An Unbiased Drug Screen for Seizure Suppressors in Duplication 15q Syndrome Reveals 5-HT_{1A} and Dopamine Pathway Activation as Potential Therapies

Supplement 1

Supplemental Methods and Materials

Fly Stocks

Flies were maintained at 25°C on a 12-hour light/dark cycle and raised on standard corn meal media (Bloomington Stock Center). Several stocks were obtained from the Bloomington Drosophila Stock Center (BDSC) (Bloomington, IN). The UAS-*ATPα* line was obtained from FlyORF (1). The *Dube3a* and human *UBE3A* UAS lines used in this study have been described previously (2). A complete list of stocks used can be found in **Table S1**.

Chemical Compound Preparation

The Prestwick Library of 1280 approved drugs was purchased from Prestwick Chemicals (Illkirch, France). This library has high chemical and pharmacological diversity with known bioavailability and safety in humans. All compounds were dissolved in DMSO (Sigma-Aldrich; St. Louis, MO) and mixed with standard corn meal medium to make drug food (0.1% final DMSO concentration; Drug Concentration: 1μM) for the primary screening. For the secondary screening and the other pharmacological interventions, drugs were dissolved in water, ethanol or DMSO (based on their highest solubility) and mixed with standard corn meal medium to make drug food (DMSO concentration used: 0.02% in all drug food except for Ceforanide = 0.05%). Drugs were serially diluted to concentrations: 0.04μM, 0.2μM, 1.0μM and 5.0μM depending on the experiment.

Primary Seizure Susceptibility Screen

For all behavior tests, flies were not exposed to CO_2 for at least two days prior to testing to avoid issues related to seizure resistance after exposure to CO_2. The bang sensitivity assay (BSA) was performed as previously described (3-5). Both drug and non-drug (DMSO alone) fed flies were transferred to empty standard fly vials and subjected to mechanical stress (“bang”) by vortexing the vial at full speed on a standard laboratory vortexer (LabNet) for 10 seconds. After vortexing, but within a 30 second critical window, the flies were separated (seizing from non-seizing) using the apparatus described in **Figure 1B**. This apparatus was constructed by placing a funnel over a falcon tube cut at both ends and placed on top of a Petri dish without a lid. The funnel was subsequently removed and replaced with an empty vial. A timer was started and after 30 seconds, the Petri dish containing the immobile seizing flies was covered with the lid and the non-seizing flies recovered in the empty vial. Flies of both the genotypes: *repo>Dube3a* and

UAS-*Dube3a;TM3,Sb* were counted in the seizure group (Petri dish) and the non-seizure group (empty vial over the falcon tube). Since seizure was induced only in the *repo>Dube3a* flies, we used these counts in the final analysis of drug induced seizure recovery. All primary screen flies were 3-4 days old.

Secondary Validation Seizure Assays

Based on our previous study, we used 4 day old *repo>Dube3a* and 9 day old *repo>UBE3A* flies (6) in subsequent experiments. Flies were sorted and separated at least two days prior to testing to avoid complications from CO_2 anaesthesia. Flies of each genotype, *repo>Dube3a* or *repo>UBE3A* (both with and without drug) were sorted by sex post eclosion and transferred to freshly prepared drug containing food or solvent-only food.

For seizure behavior studies, flies were anesthetized briefly by exposing the vials to ice for 2-3 minutes. Three to five flies were distributed to empty vials for the bang sensitivity assay. After a 1-hour recovery from cold-anesthesia, the flies were subjected to mechanical stress ("bang") by vortexing the vial using a standard laboratory vortexer (LabNet) at top speed for 10 seconds. The total recovery time was recorded for each individual fly in the vial using a digital timer. Total recovery time in our assay was defined as the time taken by a fly to fully recover (i.e. when the fly righted itself and was able to walk freely around on the base or on the walls of the vial). For each drug at each concentration, or combination of drugs, at least 40 flies of the correct genotype were tested in the bang sensitivity assay.

Glial Cell K^+ Content Assay

Stock solutions of 1 mM Asante Potassium Green 2 (APG-2, TEFlabs), a fluorescent cell membrane permeable K^+ indicator, were prepared in DMSO. Three-day old *repo>tdTomato* or *repo>Dube3a+tdTomato* flies fed on food with water or food with 0.04μM 5HT + L-dopa were briefly anesthetized with CO_2 and their heads were removed. Heads were submerged in room temperature Drosophila saline consisting of the following (in mM): 128 NaCl, 1.8 $CaCl_2$, 2 KCl, 5 $MgCl_2$, 36 sucrose, 5 HEPES, pH 7.2. Brains were dissected from the head case and incubated with 7.5μM APG-2 in Drosophila saline at

room temperature with gentle agitation for 1-hour. Brains were then washed 2X with Drosophila saline and wet mounted on a microscope slide with Drosophila saline. Images were captured from the optic lobe on a Leica DM6000B microscope (Leica) using a 63X oil immersion lens and filters for tdTomato and APG-2 respectively. Exposure times were calibrated on *repo>tdTomato* brains and microscope settings remained constant between *repo>tdTomato and repo>Dube3a+tdTomato* groups to allow for direct comparison of fluorescence intensity. APG-2 fluorescence intensity was calculated using Adobe Photoshop CC2018 and ImageJ (7). The APG-2 channel was converted to a grayscale image. Brightness and contrast parameters were kept constant in all images across various groups. Only tdTomato-positive glial cells were selected for analysis. Cells were selected using the "freeform" tool to outline the cell body in the APG-2 channel. Using the "measure" command, the mean fluorescence value for each cell was recorded as previous described (6). A minimum of 35 cells was analyzed per group from at least 3 images each from 3 different brains per group.

Statistical Analysis and Graphing

All graphs and statistical tests were performed using Prism version 7.0 (GraphPad). All histograms and measurements are shown as mean ± SEM. To test for statistical significance between two samples, Student's *t*-test with two tailed comparison was used. To determine statistical significance among multiple samples, we used a one-way ANOVA with Tukey's multiple comparison correction. Statistical significance is indicated on all graphs by convention: $p_{value} \leq 0.05$ (*), $p_{value} \leq 0.01$ (**), $p_{value} \leq 0.001$ (***) and $p_{value} \leq 0.0001$ (****). All analyses were performed with the experimenter blinded to genotype.

Supplemental References

1. Bischof J, Bjorklund M, Furger E, Schertel C, Taipale J, Basler K (2013): A versatile platform for creating a comprehensive UAS-ORFeome library in Drosophila. *Development*. 140:2434-2442.
2. Reiter LT, Seagroves TN, Bowers M, Bier E (2006): Expression of the Rho-GEF Pbl/ECT2 is regulated by the UBE3A E3 ubiquitin ligase. *Hum Mol Genet*. 15:2825-2835.
3. Benzer S (1971): From the gene to behavior. *JAMA*. 218:1015-1022.
4. Ganetzky B, Wu CF (1982): Drosophila mutants with opposing effects on nerve excitability: genetic and spatial interactions in repetitive firing. *J Neurophysiol*. 47:501-514.

5. Stone B, Burke B, Pathakamuri J, Coleman J, Kuebler D (2014): A low-cost method for analyzing seizure-like activity and movement in Drosophila. *J Vis Exp*.e51460.
6. Hope KA, LeDoux MS, Reiter LT (2017): Glial overexpression of Dube3a causes seizures and synaptic impairments in Drosophila concomitant with down regulation of the Na+/K+ pump ATPalpha. *Neurobiol Dis*. 108:238-248.
7. Schneider CA, Rasband WS, Eliceiri KW (2012): NIH Image to ImageJ: 25 years of image analysis. *Nat Methods*. 9:671-675.

Supplemental Tables and Figure Legends

Table S1. Drosophila stocks used from BDSC.
Table S2. All validated compound descriptions.
Table S3. Serotonin Receptor Agonists/Antagonists and Predicted Effects on Seizure.
Table S4. DIOPT Homology Scores Between Drosophila and Human Genes.

Figure S1. Classes of drug compounds in the Prestwick library. A) A breakdown of the classes of the 1280 compounds in the library and how many drugs from each class are represented. B) The classes of drug that could suppress seizures in the primary screen and the percentage of each compound class that those hits represent.

Figure S2. Variance spread for males vs females in different solvents. Compounds in this study were dissolved in DMSO for the primary screen, but some compounds were soluble in either water or ethanol so the recovery times were assayed in both males and females in all three solvents. Males of the *repo>Dube3a* genotype showed a higher variance in all three solvents as compared to females. Red bars are SEM and black bar is the mean recovery time in that solvent. Error bars (red) are SEM. N > 40 animals per data point.

Figure S3. Drugs that Passed Secondary Screening Validation. All 17 compounds that were identified in the primary screen were tested again using a modified single fly bang sensitivity test that measures recovery time after vortexing. Eight compounds were able to suppress seizures at concentrations lower than the primary screen (1μM) and as low as 0.04μM for some compounds. All compounds suppressed seizures by $\geq$50% of solvent alone (DMSO or Water) in males and female, at $p_{value} \leq 0.01$. Four of the 8 compounds are associated with serotonin and/or dopamine

signaling (serotonin HCl, mirtazapine, minaprine HCl and Levodopa). Error bars (red) are SEM. N>40 animals per data point. Red dotted line is 50% of the recovery time for *repo>Dube3a* alone.

Figure S4. Drugs that failed to meet secondary screening go/no-go criteria. Error bars (red) are SEM. N > 40 animals per data point.

Figure S5. Drug Combinations that failed to significantly increase suppression over serotonin alone in both males and females. Error bars (red) are SEM. N>100 animals per data point.

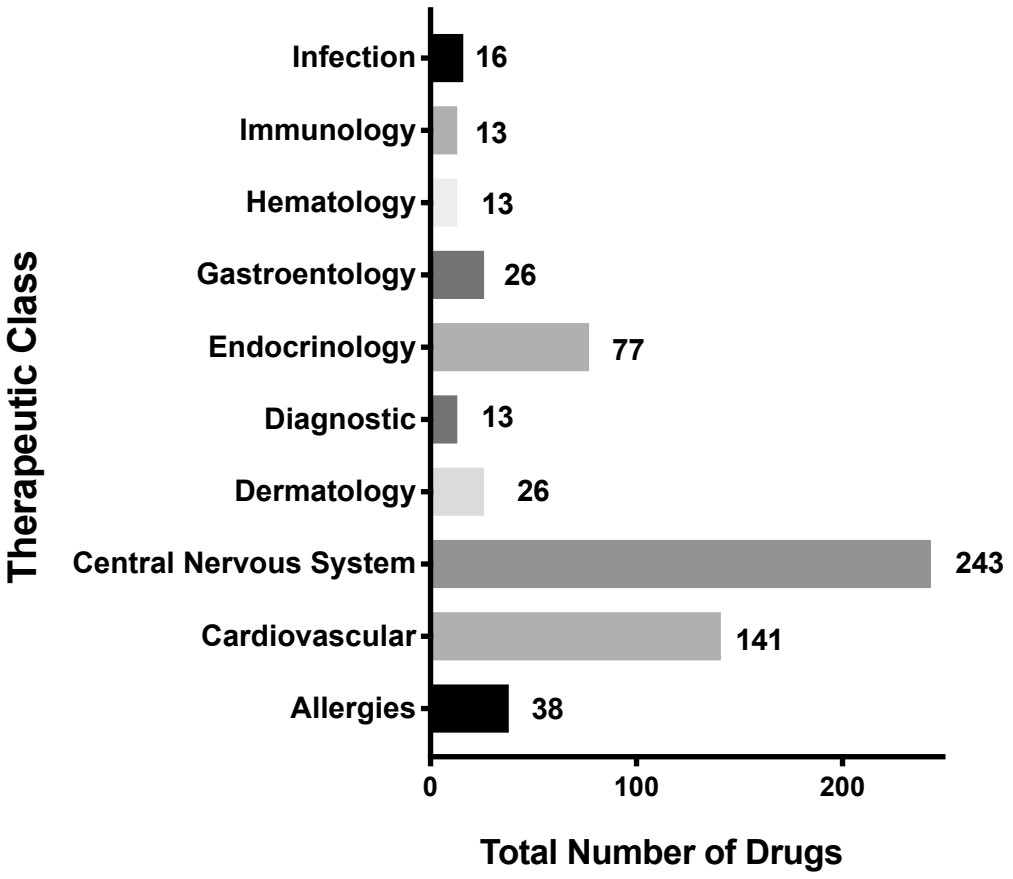
Figure S6. Male fly results with 5-HT_{1A} agonists and antagonists. Error bars (red) are SEM. N>60 animals per data point. Green dotted line represents control recovery time for *repo>Dube3a* alone.

Figure S7. Seizures in *repo>UBE3A* Animals also Respond to 5-HT_{1A} Agonists. As with flies expressing fly *Dube3a*, flies expressing human *UBE3A* in glial cells also show decreased seizure recovery time when treated with 5-HT_{1A} agonists (A), but increased seizure recovery times when treated with 5-HT_{1A} antagonists (B). Females are black columns and males are grey. Error bars (red) are SEM. N>70 animals per data point.

Figure S8. Mutations in 5-HT_{2A} Have no Effect on Seizure Suppression by Serotonin. A) While mutations in 5-HT_{1A} alone do not show a bang sensitive phenotype in *repo>Dube3a* animals, the addition of a 5-HT_{1A} loss of function mutation significantly enhances the seizure recovery time even in the presence of serotonin in the food. Loss of function mutations in 5-HT_{2A} showed no bang sensitive phenotype on their own and did not affect seizure recovery time in *repo>Dube3a* flies. Error bars are SEM. N>18 animals per data point. B) qRT-PCR gene expression data indicates that the transcription levels of *5-HT_{1A}* decrease and the transcription of *5-HT_{2A}* increases in the brains of *repo>Dube3a* animals relative to *repo*-GAL4 control brains.

Figure S9. Dup15q anti-seizure medications valproic acid and felbamate also suppress seizures in *repo>Dube3a* flies, but not as much as the 5HT_{1A} agonist vortioxetine. Error bars (red) are SEM. N>50 animals per data point. Red dotted line is 50% of the recovery time for *repo>Dube3a* alone.

A


Therapeutic Class
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Hematology
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Gastroentology
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Endocrinology
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Diagnostic
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Dermatology
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Central Nervous System
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Cardiovascular
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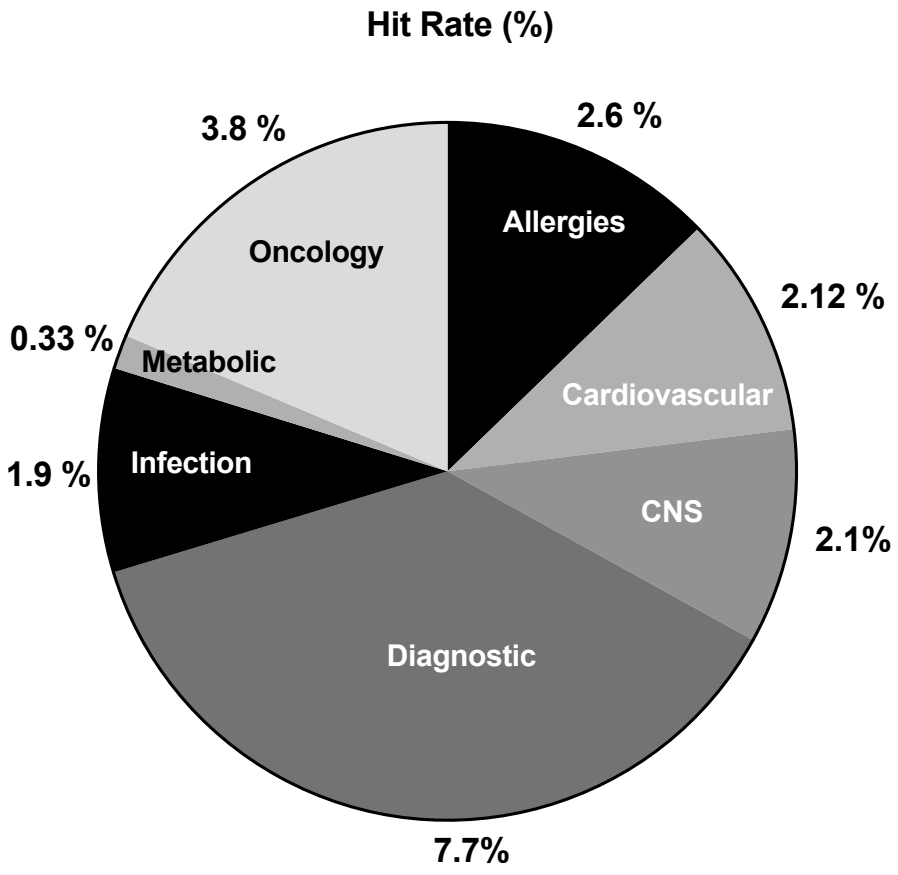
Allergies
38
0
100
200
Total Number of Drugs

B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2.6 %
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Allergies
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0.33 %
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Cardiovascular
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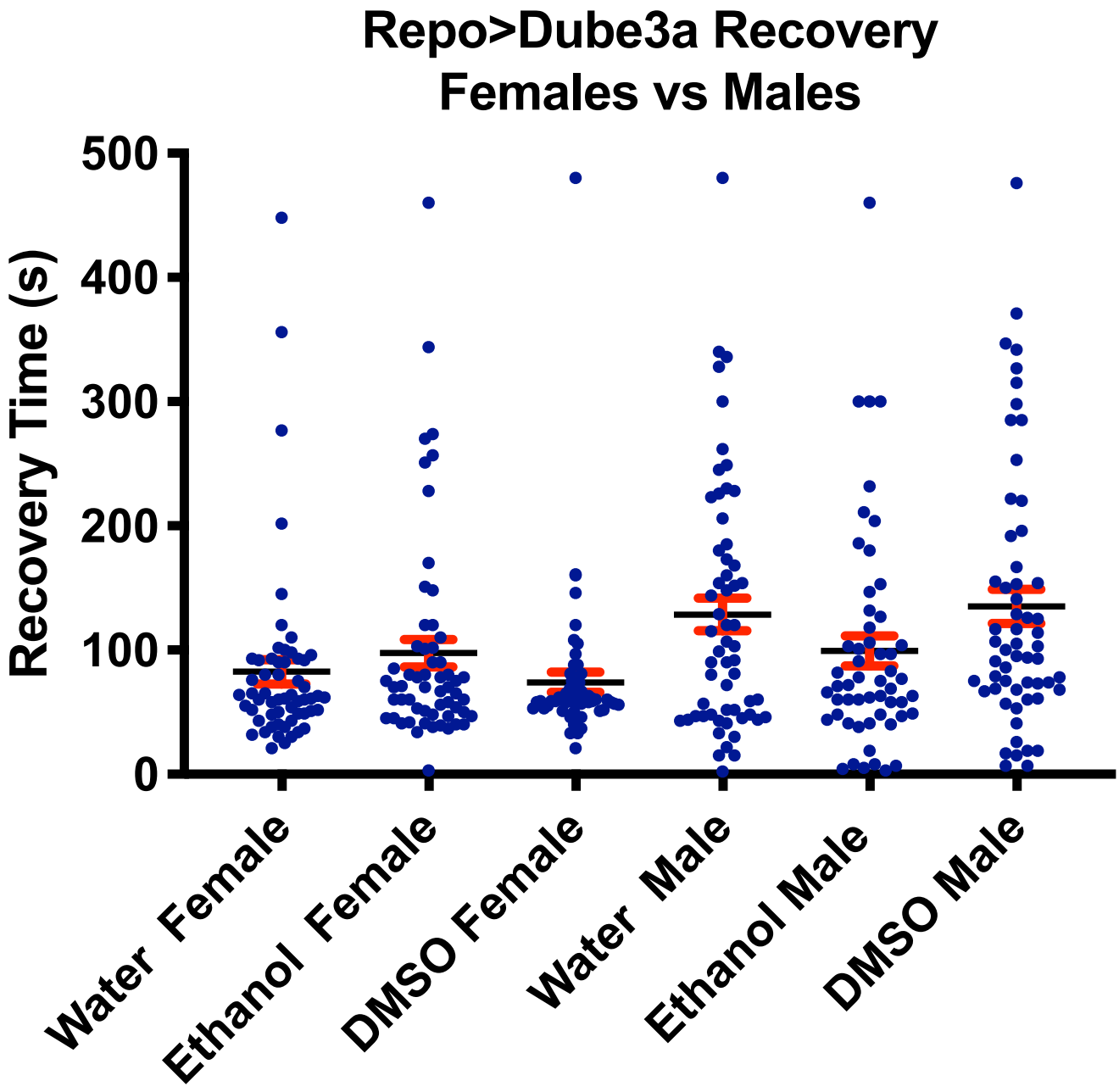
Infection
CNS
2.1%
Diagnostic
7.7%


Repo>Dube3a Recovery
Females vs Males
Recovery Time (s)
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400
300
200
100
0
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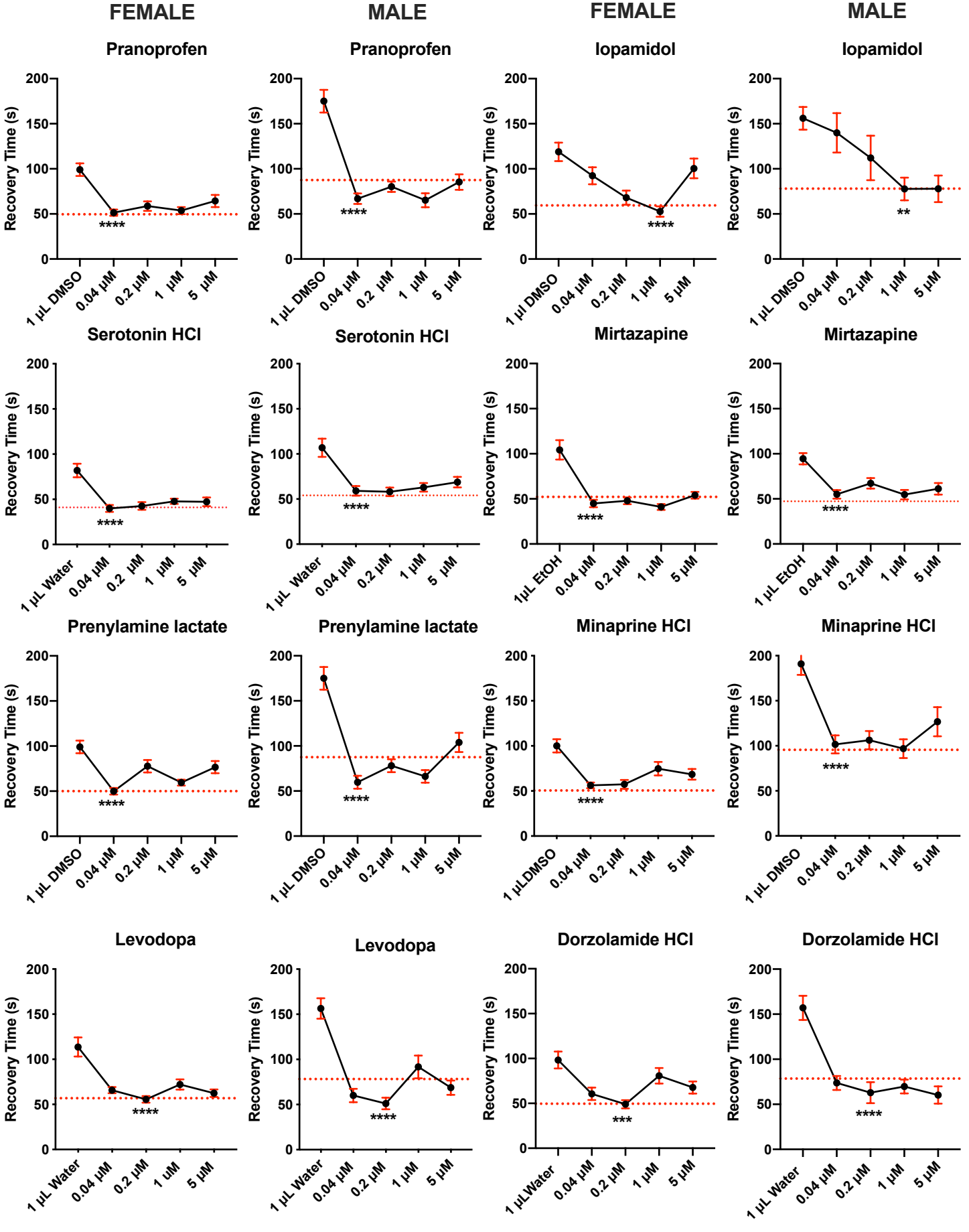
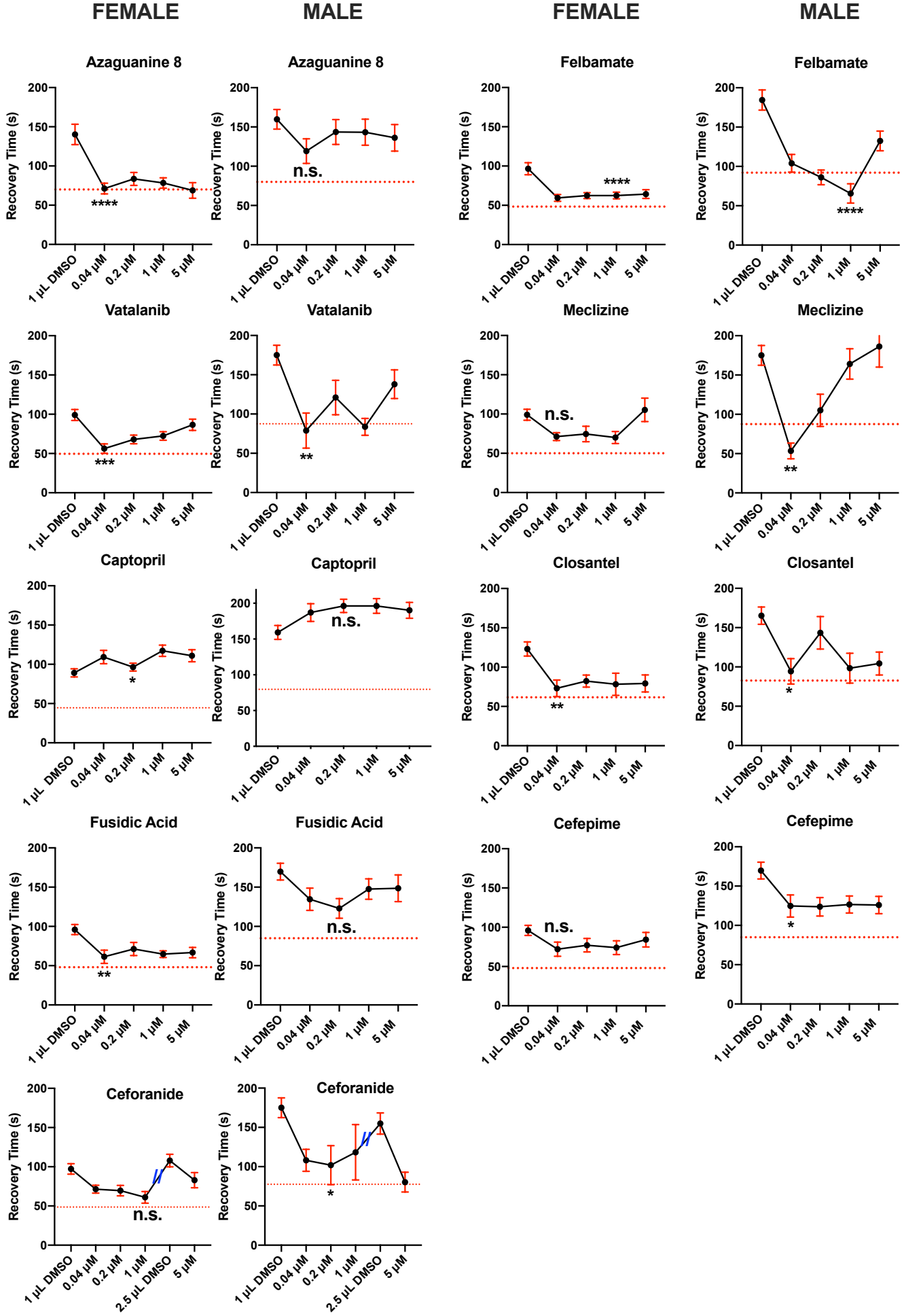
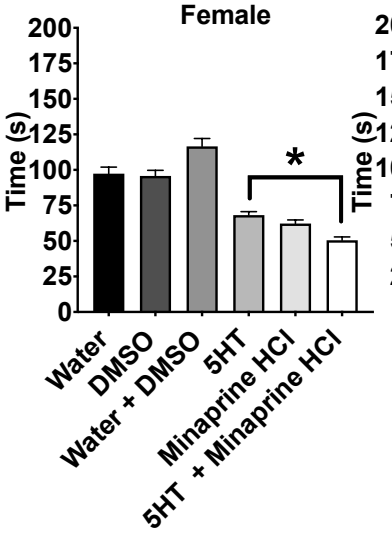
Ethanol Female
DMSO Female
Water Male
Ethanol Male
DMSO Male


FEMALE
MALE
FEMALE
MALE
Pranoprofen
Pranoprofen
Iopamidol
Iopamidol
Serotonin HCl
Serotonin HCl
Mirtazapine
Mirtazapine
Prenylamine lactate
Prenylamine lactate
Minaprine HCl
Minaprine HCl
Levodopa
Levodopa
Dorzolamide HCl
Dorzolamide HCl
Recovery Time (s)
200
150
100
50
0
****
**
***
1 µL DMSO
1 µl DMSO
1 µL Water
1µL EtOH
1 µLDMSO
1 µLWater
0.04 µM
0.2 µM
1 µM
5 µM
1 uM
5 µM


FEMALE
MALE
FEMALE
MALE
Azaguanine 8
Azaguanine 8
Felbamate
Felbamate
Vatalanib
Vatalanib
Meclizine
Meclizine
Captopril
Captopril
Closantel
Closantel
Fusidic Acid
Fusidic Acid
Cefepime
Cefepime
Ceforanide
Ceforanide
Recovery Time (s)
200
150
100
50
0
****
n.s.
****
****
***
**
n.s.
**
n.s.
*
**
*
**
n.s.
n.s.
*
1 µL DMSO
0.04 µM
0.2 µM
1 µM
5 µM
2.5 µL DMSO


Female
Time (s)
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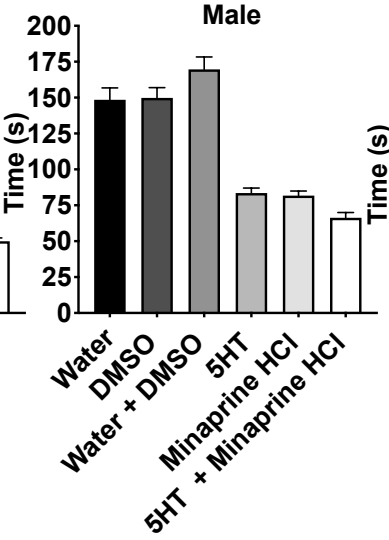
175
150
125
100
75
50
25
0
*
Water
DMSO
Water + DMSO
5HT
Minaprine HCl
5HT + Minaprine HCl


Male
Time (s)
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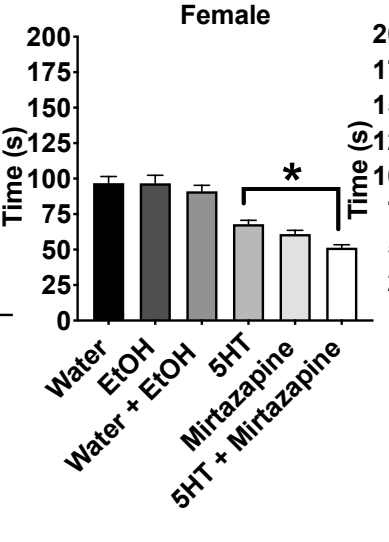
150
125
100
75
50
25
0
Water
DMSO
Water + DMSO
5HT
Minaprine HCl
5HT + Minaprine HCl


Female
Time (s)
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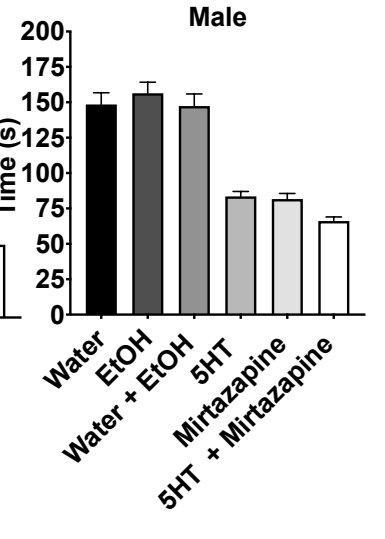
175
150
125
100
75
50
25
0
*
Water
EtOH
Water + EtOH
5HT
Mirtazapine
5HT + Mirtazapine


Male
Time (s)
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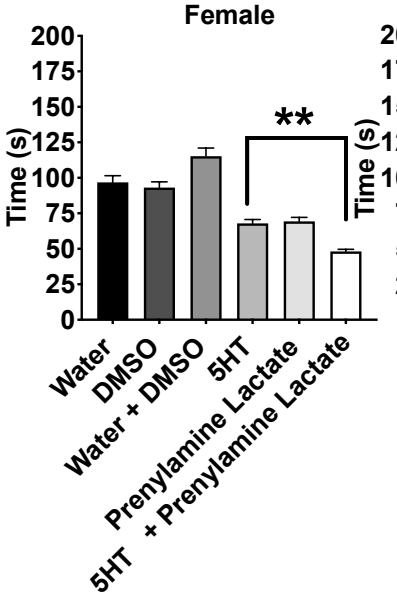
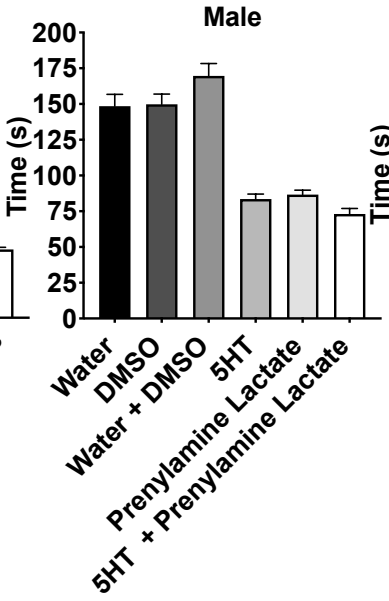
175
150
125
100
75
50
25
0
Water
EtOH
Water + EtOH
5HT
Mirtazapine
5HT + Mirtazapine


Female
Time (s)
200
175
150
125
100
75
50
25
0
**
Water
DMSO
Water + DMSO
5HT
Prenylamine Lactate
5HT + Prenylamine Lactate


Male
Time (s)
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175
150
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50
25
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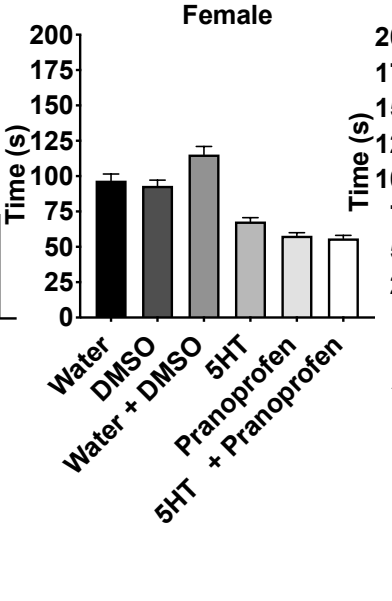
DMSO
Water + DMSO
5HT
Prenylamine Lactate
5HT + Prenylamine Lactate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Time (s)
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175
150
125
100
75
50
25
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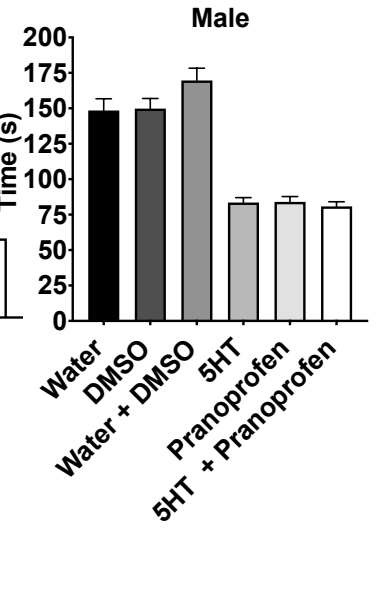
DMSO
Water + DMSO
5HT
Pranoprofen
5HT + Pranoprofen


Male
Time (s)
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150
125
100
75
50
25
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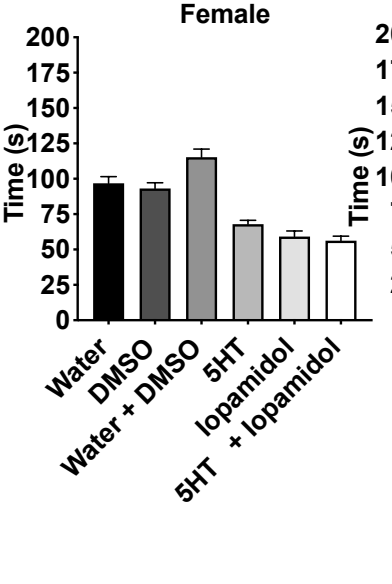
Water
DMSO
Water + DMSO
5HT
Pranoprofen
5HT + Pranoprofen


Female
Time (s)
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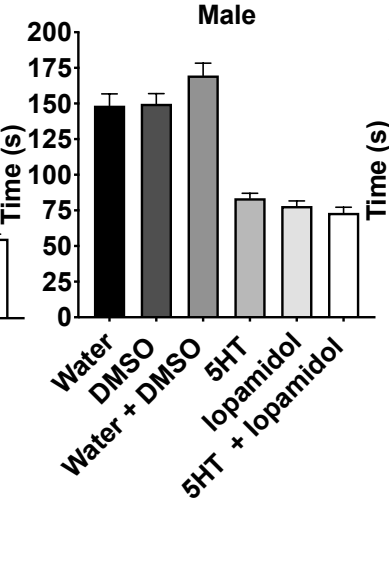
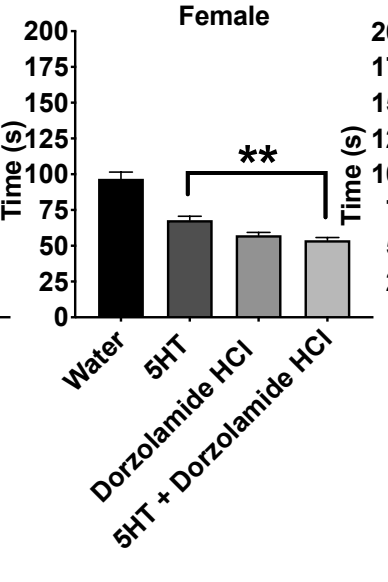
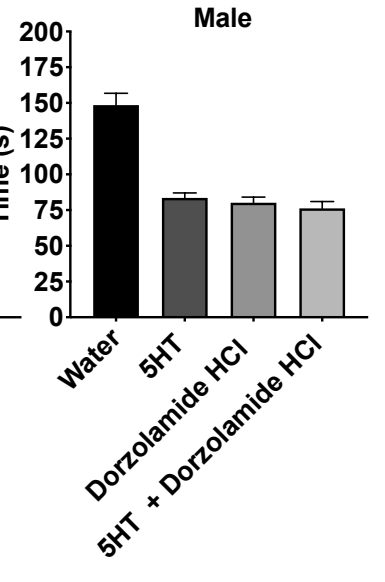
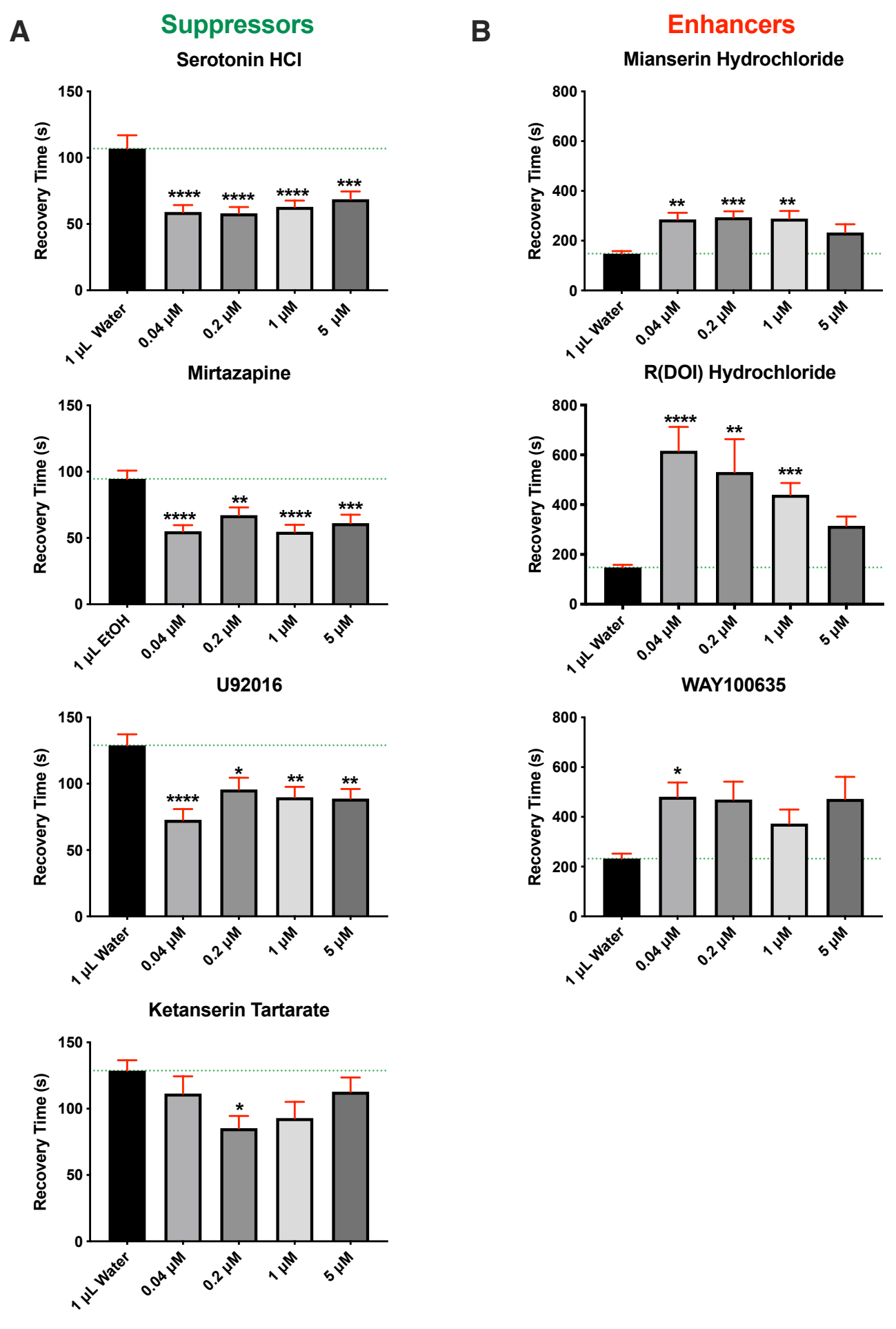
175
150
125
100
75
50
25
0
Water
DMSO
Water + DMSO
5HT
Iopamidol
5HT + Iopamidol


Male
Time (s)
200
175
150
125
100
75
50
25
0
Water
DMSO
Water + DMSO
5HT
Iopamidol
5HT + Iopamidol


Female
Time (s)
200
175
150
125
100
75
50
25
0
**
Water
5HT
Dorzolamide HCl
5HT + Dorzolamide HCl


Male
Time (s)
200
175
150
125
100
75
50
25
0
Water
5HT
Dorzolamide HCl
5HT + Dorzolamide HCl


A
Suppressors
Serotonin HCl
Recovery Time (s)
150
100
50
0
****
****
****
***
1 µL Water
0.04 µM
0.2 µM
1 µM
5 µM
Mirtazapine
Recovery Time (s)
150
100
50
0
****
**
****
***
1 µL EtOH
0.04 µM
0.2 µM
1 µM
5 µM
U92016
Recovery Time (s)
150
100
50
0
****
*
**
**
1 µL Water
0.04 µM
0.2 µM
1 µM
5 µM
Ketanserin Tartarate
Recovery Time (s)
150
100
50
0
*
1 µL Water
0.04 µM
0.2 µM
1 µM
5 µM
B
Enhancers
Mianserin Hydrochloride
Recovery Time (s)
800
600
400
200
0
**
***
**
1 µL Water
0.04 µM
0.2 µM
1 µM
5 µM
R(DOI) Hydrochloride
Recovery Time (s)
800
600
400
200
0
****
**
***
1 µL Water
0.04 µM
0.2 µM
1 µM
5 µM
WAY100635
Recovery Time (s)
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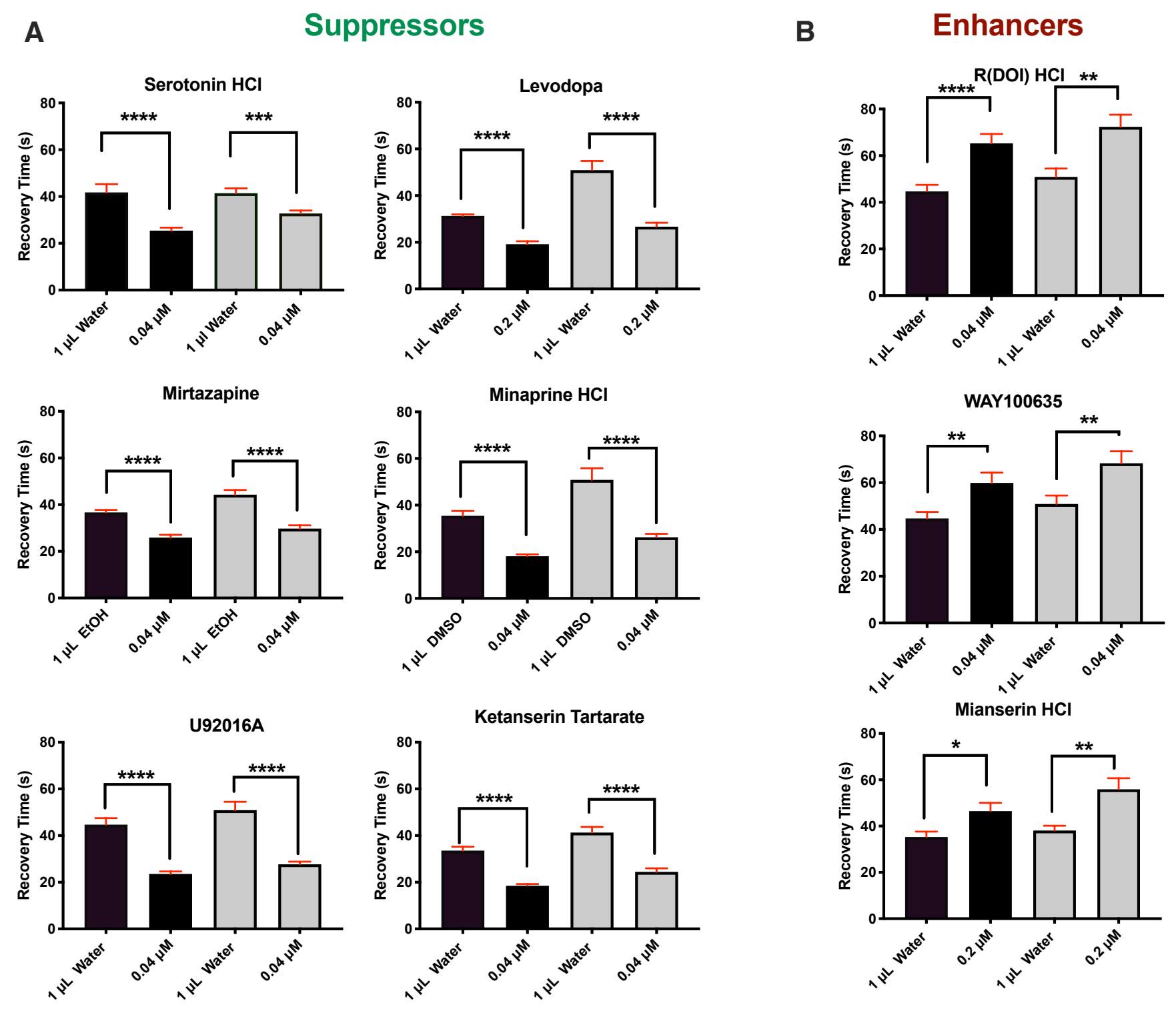
600
400
200
0
*
1 µL Water
0.04 µM
0.2 µM
1 µM
5 µM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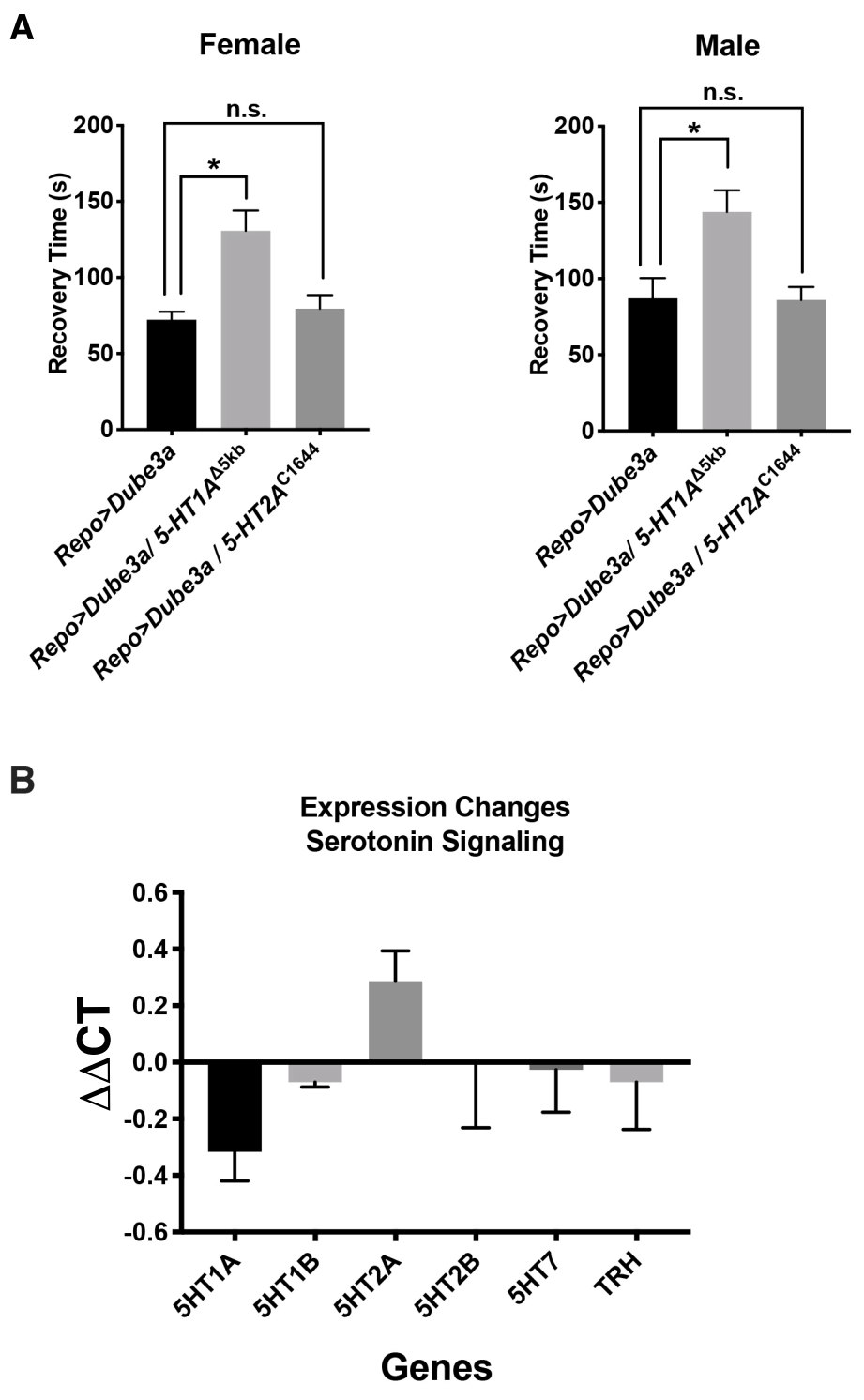
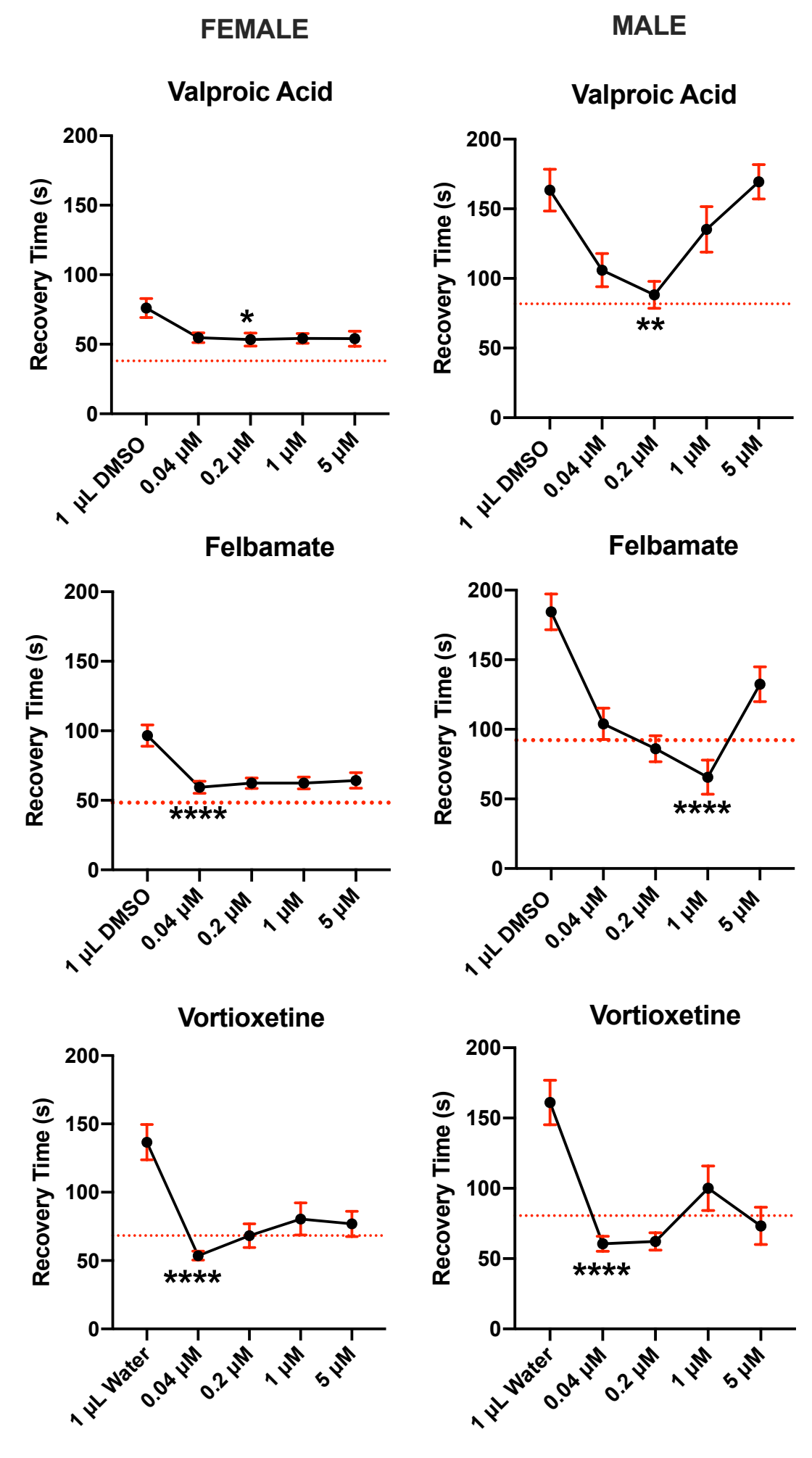
Suppressors
B
Enhancers
Serotonin HCl
Levodopa
R(DOI) HCl
Mirtazapine
Minaprine HCl
WAY100635
U92016A
Ketanserin Tartarate
Mianserin HCl
Recovery Time (s)
1 µL Water
0.04 µM
1 µl Water
0.2 µM
1 µL EtOH
1 µL DMSO
****
***
**
*


A
Female
Male
n.s.
*
Recovery Time (s)
200
150
100
50
0
Repo>Dube3a
Repo>Dube3a/ 5-HT1A Δ5kb
Repo>Dube3a / 5-HT2A C1644
B
Expression Changes
Serotonin Signaling
ΔΔCT
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
5HT1A
5HT1B
5HT2A
5HT2B
5HT7
TRH
Genes


FEMALE
MALE
Valproic Acid
Valproic Acid
Felbamate
Felbamate
Vortioxetine
Vortioxetine
Recovery Time (s)
200
150
100
50
0
*
**
****
****
****
1 µL DMSO
1 µL Water
0.04 µM
0.2 µM
1 µM
5 µM