### Results

The goal of this research was to identify transcriptional changes in FMR1-KO mice that might explain impaired memory discrimination. I used the active place avoidance task with conflict learning to observe initial avoidance learning and cognitive discrimination in WT and FMR1-KO mice. Given that FMRP is a translational modifier, little research has been done to investigate transcriptional changes upstream that might occur through regulatory feedback processes.

In the Active Place Avoidance Task, place learning and memory are evidenced by examining multiple aspects of behavior. I focused on the proportion of time spent in different quadrants of the arena, the number of entrances into the shock zone, and path to the first entrance.

#### No significant pre-training group differences

First I examined the data to determine if the groups were different prior to experiencing shock. I found that all groups where equal in the proportion of time spent in four quadrants of the arena (**Figure 2.2A**). There was no significant geneotype or treatment group on pre-training proportion of time spent in the shock zone (mean = 0.24; genotype: F(1,38) = 0.438, p = 0.512; group: F(3,38) = 0.438, p = 0.512), clockwise (mean = 0.26; genotype: F(1,38) = 0.153, p = 0.698; group: F(3,38) = 0.507, p = 0.680), opposite (mean = 0.21,; genotype: F(1,38) = 0.008, p = 0.929; group: F(3,38) = 1.051, p = 0.381), or counter clockwise (mean = 0.28, ; genotype: F(1,38) = 0.012, p = 0.913; group: F(3,38) = 0.979, p = 0.413).

There was also no significant effect of geneotype, training, or the interaction on pre-training number of entrances (**Figure 2.2B**) or path to the first entrance (**Figure 2.2C**), which are two measures that are used to identify the avoidance strategy. The was no significant main effect of geotype or training on the number of entrances (mean = 28.58, genotype: F(1,35) = 0.106, p = 0.747; training: F(3,35)= 1.717, p = 0.181) or path to the 1st entrance (mean = 0.42, genotype: F(1,35) = 0.165, p = 0.92; training: F(3,35)= 1.583, p = 0.211).

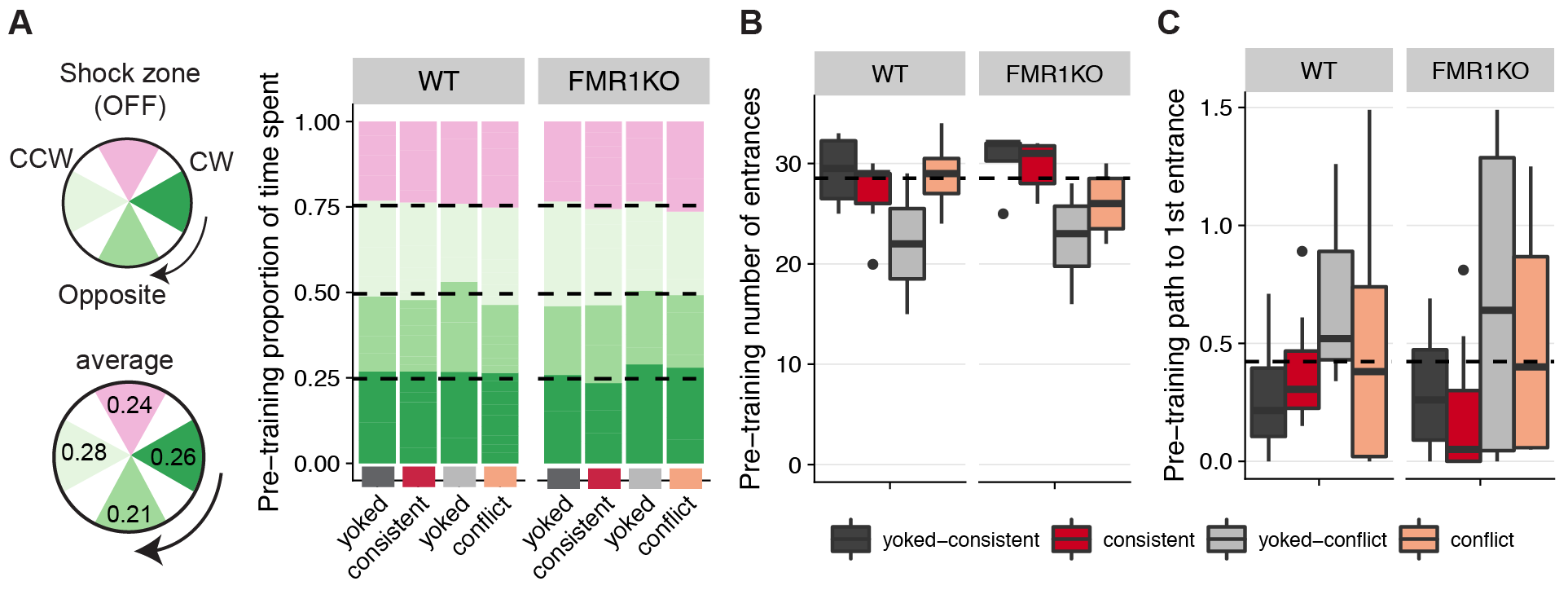


Fig. 2.2: No group differences before behavioral manipulation.

##### A) This graph shows that all groups of mice spend ~ 25% of their time equally across four quadrants of the arena during the pre-training session (pink: future shock zone, dark green: clockwise, green: opposite the shock zone, light green: counterclockwise). B) Pre-training number of entrances into the shock zone and C) path to the first entrance are not significantly different between treatment groups and genotypes (dark grey: yoked-consistent, red: consistently-trained, light grey: yoked-conflict, peach: conflict-trained).

#### Training has more substantial effect than genotype on avoidance behaviors

After confirming equal variation among groups during pre-training, I asked if there were groups differences in the distribution of time spent during training, retest, conflict session (**Fig. 2.3**). Using a linear model I found that time spent in the shock zone is not significantly influenced by genotype (F(1,286) = 1.49, p = 0.22) by is influenced by training (F(2,286) = 128.58, p < 0). This linear model with training, genotype, and the interaction explains 73% of the variation in time spent in the shock zone. Among only the yoked groups, there is no effect of genotype (F(1,80) = 0.040, p = 0.84) or training (F(1,80) = 3.438, p = 0.067) on time spent in the shock zone.



Fig. 2.3. Proportion of time spent in in the arena with the shock on.

##### The average proportion of time spent in each 60 degrees quadrant of the arena was calculated or each group for each session with the shock was on (T1, T2, T3: training sessions 1-3; R1: retest; C1, C2, C3: conflict training sessions; pink: future shock zone; dark green: clockwise; green: opposite the shock zone; light green: counter clockwise ). For trained mice, mice are expected to spend very little time in the shock zone (<0.4%) and to equally split their time between the three remaining quadrants (~32% each). For yoked mice, time spent is expected to be evenly distributed across all four quadrants (~25% each).

The differences between the conflict and consistently trained mice are apparent during the three conflict training sessions (**Fig 2.4**). Both consistent and conflict groups avoid the shock zone, spending less than 2% of their time in the shock zone, but there is no difference between groups (mean = 0.019, F (1,78) = 1.2166, p = 0.27). Consistently trained groups spend significantly less time clockwise of the shock zone than conflict trained groups (F (1,78) = 23.3405, p < 0.001). Consistently trained groups spend more time in the counterclockwise zone than conflict trained mice (F (1,78) = 8.2837, p = 0.005).

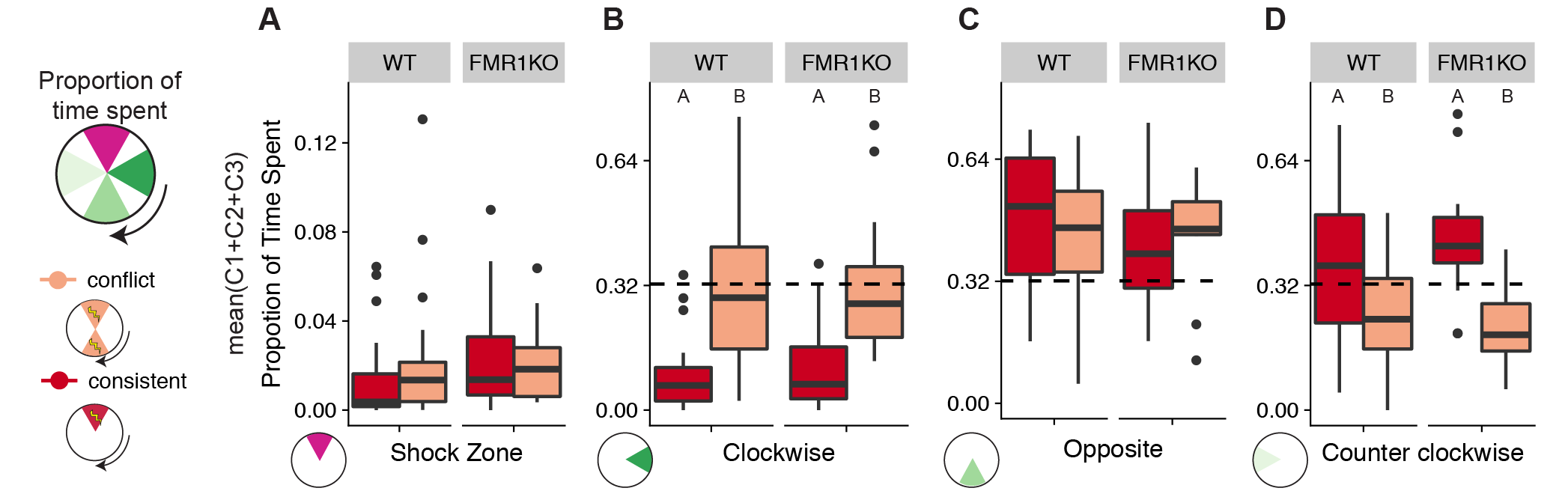


Fig. 2.4. Consistent and conflict trained mice use space differently during conflict training sessions.

#### A) During the conflict training sessions, consistent and conflict mice both avoid the shock zone but there is not a difference between groups. B) Consistently trained mice spend significantly less time in space clockwise to the shock zone. C) All groups spend more time on average in the space opposite the shock zone, but there are no group differences. D) Consistently trained mice spend more time in the counterclockwise zone than conflict mice. Legend) dark grey: yoked-consistent, red: consistently-trained, light grey: yoked-conflict, peach: conflict-trained.

#### Initial learning not as strong as anticipated

After establishing place avoidance behavior in the trained groups, I next investigated the extent to which punishment and memory contributed to place avoidance (**Fig. 2.5**). I unexpectedly found that mice were not using memory (path to first entrance) but were relying on punishment (number of entrances into the shocked zone by the trained groups) to avoid the shock zone. This was unexpected given the results of Chapter 1 (**Fig. 2.5C, D**). There was no effect of genotype the number of entrances into the shock zone at any given timepoint (**Fig. 2.5A, B**). However, there was an effect of genotype on the path to first entrance during the retest, but this appears to be driven by unexplained avoidance behavior driven by a yoked group (**Fig. 2.5D, E**). At this level of analysis, the results place learning was not robust in WT or FMR1-KO mice.

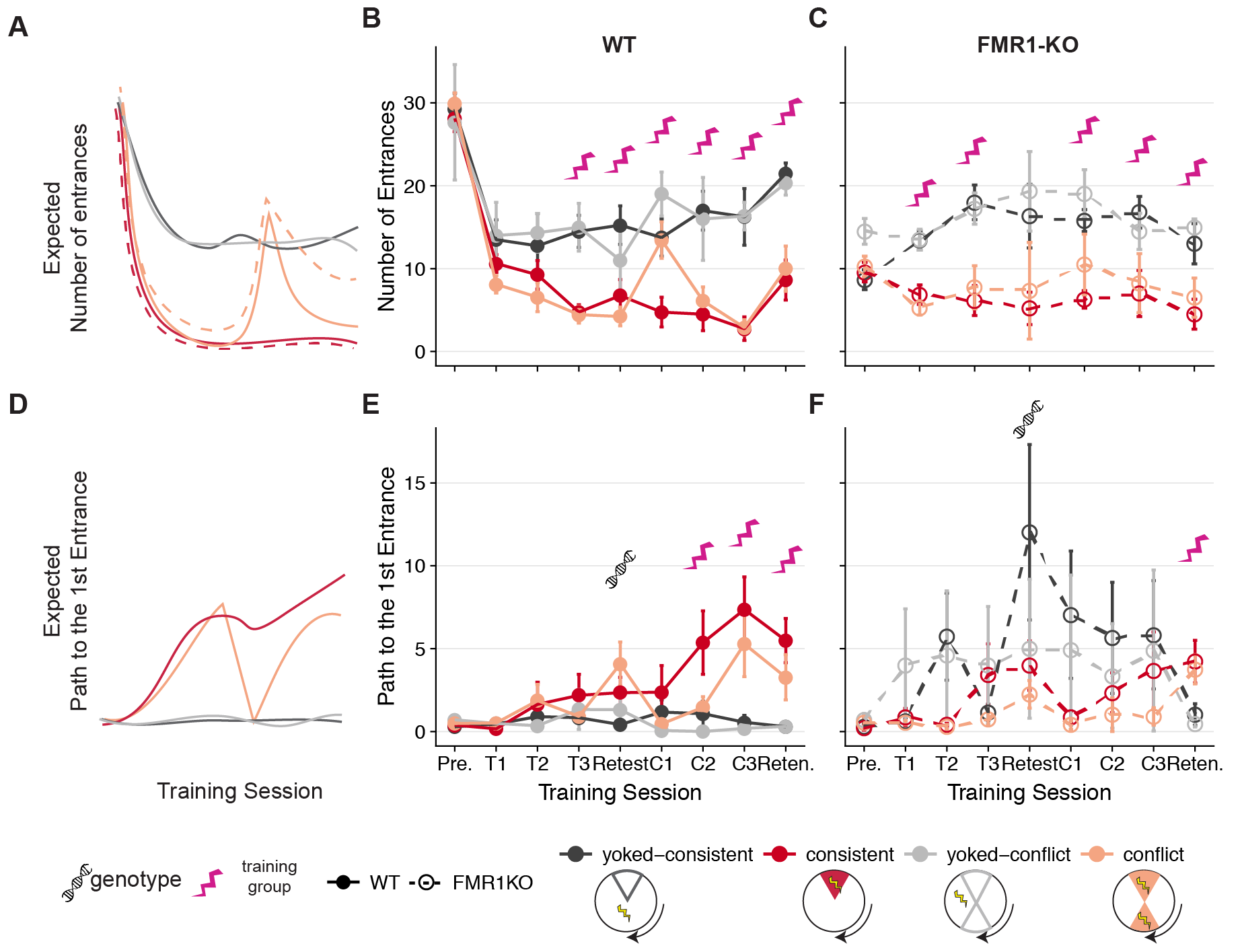


Fig. 2.5.Summary of punishment and estimates of memory in WT and FMR1-KO mice

##### A,B) Consistent and conflict trained mice from WT and FMR1-KO groups to make fewer entrances into the shock zone than yoked-mice; C) however, the pattern does not exactly match the expected results. D, E) Consistent and conflict trained mice from WT FMR1-KO do not show evidence of place memory until after the first day of initial training. F) This pattern does also not mirror the expected results. Legend) Pre: pre-training; T1,T2,T3: training sessions 1-3; C1, C2, C3: conflict training sessions; Reten: retention session; dark grey: yoked-consistent, red: consistently-trained, light grey: yoked-conflict, peach: conflict-trained. The pie-shaped shaded regions of the inserts highlight the region used to count the number of entrances.

#### Evidence for cognitive discrimination in WT mice

In the Active Place Avoidance Task with conflict training, cognitive discrimination is evidenced by a shift in the number of entrances and path to the shock zone. The number of entrances are expected to highest at C1 and path to first entrances is expected to be lowest at C1 if cognitive discrimination is used to learn to avoid the rotated shock zone (**Fig 2.5**). The difference in number of entrance by WT consistent and conflict trained groups is evidence for cognitive discrimination, but there is no evidence to support cognitive discrimination in the FMR1-KO conflict group (**Fig 2.6A, B**)

I measured the mean number of entrances into the shock zone in animals that were consistently trained and compared their performance to the yoked counterparts (Fig. 2.2). A three-way ANOVA with Tukey Honest Significant Differences (Tukey HSD) test was carried out to determine the influence of Genotype \* Treatment Group \* Training Session and the Genotype \* Treatment Group interaction on the number of entrances during the active place avoidance task with the conflict training. For this statistical analysis, I included only the three training or conflict sessions on day 2 (T4/C1, T5/C2, and T6/C3). As expected Treatment Group had a highly significant effect on number of entrances [F(1, 69)= 41.3, p < 0.001]. The interaction between the effects of Genotype and Training Group was not significant [F(1, 69)= 0.009, p = 0.924]. While there is a significant effect of genotype alone on the number of entrances [F(1, 69)= 8.17, p = 0.005], there was a significant difference between WT conflict and FMR1-KO conflict (p = 0.78) or between WT yoked-conflict and FMR1-KO yoked-conflict (p = 0.93).

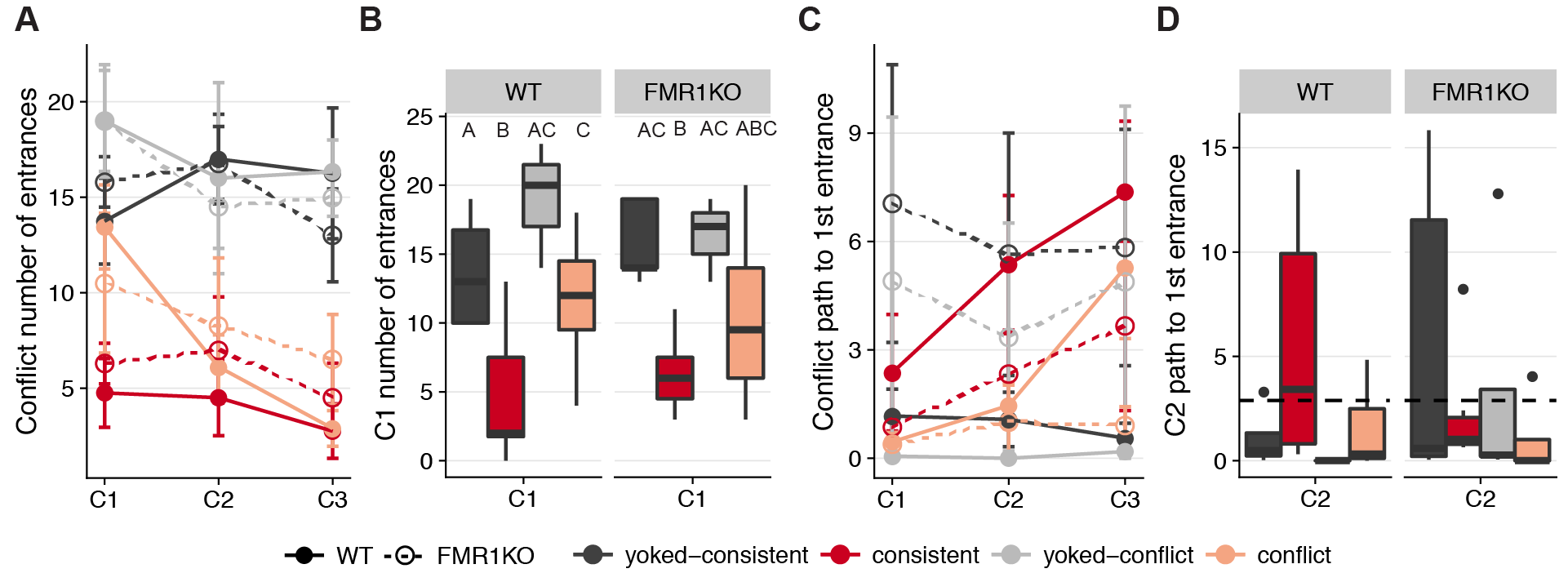


Fig. 2.6. During recall, mice avoid the shock zone using a non-place strategy.

#### Poor recall evidence of minimal place avoidance learning

During the retention session, training but not genotype influences multiple measures of place avoidance (**Fig. 2.6**). Time spent in the shock zone during the retention session is affected by training (F(3,42) = 5.5420, p = 0.002685) but not genotype (F(1,42) = 0.043, p = 0.837), which is driven by difference between consistent and yoked-consistent (p = 0.00684) but not between conflict and yoked-conflict (p = 0.125)(**Fig. 2.6A**). Trained mice also make fewer entrances into the shock zone (**Fig. 2.6B**), but their path to the shock zone is not significantly longer (**Fig. 2.6C**). THe results of a two-way ANOVA followed by Tukey HSD was are visualized on on Figure 2.C.

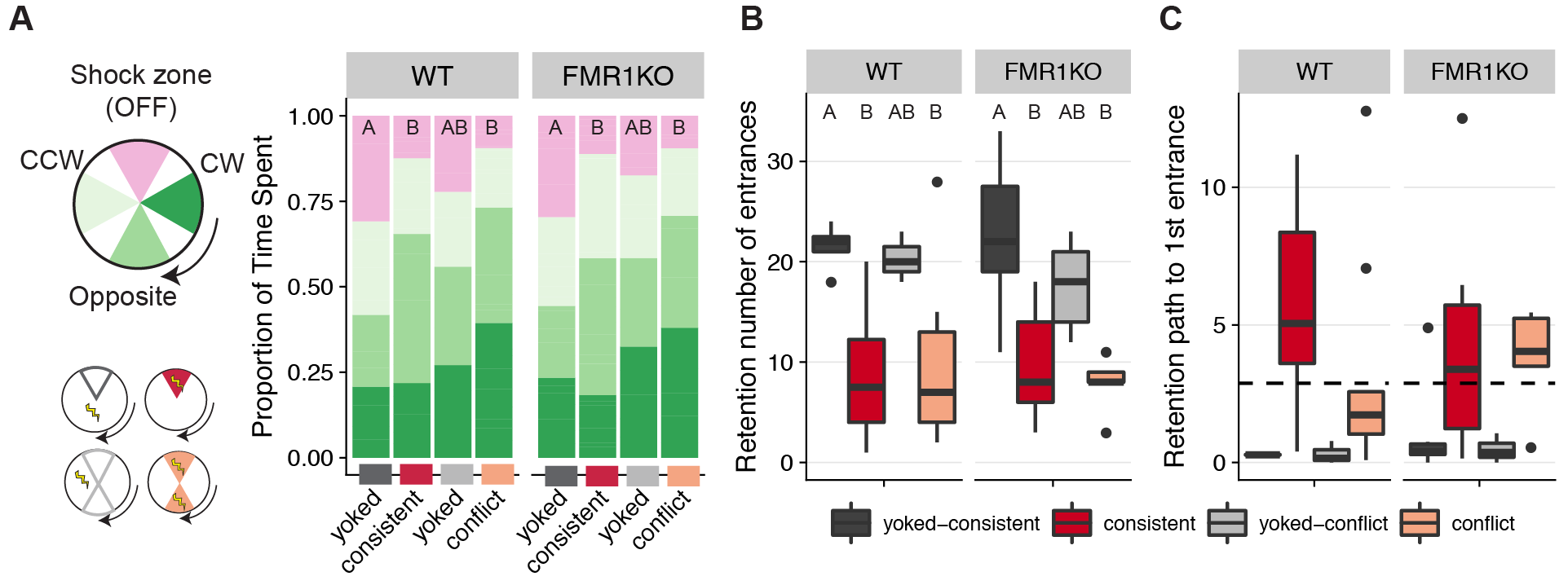


Fig. 2.6. During recall, mice avoid the shock zone.

##### A) Trained mice spend less time in the shock zone than their yoked counterparts. B) They also make fewer entrances into the shock zone, C) but their path to the shock zone is not significantly longer. Legend) dark grey: yoked-consistent, red: consistently-trained, light grey: yoked-conflict, peach: conflict-trained, pink: future shock zone, dark green: clockwise, green: opposite the shock zone, light green: counterclockwise.

Consistent with the evidence for poor place memory, I found no change in synaptic strength at the CA3-CA1 synapse (as measured by maximum fEPSP slope) due to genotype or training (**Fig. 2.7**).

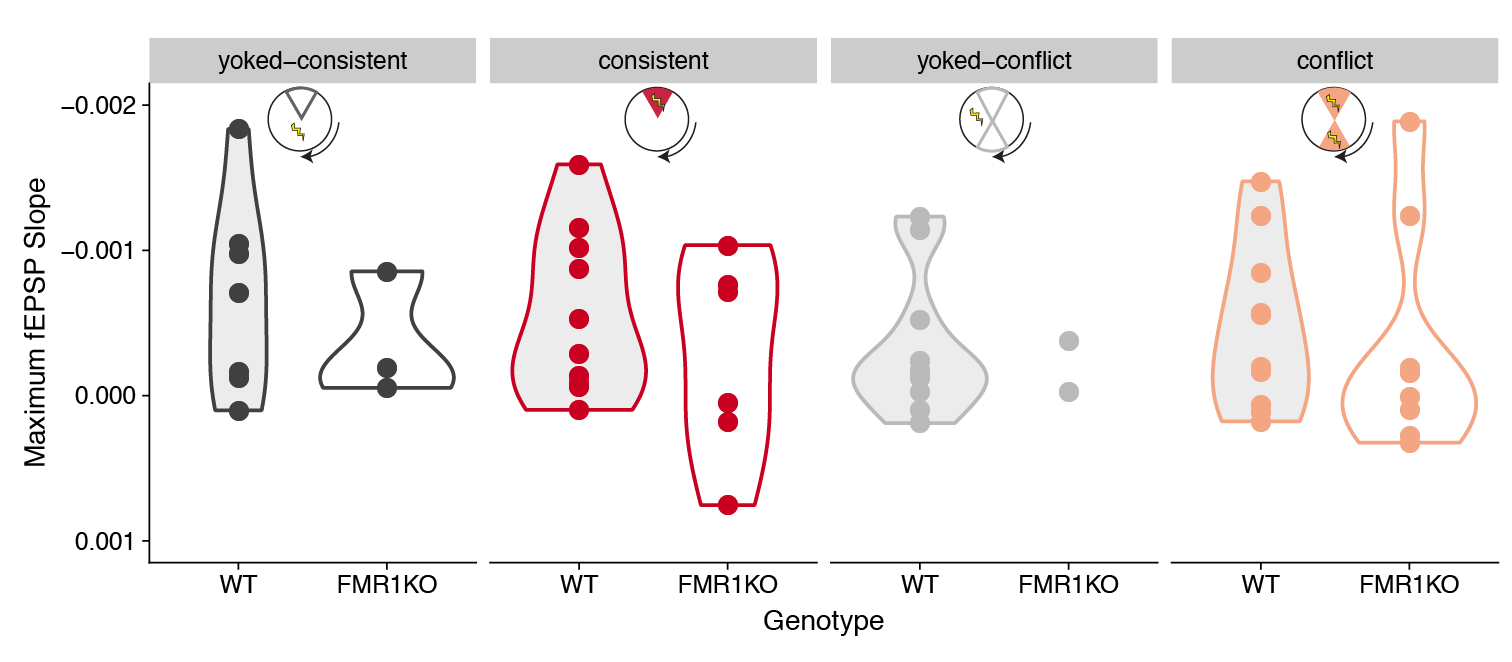


Fig. 2.7. CA3-CA1 synaptic strength is not altered by genotype or place avoidance training.

##### The maximum fEPSP slope is no different between groups indicating that neither training or genotype influence synaptic strength at CA3-CA1 synapses. WT: filled violin plot, FMR1-KO, open violin plots, dark grey: yoked-consistent, red: consistently-trained, light grey: yoked-conflict, peach.

### CA1 transcriptional response to constitutive FMR1 knockout

Given the lack a strong and robust signal of hippocampus-dependent place learning, I elected not to continue looking for the molecular underpinnings of impaired cognitive functions in the FMR1-KO mouse. Instead, I decided to investigate whether there are molecular differences between the WT and FMR1-KO mice when the internal representations of the world are equivalent, as far as I can tell from behavior. Thus, I sequenced the transcriptome the CA1 subfield of the dorsal hippocampus from the mice in the yoked-consistent treatment group (**Fig. 2.8A**).

RNA was isolated from a tissue sample (250 μm in diameter x 300 μm thickness) from the CA1 subfield of the dorsal hippocampus. Briefly, the transcriptome was constructed from mRNA-enriched Illumina libraries, transcript levels were estimated with Kalliso18 using the Gencode Mouse reference transcirptome19, the statistical significance of enriched genes and molecular functions was inferred using DESeq221 and GO\_MWU30, respectively. I identified 20 genes whose expression in the CA1 subfield was altered by the constitutive elimination of FMRP (**Fig 2.8B**). About half of these genes are upregulated in FMR1-KO mice (Apc2, Arel1, Brf1, Cry, Fibcd1, Grin1, Ncdn, Pnmal2, Prpf8, Sidt1, Slc8a2, Tnik, and Wipf3) while the other half are down-regulated in FMR1-KO mice (Cacna1g, Car4, Ccnd1, Cpne7, Dlx1, Efcab6, Fgfr1, Fmr1, Kcnt1, Mtus1, Plat, Serpina3n, Slc29a4, Sstr3, and Xbp1). Ccnd2 and Fmr1 are highly upregulated in WT compared to FMR1-KO mice (Fig 2.3C).

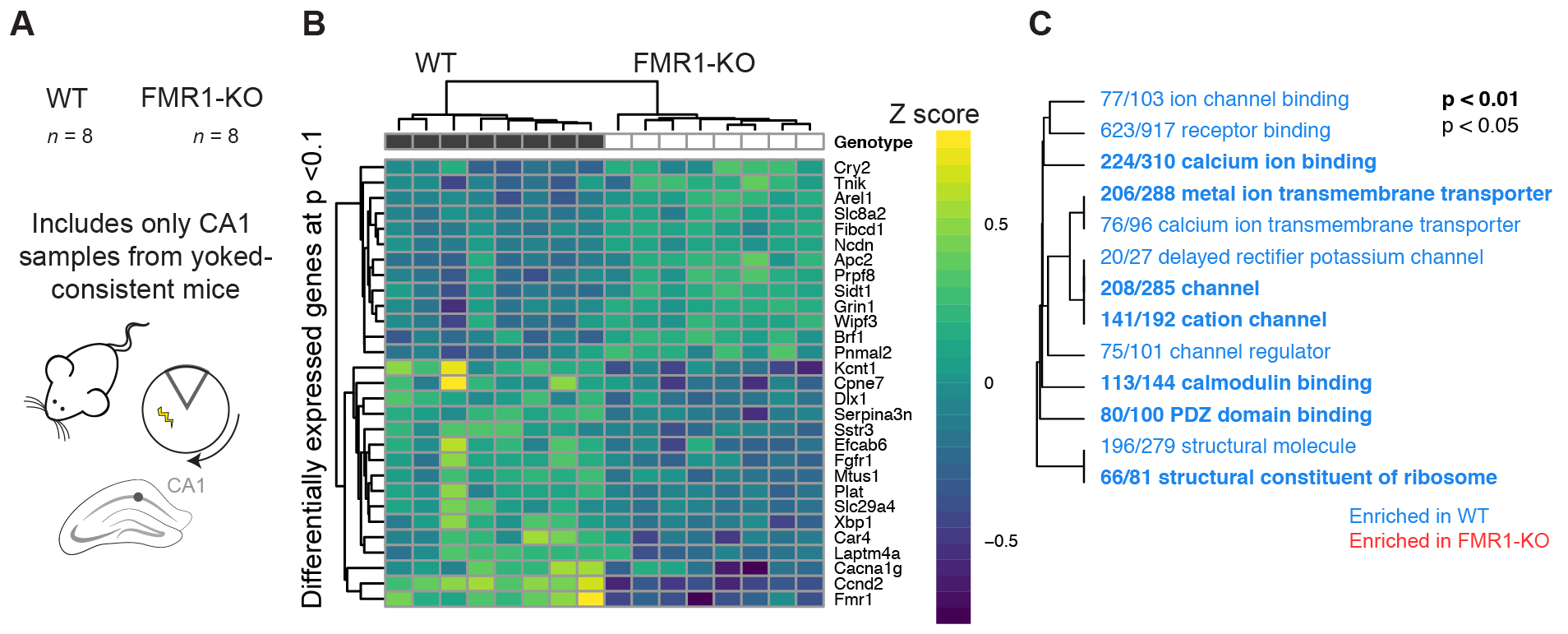


Fig. 2.8. FMR1-KO show downregulation of calcium ion signaling in the the the CA1 subfield.

##### A) The sample size for RNA-sequencing is 8 WT and 8 FMR1-KO tissues from the CA1 subfield from only the consistent-yoke group. B) Hierarchical clustering of differentially expressed genes shows that only 13 genes are upregulated in response to FMR1KO while 16, including Fmr1, were downregulated in the CA1 subfield of yoked-consistent mice. C) Down-regulation of ion channel binding, receptor binding, calcium binding, metal ion membrane transport, calcium ion transmembrane transporter, delayed rectifier potassium channel, channel, cation channel, channel regulator, calmodulin binding, PDZ domain binding, structural molecular, and structural constituent of ribosome. On the plot, different fonts are used to indicate significance (bold: p < 0.01, regular: p < 0.05) and color indicates enrichment with either up (red) or down (blue) regulated genes. The fraction next to GO category name indicates the fraction of "good" genes that exceed the p-value cutoff.

#### Down-regulation of ion transport in the the the CA1 subfield

Down-regulation of ion channel binding, receptor binding, calcium binding, metal ion membrane transport, calcium ion transmembrane transporter, delayed rectifier potassium channel, channel, cation channel, channel regulator, calmodulin binding, PDZ domain binding, structural molecular, and structural constituent of ribosome. On the plot, different fonts are used to indicate significance (bold: p < 0.01, regular: p < 0.05) and color indicates enrichment with either up (red) or down (blue) regulated genes. The fraction next to GO category name indicates the fraction of "good" genes that exceed the p-value cutoff. The tree on the plot is hierarchical clustering of GO categories based on shared genes. Categories with no branch length between them are subsets of each other.

#### Reproduction of and comparison to the Ceolin et al. 2017 study.

Next, I reproduced the data from the Ceolin et al. 2017 which used Ceolin's fluorescence labeling to selectively sequence pyramidal neurons in the CA1 subfield of the hippocampus from WT and FMR1-KO mice (**Fig 2.9A, B**). My reproduction of their data produced a very similar pattern of gene expression and list of differentially expressed genes (**Fig 2.9C, D**). I found roughly the same scale of gene expression changes. Of list of "replicated" 39 differentially expressed genes, two genes (*Serpina3a* and *Efcab6*) were also identified in my analysis of differential expression (**Fig. 2.8D**). However, my list does have fewer significant genes. Most of the genes were identified by both analytical methods indicating a robust response. Our GO analysis highlighted different patterns. The Ceolin study highlights the molecular function enriched pathways in FMR1-KO mice, but my analysis provided stronger evidence for a deletion of calcium receptor-related functions (**Fig 2.9E**). This suggests a role for dysregulation of calcium signalling in the hippocampus of Fragile X Syndrome patients and is consistent with my research findings.

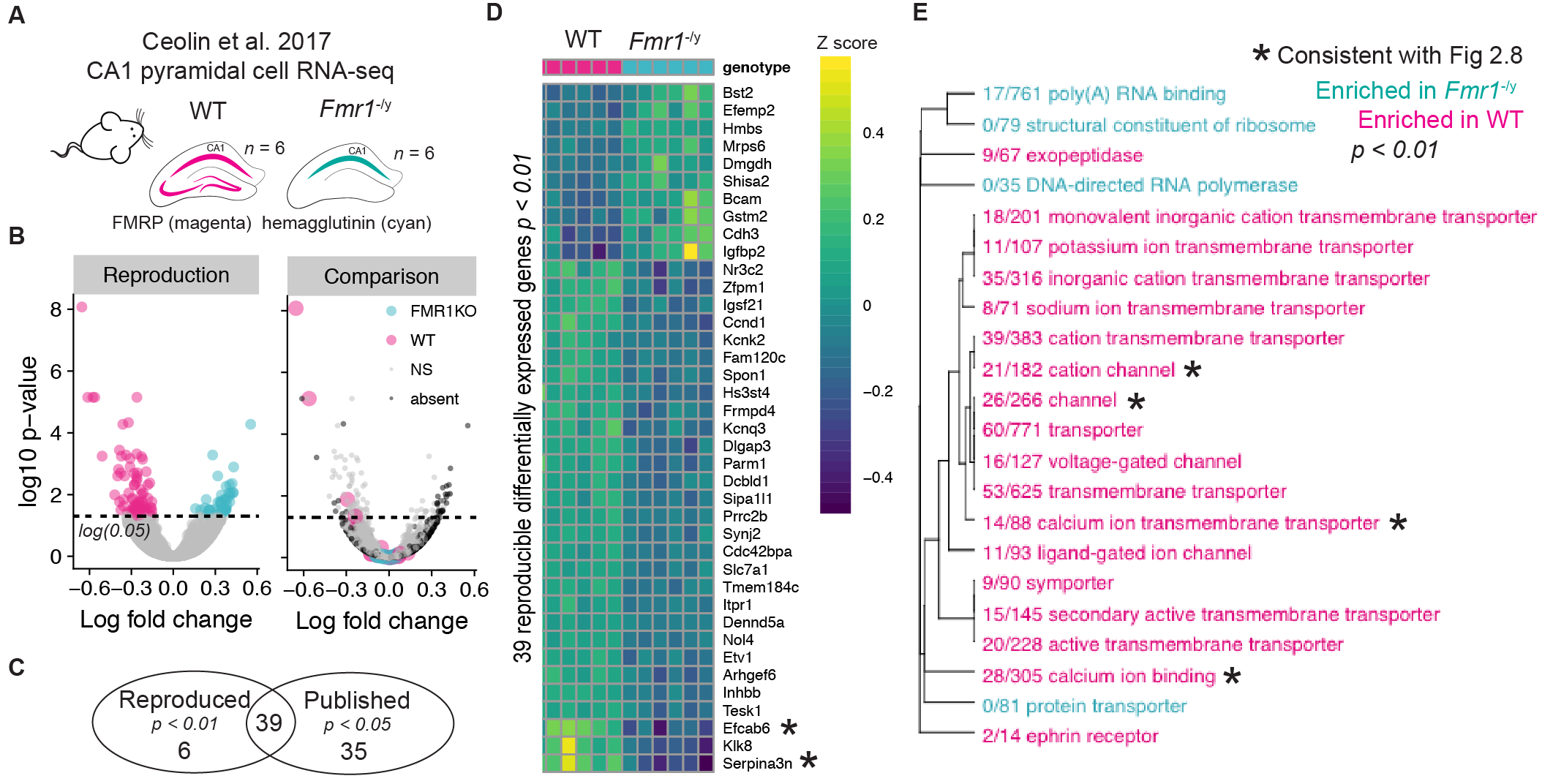


Fig. 2.9. Reproducing the Ceolin study for direct comparison of results.

##### A) Graphical representation of the samples for the Ceolin et al. 201732 study examining CA1 expression in WT and FMR1-KO mice. B) Volcano plot showing enrichment of 88 genes that are up-regulated in FMR1-KO mice and the 146 genes that are up-regulated in WT mice. C) Hierarchical clustering shows the names and expression patterns of those same significant genes. D) GO analysis showing a very similar pattern of depletion of calcium channel activity as was shown in Fig. 2.4C). In contrast, Ceolin detected enrichment of ribosomal processes in response to FMR1-KO in CA1 pyramidal neurons.A) Graphical representation of the samples for the Ceolin et al. 201732 study examining CA1 expression in WT and FMR1-KO mice. B) Volcano plot showing enrichment of 88 genes that are up-regulated in FMR1-KO mice and the 146 genes that are up-regulated in WT mice. C) Hierarchical clustering shows the names and expression patterns of those same significant genes. D) GO analysis showing a very similar pattern of depletion of calcium channel activity as was shown in Fig. 2.4C). In contrast, Ceolin detected enrichment of ribosomal processes in response to FMR1-KO in CA1 pyramidal neurons.