid (v/v). Solvent B was HPLC grade acetonitrile. The flow rate was 600 µL per minute and the column was keep at a constant temperature of 60°C. The gradient was 100% A to 45% B over 5.5 minutes then to 51% B 4.5 minutes then a stepped to 95% B in 0.15 minutes and held at 95% B for 1 minutes. The column was then re-equilibrated at 0% percent A for 5 minutes. The mass

spectrometry parameters were Gas 1 30, Gas 2 20, CUR 30, Temp 450°C, collision gas 6, IonSpray Voltage 5500. The MRM for dopamine is 466.2-105.1 and for dopamine IS 470.2-105.1 DP 75, CE 40 and CXP 10. Dopamine eluted at 7.9 minutes. The data was processed using MutiQuant™. Dopamine concentrations were divided by the brain weights for normalization and then compared using JMP™ software by performing a t-test.

Supplementary Reference

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