### 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 proportion of time spent in different quadrants of the arena, number of entrances into the shock zone, and path to 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).

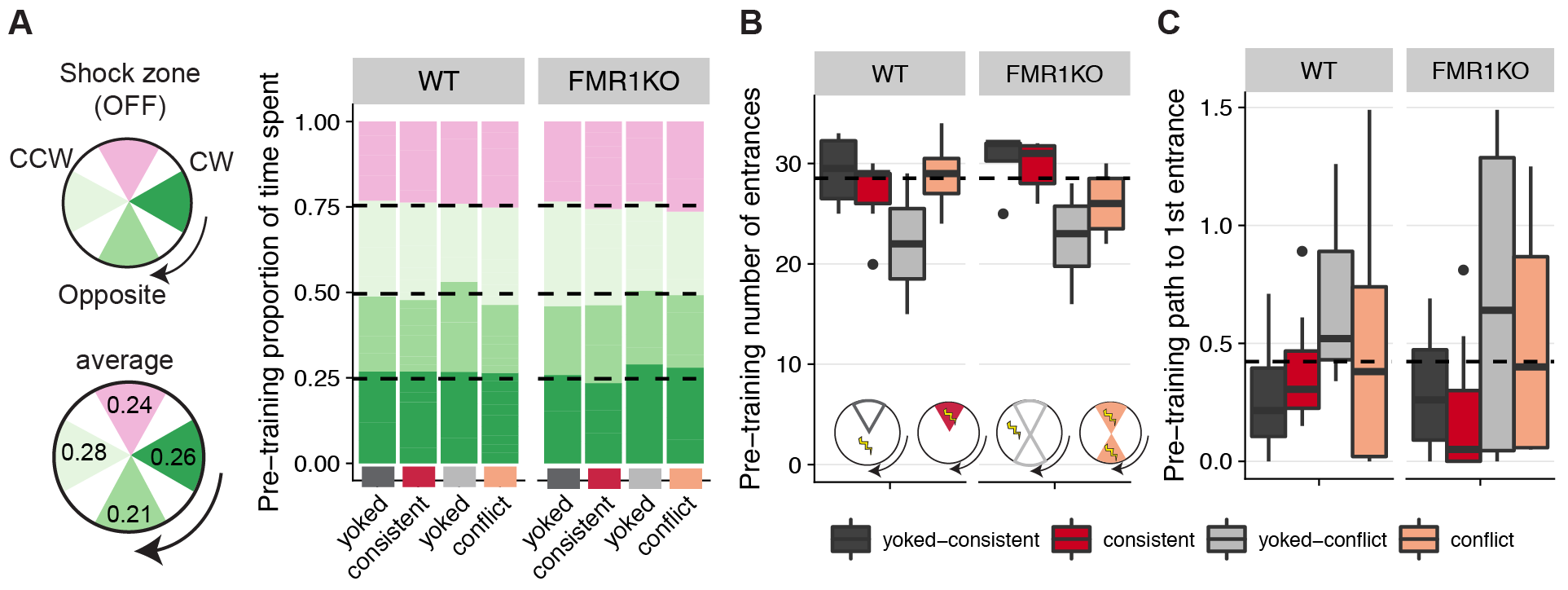


Fig. 2.2: No group differences prior to behavioral manipulation.

##### 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: counter clockwise). B) Pre-training number of entrances into the shock zone and C) path to first entrance are not sinificantly different between treatment groups and genotypes (dark grey: yoked-consistent, red: consistently-trained, light grey: yoked-conflict, peach: conflict-trained).

#### Training has larger effect than genotype on avoidance behaviors

After confirming equal variation amoung 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 interactino expalins 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.

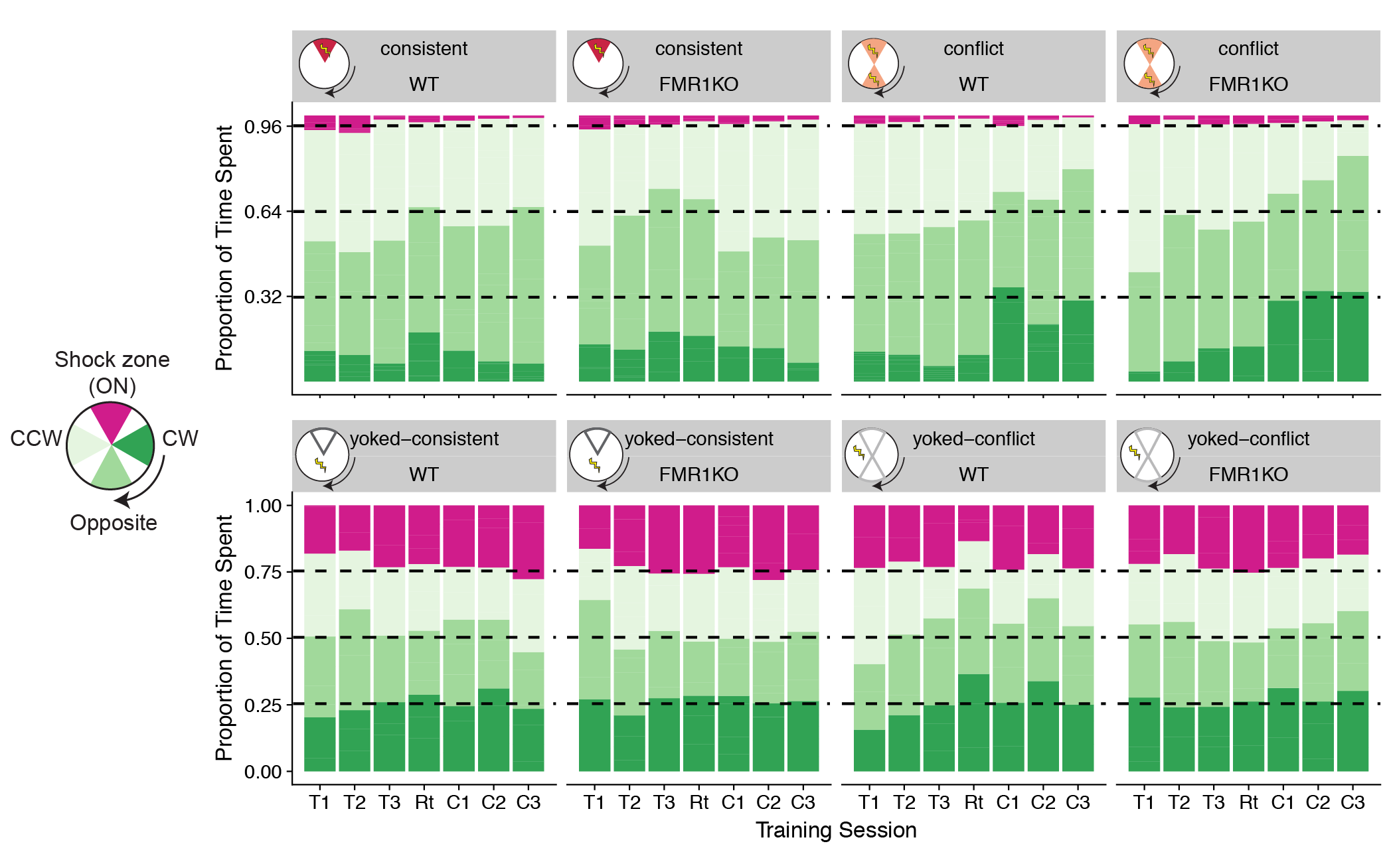


Fig. 2.3. Proportion of time spent in in the arena with the shock on.

##### The aveage proportion of time spent in each 60 degree guadrant of the arena was caluculated 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 clockwis ). 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 equally distributed across all four quatrants (~25% each).

The differences between the conflict and consistnetly trained mice are appearent during the three conflict training sessions (**Fig 2.4**). Both consitent 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). Consiently trained groups spend significantly less time clockwise of the shock zone than conflict trained groups (F (1,78) = 23.3405, p < 0.001). Consistnetly trainined groups spend more time in the counter clockwise zone than conflict trained mice (F (1,78) = 8.2837, p = 0.005).

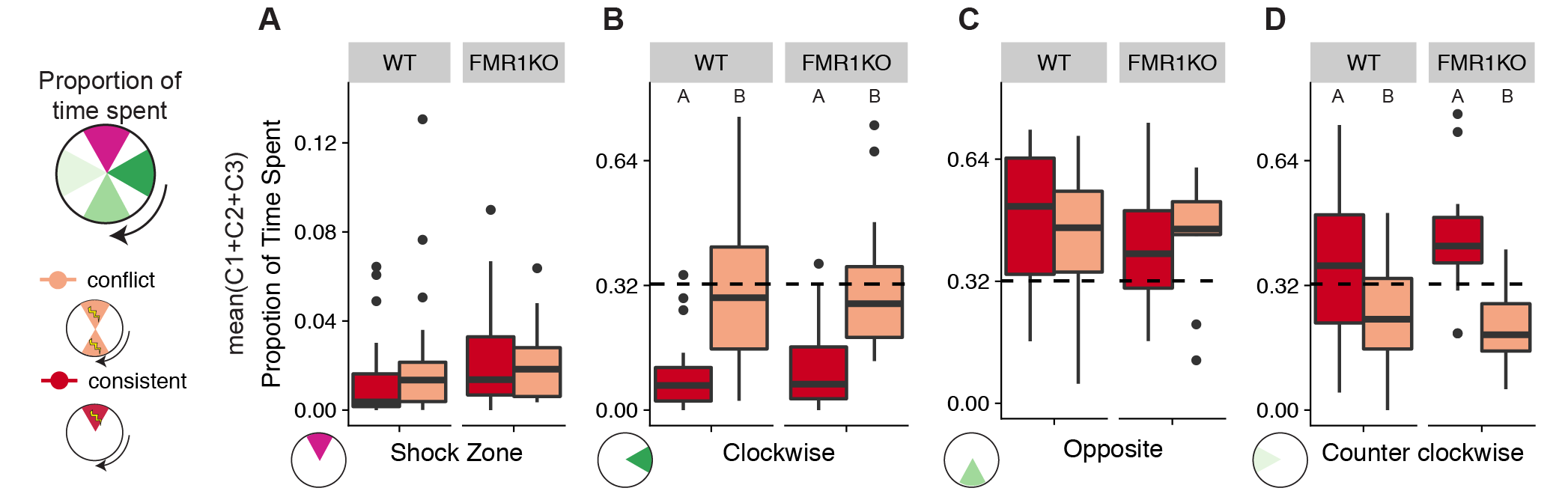


Fig. 2.4. Consistent and conflict trained mice use space differently during conflict training sessions.

#### A) During the conflict training sessions, consistent and conflict mice both avoid the shock zone but there is not 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 counter clockwise 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 entent to which punishment and memory contributed to place avoidance (**Fig. 2.5**). I unexpectly 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 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 anlaysis, the results place learning was not robust in WT or FMR1-KO mice.

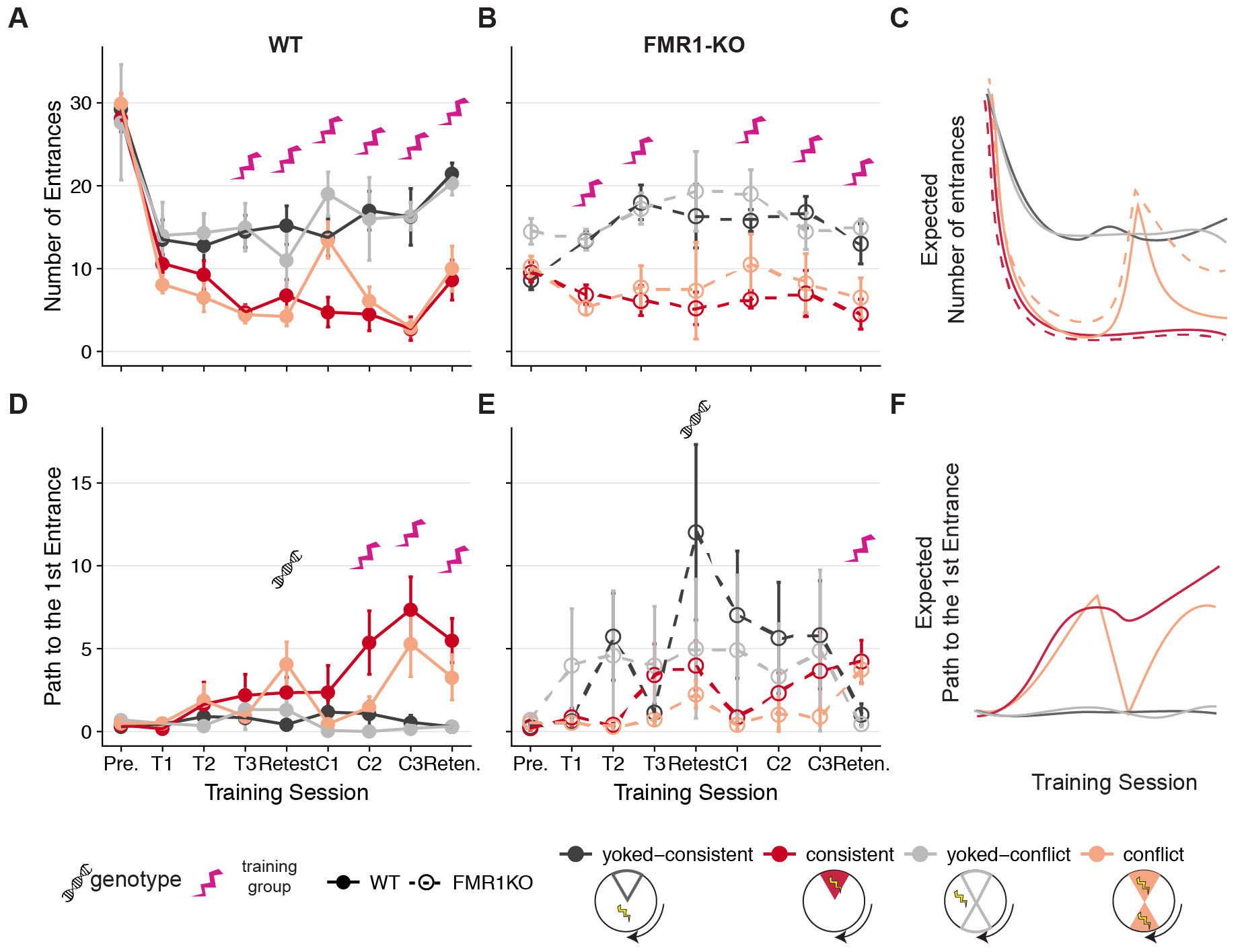


Fig. 2.5.Summary of punishment and estimates of memory in WT and FMR1-KO mice

##### A,B) Consistent and conflict trained mice from WT and FMR1-KO groups 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 th expected results. Legend) Pre: pre-training; T1,T2,T3: training sessions 1-3; C1, C2, C3: conflict training sessions; Reten: retetenion 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. 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 (TukeyHSD) 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 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).

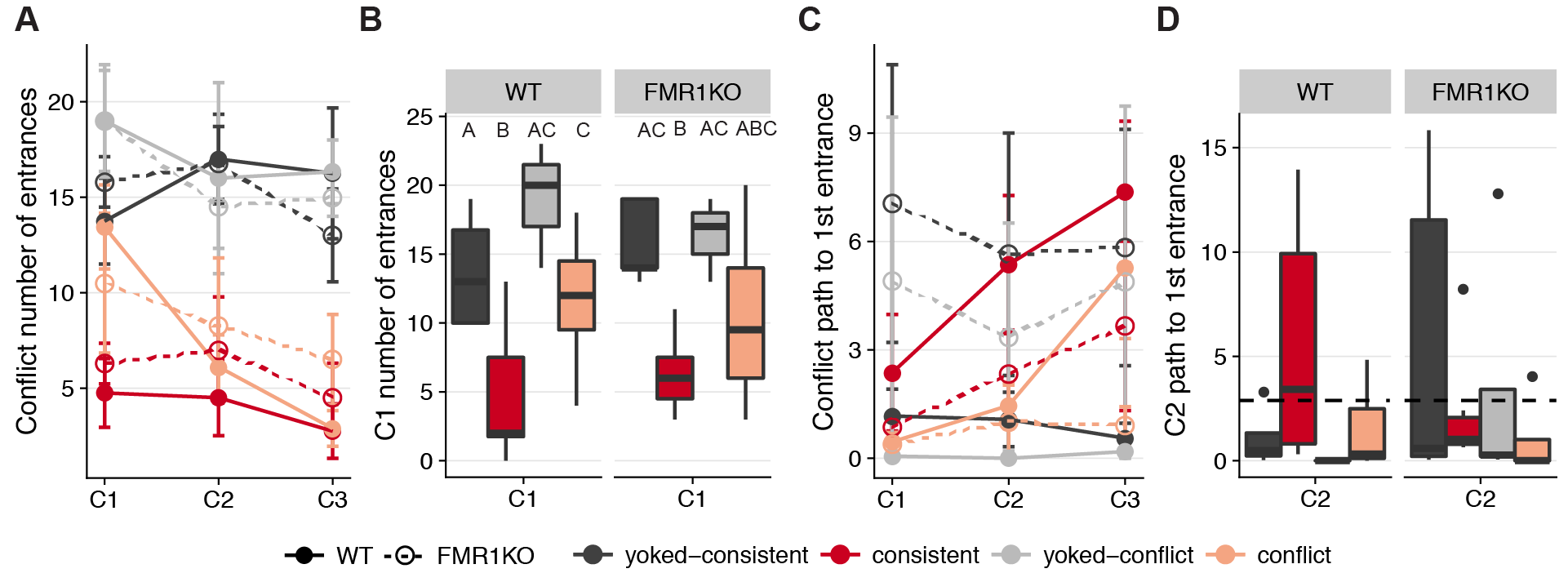


Fig. 2.6. During recall, mice avoid the shock zone using a non-place strategy.

#### 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-consisent (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 there path to the shock zone is not significantly longer (**Fig. 2.6C**). THe results of a two-way Anova followed by TukeyHSD was are visualized on on Figure 2.C.

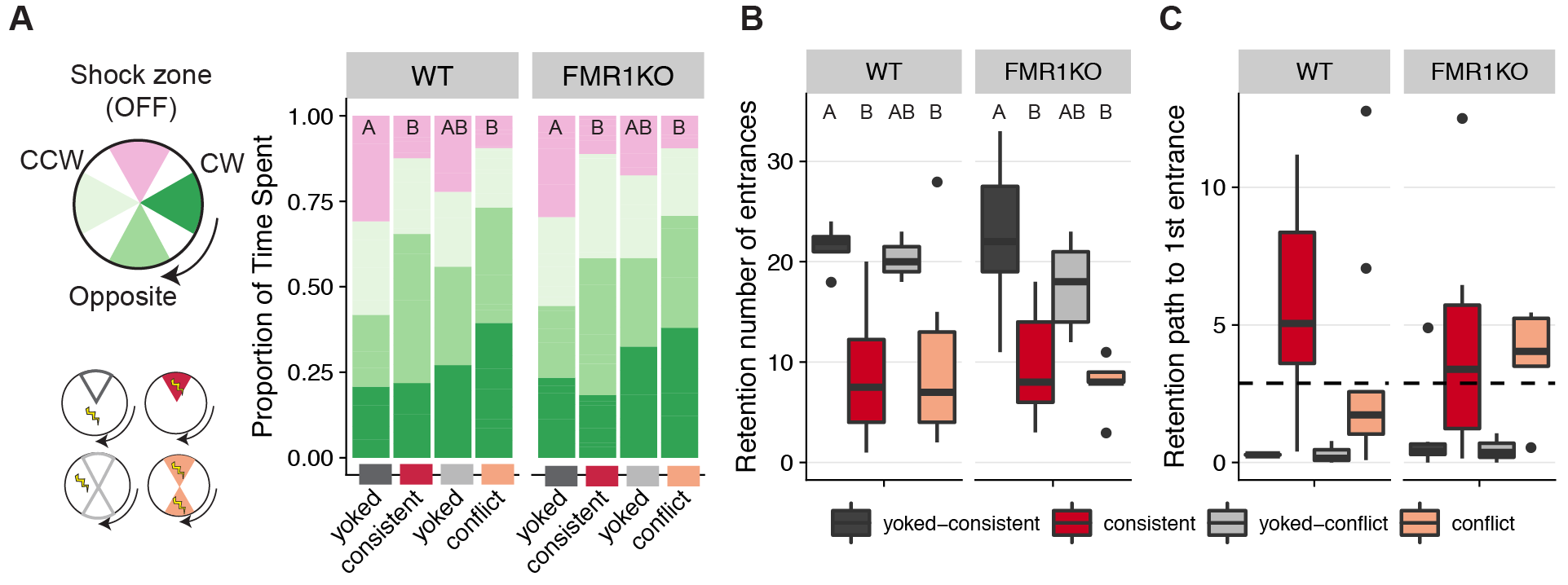


Fig. 2.6. During recall, mice avoid the shock zone.

##### 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 there 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: counter clockwise.

Consisent 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**).

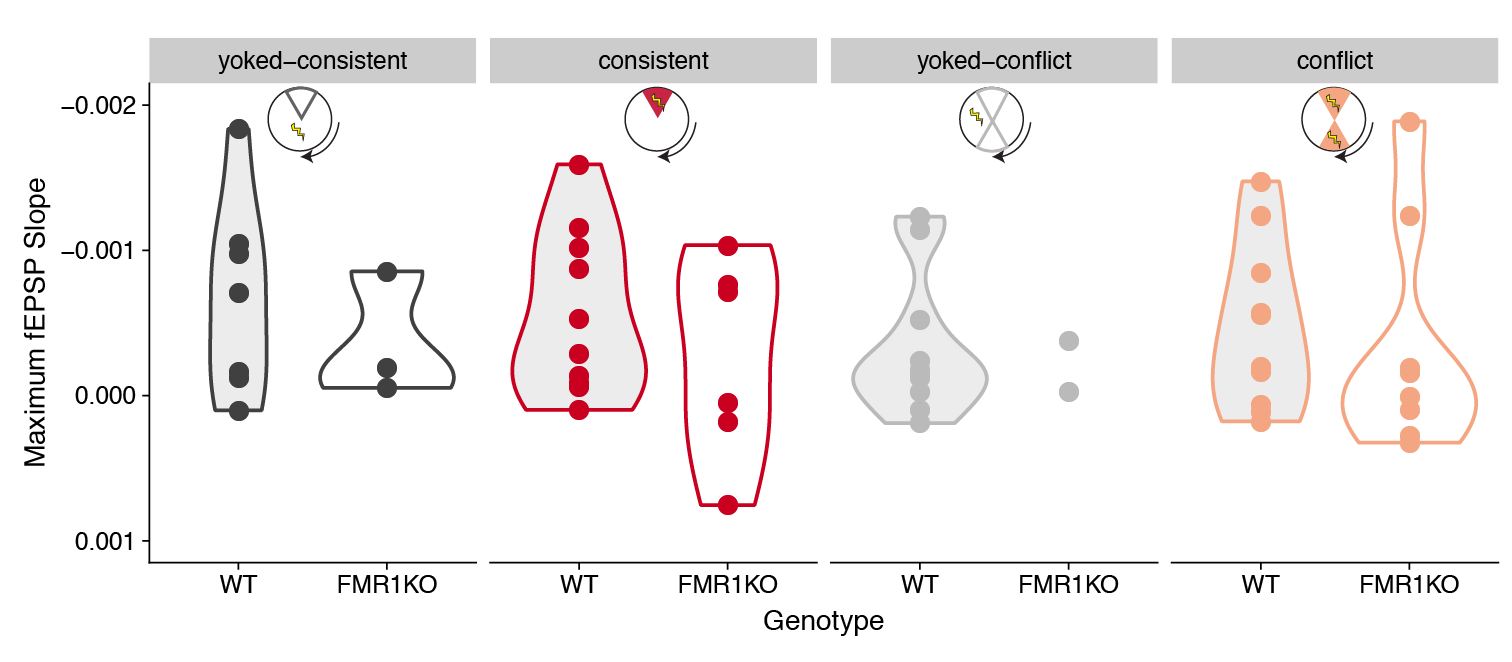


Fig. 2.7. CA3-CA1 synaptic strength is not altered by genotype or place avoidance training.

##### The maximum fEPSP slope is no different between groups indicating that neither training or genotype influence synaptic strenght at CA3-CA1 synapses. WT: filled violin plot, FMR1-KO, open violoin 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 rubust signal of hippocampus dependent place learnig, 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.

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 constitutive elimination of FMRP (Fig 2.3B). 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).

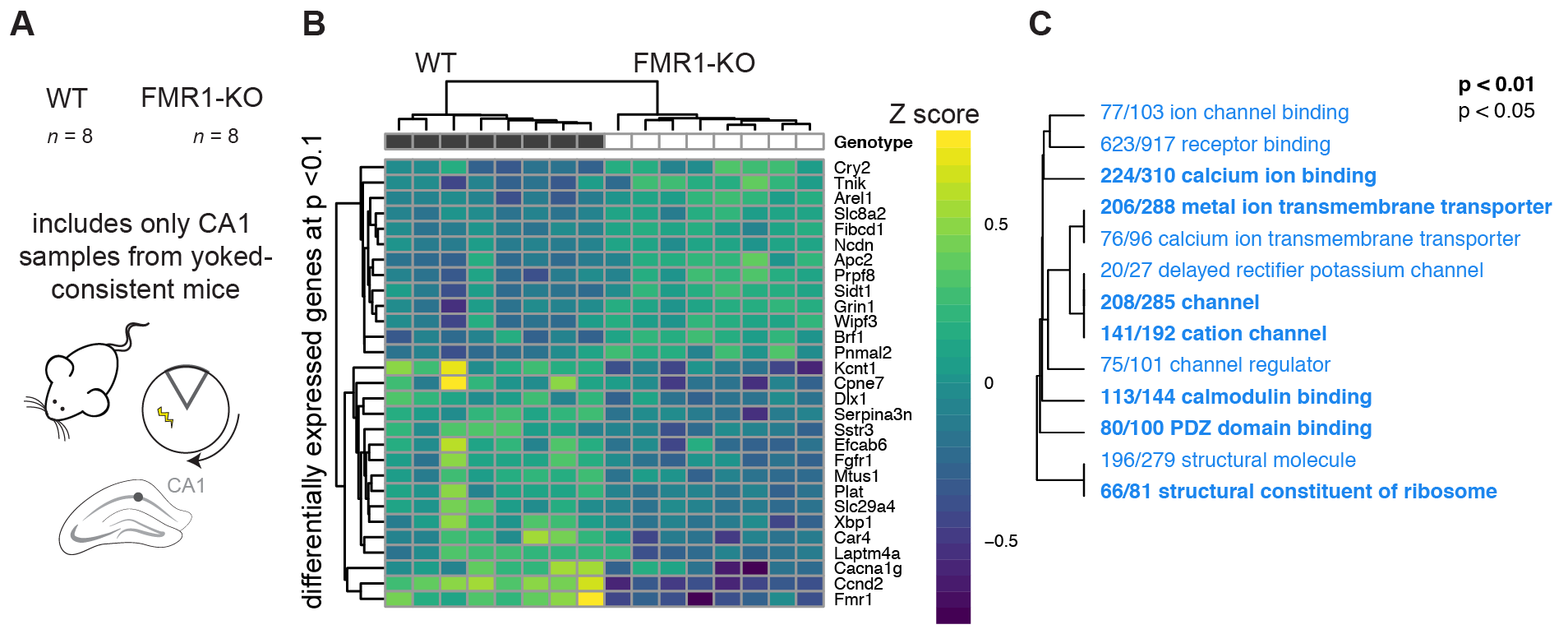


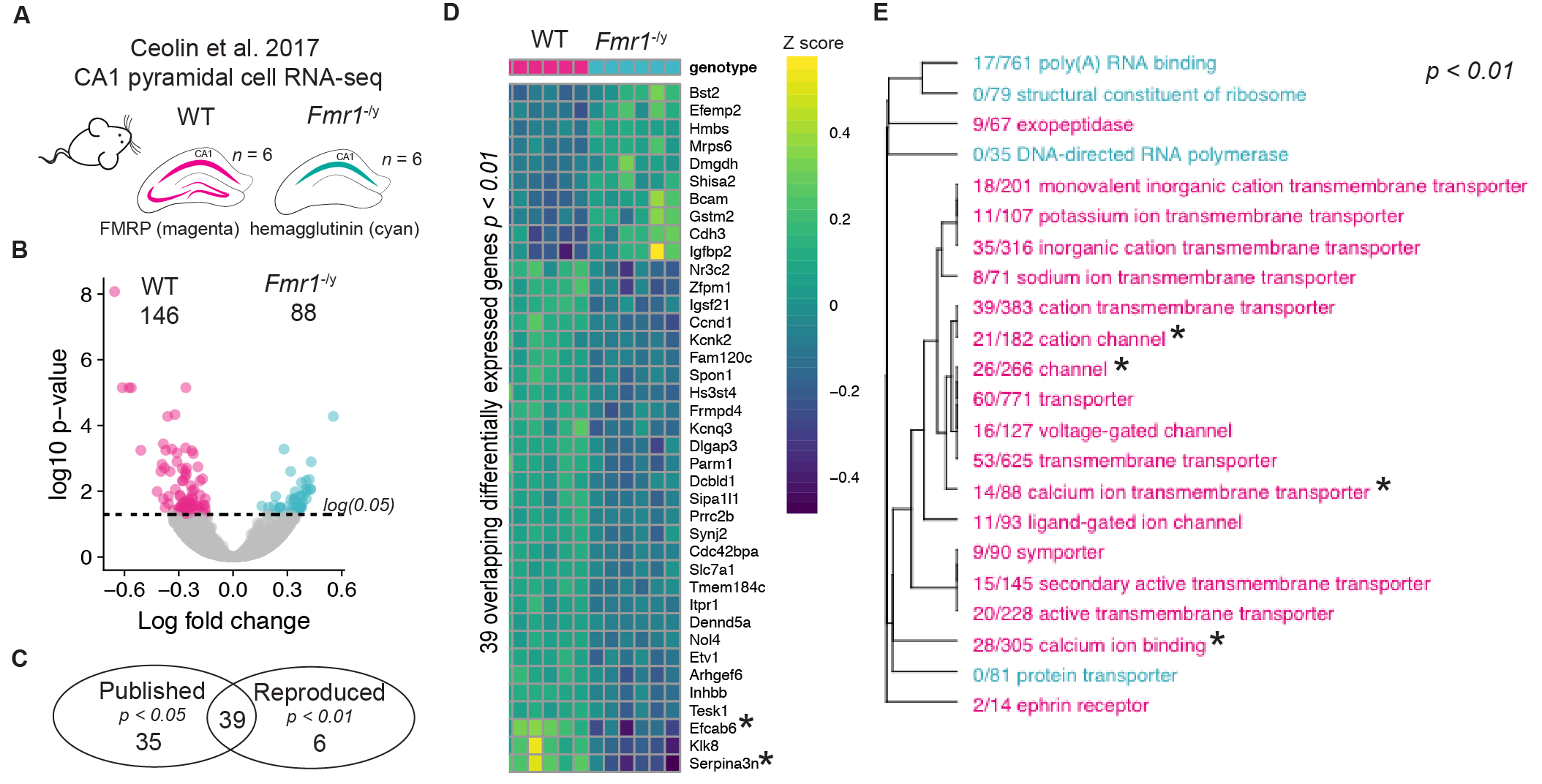
Fig. 2.8. FMR1-KO show downregulation of calcium ion signaling in the the the CA1 subfield.

##### 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) A volcano plot shows that expression Ccnd2 and Fmr1 are highly upregulated in WT compared to FMR1-KO mice. Genes with negative log fold change value are more highly expressed in WT (dark grey filled circles) while genes with a positive log fold change value are more highly expressed in the Fmr1 mice (open circles). Genes that are not significantly expressed are shown in light grey.

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.

CA3-CA1 synaptic strength is not altered by genotype or place avoidance training. I did not observe reduction in synaptic strength at the CA3-CA1 synapse (as measured by maximum fEPSP slope) with avoidance learning or with FMR1-KO. The interaction between Genotype and Training Group was not significant, and Genotype alone was not significant. Violin plots are colored by treatment group and shaded according to genotype.

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



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 showing 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 showing 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.

### Discussion

I found no major groups different prior to experiencing shock when examining all the data together (**Fig 2.2**). Some measusrs appear to be more variabilty, and that may affect our ability to detect effects of small size.

Do the genotypes differ in initial learning of the shock zone? On training sesssions 1-3, differences in path to the 1st entrance between yoked and trained are minimal indicanting that there is no evidence of acquiring place memory. Because entrances to the shock zone decrease in trained compared to yoked, these data suggest the mice are not relying on place memory from before the trial and are adopting a non-place avoidance strategy to avoid being shocked (**Fig 2.3**, \*\* Fig. 2.5\*\*). This lack of initial learning allows me to interpret subsequent learning differences when shock location is rotated as due to the rotation rather than to a preexisting difference in place avoidance. One explanation for the lack of cognitive impairment is a failure to tightly control the physical environment. The mean number of entrances into the shock zone by the yoked-consistent groups (mean entrances at T1= 13.5, see figure Fig 1.2A) is much lower than a previous study (mean entrances at T1= 21.5, see figure Fig 1.2A). This indicated that there might have been a confounding factor in the environment that cause mice to develop a preference for or aversion to one particular place in its environment.

Do the genotypes differ in 24-h recall of the shock zone? Yes. There was an effect of genotype on path to first entrance during the retest, but this appears to be driven by unexplained avoidance behavior driven by a yoked group (**Fig. 2.5**).

Do the genotypes differ in extinction learning? Trained mice also make fewer entrances into the shock zone, but there path to the shock zone is not significantly longer (**Fig. 2.6**).

Are the trained groups different when the shock is rotated compared to when it is consistent? Both consitent and conflict groups avoid the shock zone, spending less than 2% of their time in the shock zone. But where they spend their time avoiding the shock zone is differnt. Consiently trained groups also avoid the clockwise region of the arean, but conflict mice utilize the three quadrants equally (**Fig. 2.4**). There is also a genotype effect, with only WT expressing a place avoidance strategy as evidence by an incrase in path to first entrance of the shock zone (**Fig. 2.5**). This is evidence of a differnt mechansms for cognitive discrimination between genotypes

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. 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**)

q9) Did changing the shock zone make avoidance worse? Both consistent and conflict do worse on T4/C1 compared to the retest. So, it doesn't seem like it was the changing of the shock zone that made avoidance worse.

"Both consistent and conflict do worse on T4/C1 compared to the retest.” is only true for KO suggesting they did not adopt a place strategy but WT consistent is not worse on T4 whereas conflict is worse on C1 compared to the retest. There is a clear disruption in WT compared to consistent but not in KO. Here is the cognitive discrimination evidence in WT but not in KO (you actually have replicated prior work :-)

q10) Do the genotypes differ in cognitive discrimination? Maybe. Both WT and FMR1 KO conflict animals do worse. WT consistent animals do not perform worse but FMR1 consistent animals do worse.

In light of all the data that is not the correct conclusion that KO perform worse on conflict, they perform worse on conflict but no worse than can be expected by chance estimated by the consistent KO mice. As a group KO animals do no worse than their yoked controls (error bars clearly overlap). They did not have a place response.

q11) Do the genotypes differ in cognitive discrimination? save for later.

CONCLUSION: Place learning was not robust, but it was established by the retest trial. While WT show clear cognitive flexibility, KO do not. My ability to detect cognitive flexibilty may not have been properly assessed give the week learning on Day 1.

Animals that use a non-place strategy are, in essence, indifferent to shock zone change and therefore did not get challenged to demonstrate behavioral flexibility. KO animals by preferring to use non-place strategies are unlikely to depend on hippocampus for their adaptive behavior and instead demonstrate adaptive but fixed (inflexible) behavior that can be an effective general purpose solution to their cognitive challenges although it is not optimal.

THIS INTERPRETATION PREDICTS THAT ACROSS THE ENTIRE CONFLICT PROTOCOL THE KO AND WT GROUPS WILL RECEIVE SIMILAR AMOUNTS OF PUNISHMENT. THE KO PUNISHMENT IS CONSTANT aAND INTERMEDIATE ACROSS TRIALS WHEREAS THE WT IS LARGE AT THE START AND LOWER AT THE END.