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Supplementary Information for

Fear circuit-based neurobehavioral signatures mirror resilience to chronic social stress in mouse

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Supplementary Information Text

Methods

Pain Threshold Test

Following chronic social defeat (CSD) and social threat-safety test (STST), a cohort of animals from each of the three *Defeated* subgroups underwent the Pain Threshold Test. The test was performed in Fear Conditioning boxes by TSE, Bad Nauheim, Germany. The arenas were rectangle and had metal grid floors. One by one, animals were introduced into the arenas and an electrical current was gradually increased from zero. Once the animal reacted by raising at least one limb, the current was recorded and the test was terminated. The test was conducted on two consecutive days and the average of both values was calculated for each animal.

CSD

Every day for 10 straight days, experimental mice (*Defeated* n=165) were introduced to the home cage of a larger, older, and retired male breeder from the CD-1 strain (aggressors' strain, pre-existing in the facility). Each day, the aggressor was a different animal. After physical defeat of the experimental mouse, a mesh wall was introduced between the two mice overnight, allowing only sensory contact. During the same period, age-matched experimental mice maintained in the same conditions but randomised to the non-defeated control group (*Control* n=42) were placed for 90s in an empty cage before being returned to their individual cages, separated in half by identical mesh walls used for the *Defeated* group. On the last day of the procedure, all experimental mice were housed individually in new cages and left to rest overnight.

RNA Isolation

Frozen brains were sectioned in a cryostat microtome (Microm HM 560 M, ThermoScientific) at 100µm. Sectioning temperature of the knife was -10°C and of the specimen -11°C. The mPFC (Bregma: 2.58 to 2.22), blA (Bregma: -1.31 to -1.79), and vHC (Bregma: -2.69 to -3.27) regions were punched with a brain punch tissue needle (Leica Biosystems; diameter red 1mm and yellow 0.75mm, respectively) and stored at -80°C until processing. Total RNA was extracted according to the manufacturer's protocol using RNeasy Micro Kit (Qiagen) in combination with TRIzol (Thermo Fisher Scientific). RNA was stored at −80 °C and freshly diluted for application.

Clontech's SMART-Seq v4 Ultra Low Input RNA Kit (112219) was used for cDNA generation from 0,5ng of total RNA, according to the manufacturer's protocol. cDNA was amplified by 16 cycles of LD-PCR. NGS library preparation was performed from 1ng of cDNA with Illumina`s Nextera XT DNA library prep Kit Reference Guide (May 2019, Docu- ment: 15031942v05), and amplified in 12 PCR cycles. Libraries were profiled in a High Sensitivity DNA Chip on a 2100 Bioanalyzer (Agilent Technologies) and quantified using the Qubit dsDNA HS Assay Kit, in a Qubit 2.0 Fluorometer (Life Technologies). The 81 samples (later referred to as trancriptome profiles) were pooled in equimolar ratio and se- quenced on 8 NextSeq 500 High output Flowcells, SR for 1x75 cycles plus 2x8 cycles for the dual index read.

Transcriptome Analysis

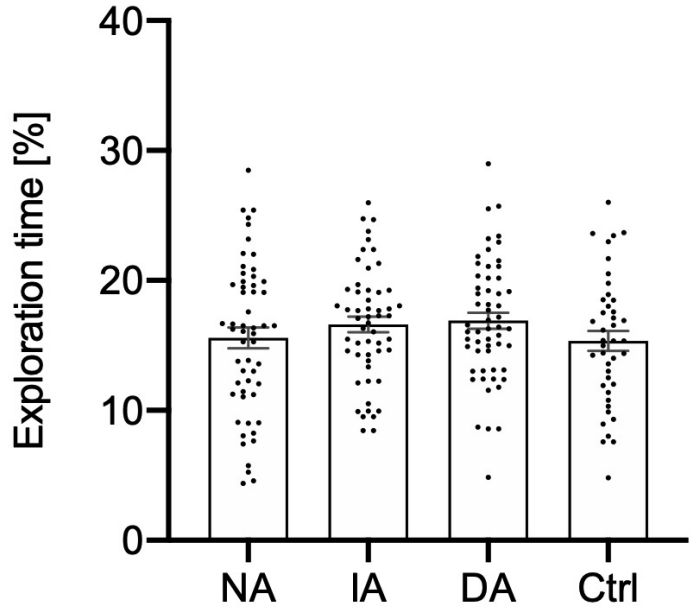
Raw sequencing read files of 81 transcriptome profiles were analysed with FastQC (version 0.11.8), mapped to the mouse genome (version GRCm38.p6) using the Star alignment software (version 2.5.3a; 1) resulting in unique mapping rates >90% with ~ 35 mio mapped reads per sample on average. Transcript quantification was performed using the FeatureCounts software of the Subread package (version 1.5.3; 2) and GENCODE mouse annotation (version M17; 3). Linear and nonlinear dimension reduction for 2-d transcriptome profile representations were performed through PCA and t-SNE implementations in the R analysis environment. Outliers were identified by large distance in sample correlation and clustering plots as well as based on gene expression heatmaps resulting in the removal of 15 transcriptome profiles. Differential gene expression analysis between all groups was conducted using the DESeq2 R package (version 1.20.0; 4). Only transcripts with at least 5 mapping reads in one of the corresponding transcriptome profiles were considered. DESeq2 with default parameters and the integrated log2-fold change shrinkage method 'normal' was used. Significantly differentially expressed genes were identified at p-adjusted≤0.05 and fold change ≥2. DEG intersection plots were generated using the 'UpSet' function of the R-package ComplexHeatmap version 2.10.0 (5). For WGCNA, normalised gene expression matrices for truly expressed genes of each subgroup across all brain regions were independently processed with the WGCNA R-package (6) using a minimum module size of 30 genes. Sample numbers for subgroups were 15 for *Control* and *Discriminating-avoiders*, 17 for *Non-avoiders* and 19 for *Indiscriminate-avoiders*. Enrichment of GO biological processes for

each module was performed via the TopGO package (7) using only truly expressed genes as background and correction for multiple testing using the Benjamini-Hochberg (FDR) method.

Tracking

Tracking of the STST was done using Ethovision software 11.0 by Noldus® (Wageningen, Netherlands). Exploration (during habituation phase of the test) and interaction (during the testing phase of the test) were scored when the nose tip of the experimental mouse was within 2cm of the area surrounding the mesh enclosures. Additionally, a blinded observer corrected for nose-tail switches.

a


Exploration time [%]
40
30
20
10
0
NA
IA
DA
Ctrl

b

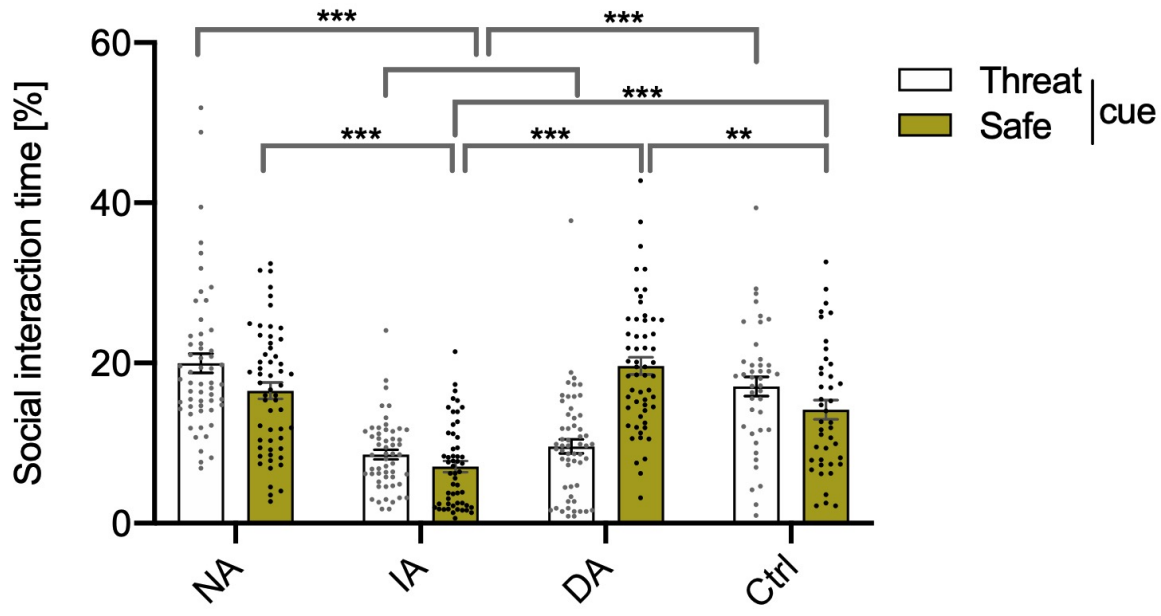

Social interaction time [%]
60
40
20
0
***
***
***
***
***
**
NA
IA
DA
Ctrl
Threat
Safe
cue

Figure S1. Similar exploration- but different social interaction times between the three *Defeated* subgroups

a. Exploration times of the empty mesh enclosures during the habituation phase of the STST was similar between the three *Defeated* subgroups and the *Control* (Ctrl) group. b. Social interaction times during the testing phase of the STST were different depending on the sub/groups and in line with the results obtained calculating the social interaction indices. Note that each animal is represented by two points one with each strain (cue). Results presented as mean±s.e.m, a. One-way ANOVA, Treatment (between subgroups): $F(3, 203)=1.179$, $p=0.3190$. b. Two-way ANOVA, Strain: $F(1, 204)=0.6341$, $p=0.4268$, Subgroup: $F(3, 204)=40.90$, $p<0.0001$***, Interaction: $F(3, 204)=21.56$, $p<0.0001$***, and Bonferroni's multiple comparisons test between the subgroups with each strain (cue), $p<0.001$**, $p<0.0001$***. *Non-avoiders* (NA) n=55, *Indiscriminate-avoiders* (IA) n=54, *Discriminating-avoiders* (DA) n=56, and *Control* (Ctrl) n=42.

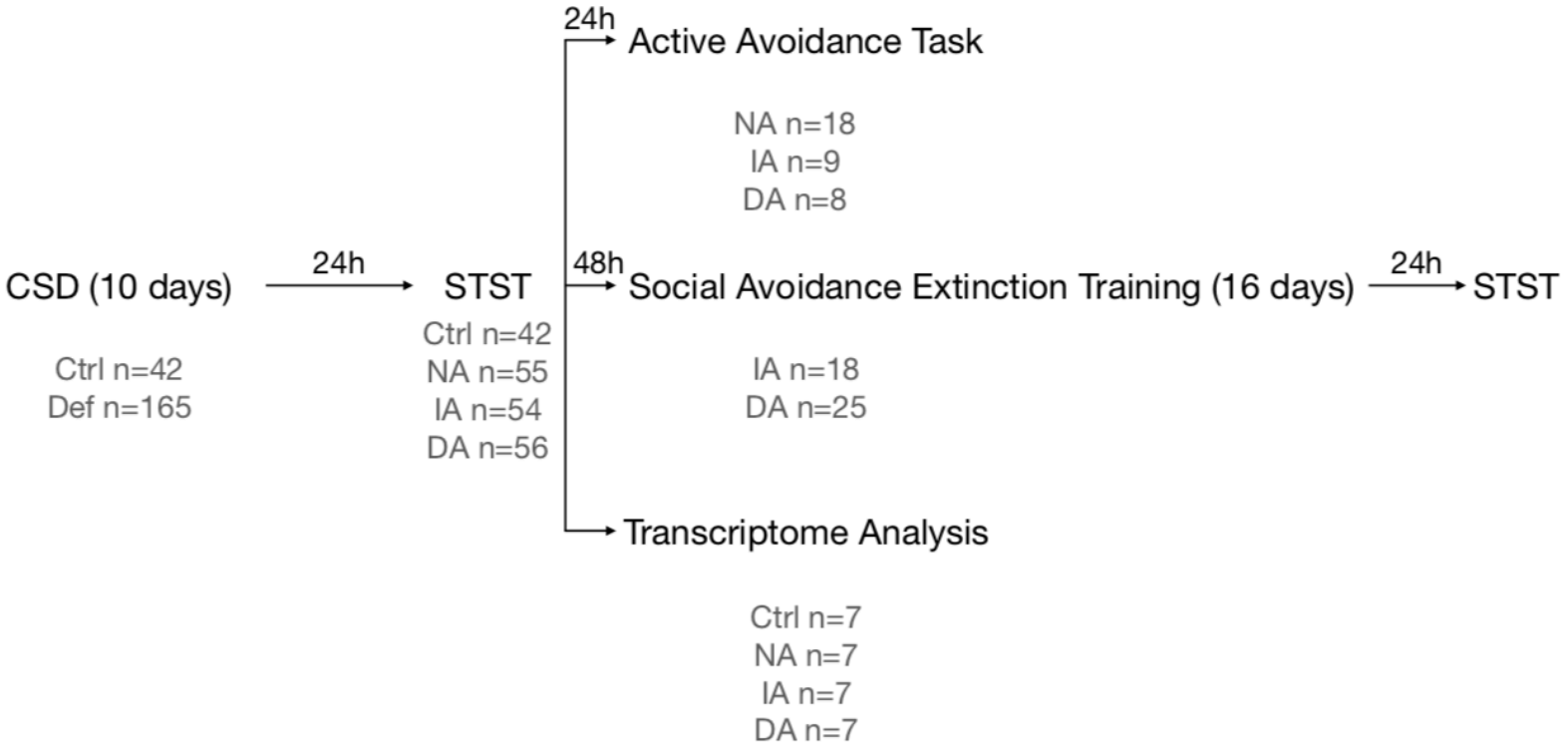
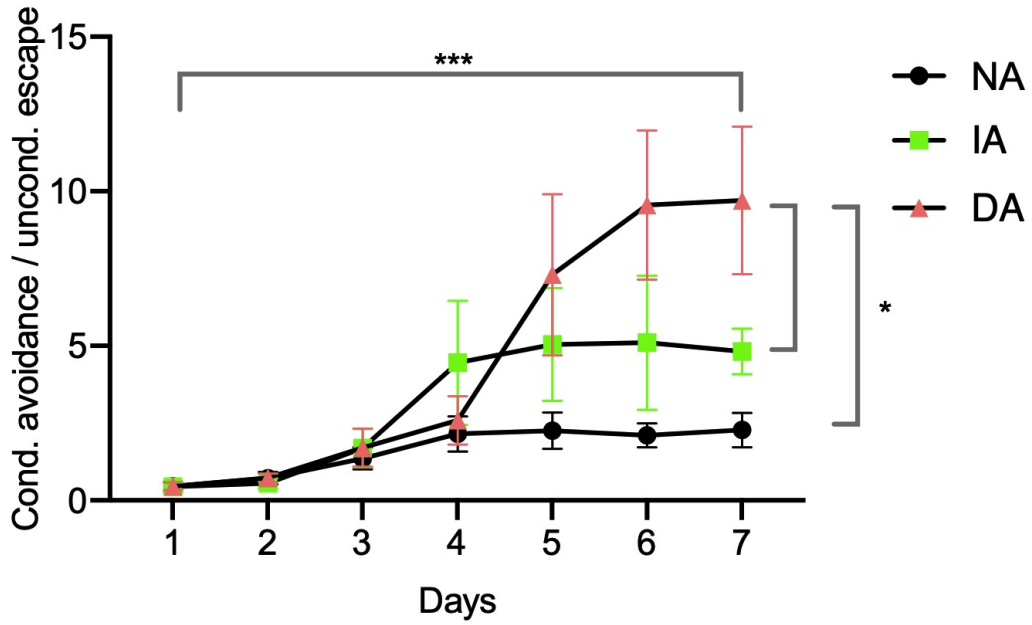

24h
Active Avoidance Task
NA n=18
IA n=9
DA n=8
CSD (10 days)
Ctrl n=42
Def n=165
24h
STST
Ctrl n=42
NA n=55
IA n=54
DA n=56
48h
Social Avoidance Extinction Training (16 days)
IA n=18
DA n=25
24h
STST
Transcriptome Analysis
Ctrl n=7
NA n=7
IA n=7
DA n=7

Figure S2. Schematic timeline
Following the last session (10th) of CSD (*Defeated* group; *Def*) or handing of the non-defeated *Control* group (*Ctrl*), the STST was performed, identifying three phenotypic subgroups among the single *Defeated* group. Following the test, different cohorts of each subgroup underwent either an *Active Avoidance Task*, a *Social Avoidance Extinction Training*, or transcriptome analysis. Following the last session of extinction (16th), the second STST took place.

a


Cond. avoidance / uncond. escape
15
10
5
0
***
*
NA
IA
DA
1
2
3
4
5
6
7
Days

b

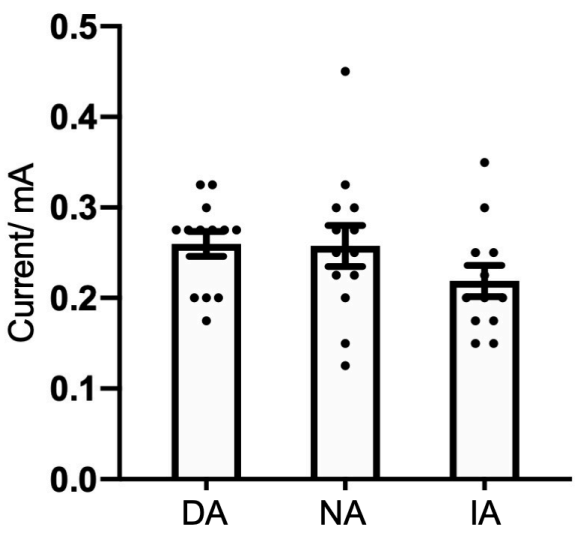
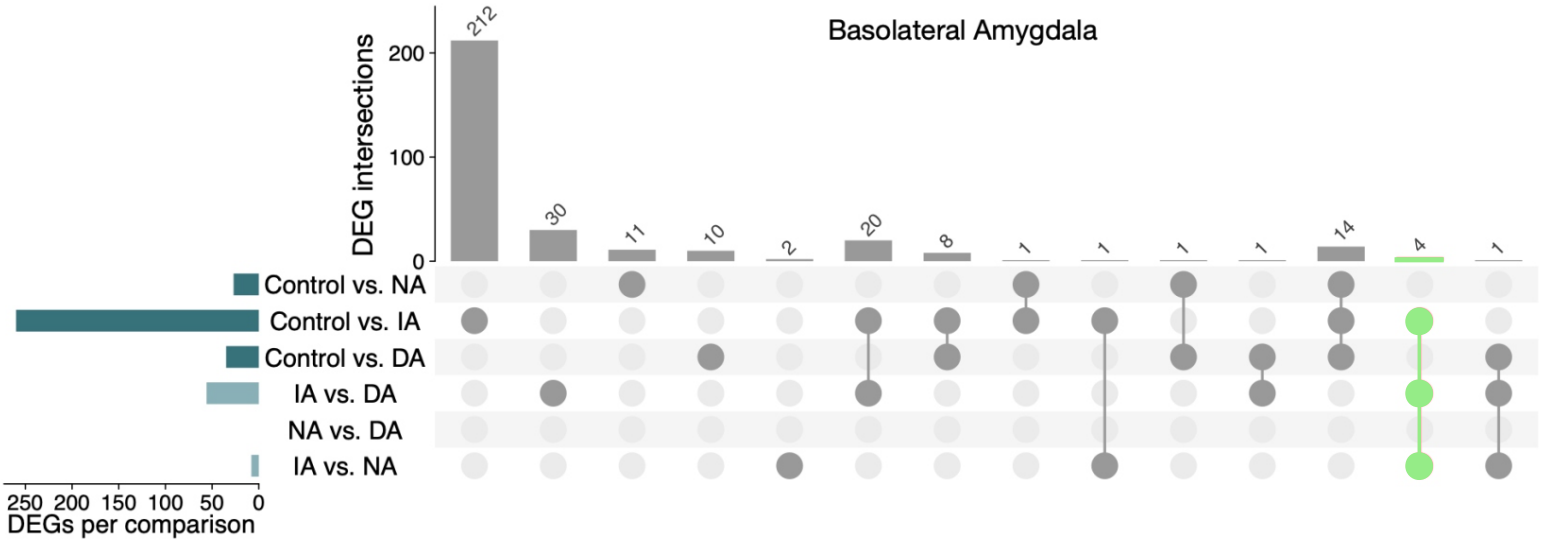
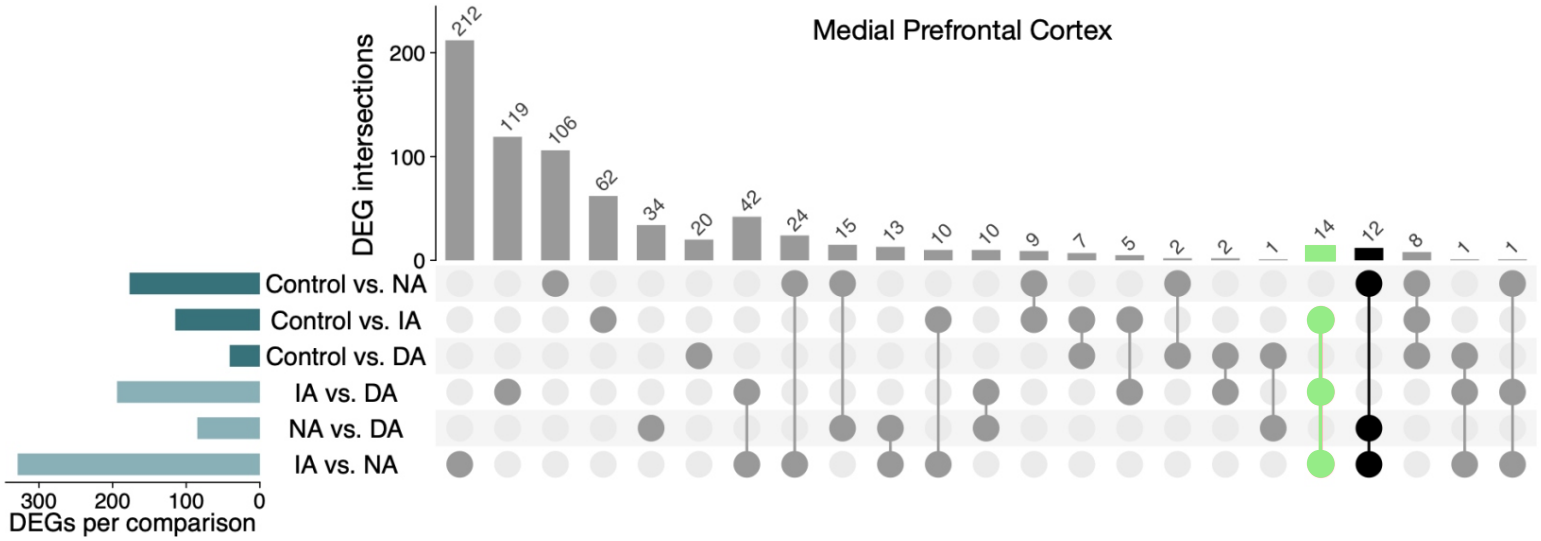

Current/ mA
0.5
0.4
0.3
0.2
0.1
0.0
DA
NA
IA

Figure S3. *Non-avoiders* show impairment in conditioned learning of aversive cues but similar pain threshold between the three *Defeated* subgroups

a. All subgroups had a significant increase in ratio between conditioned avoidance response and unconditioned escape response throughout the training days. However, the *Non-avoiders* subgroup did so to a significantly lesser extent compared to the other two subgroups on the seventh (last) day. b. On average, the pain threshold, measured as the degree of electrical current required for the individual animal to react (raising at least one limb), was similar between the three *Defeated* subgroups. Animals were tested twice and the average value was taken for each animal. Results presented as mean±s.e.m, a. Two-way ANOVA, Subgroup: $F(2, 32)=7.222$, $p=0.0026$**, Time: $F(3.517, 112.5)=18.73$, $p<0.0001$***, Interaction: $F(12, 192)=4.362$, $p<0.0001$***, and Bonferroni's multiple comparisons test on the last day: *Discriminating-avoiders* (DA; n=8) vs. *Indiscriminate-avoiders* (IA; n=9), p=ns, *Discriminating avoiders* or *Indiscriminate-avoiders* vs. *Non-avoiders* (NA; n=18), $p\leq0.05$*. B. One-way ANOVA, Treatment (between subgroups): $F(2, 35)=1.527$, $p=0.2312$, NA n=13, IA n=12, DA n=13.


Basolateral Amygdala
DEG intersections
212
30
11
10
2
20
8
1
1
1
1
14
4
1
Control vs. NA
Control vs. IA
Control vs. DA
IA vs. DA
NA vs. DA
IA vs. NA
250 200 150 100 50 0
DEGs per comparison


Medial Prefrontal Cortex
DEG intersections
212
119
106
62
34
20
42
24
15
13
10
10
9
7
5
2
2
1
14
12
8
1
1
Control vs. NA
Control vs. IA
Control vs. DA
IA vs. DA
NA vs. DA
IA vs. NA
300 200 100 0
DEGs per comparison

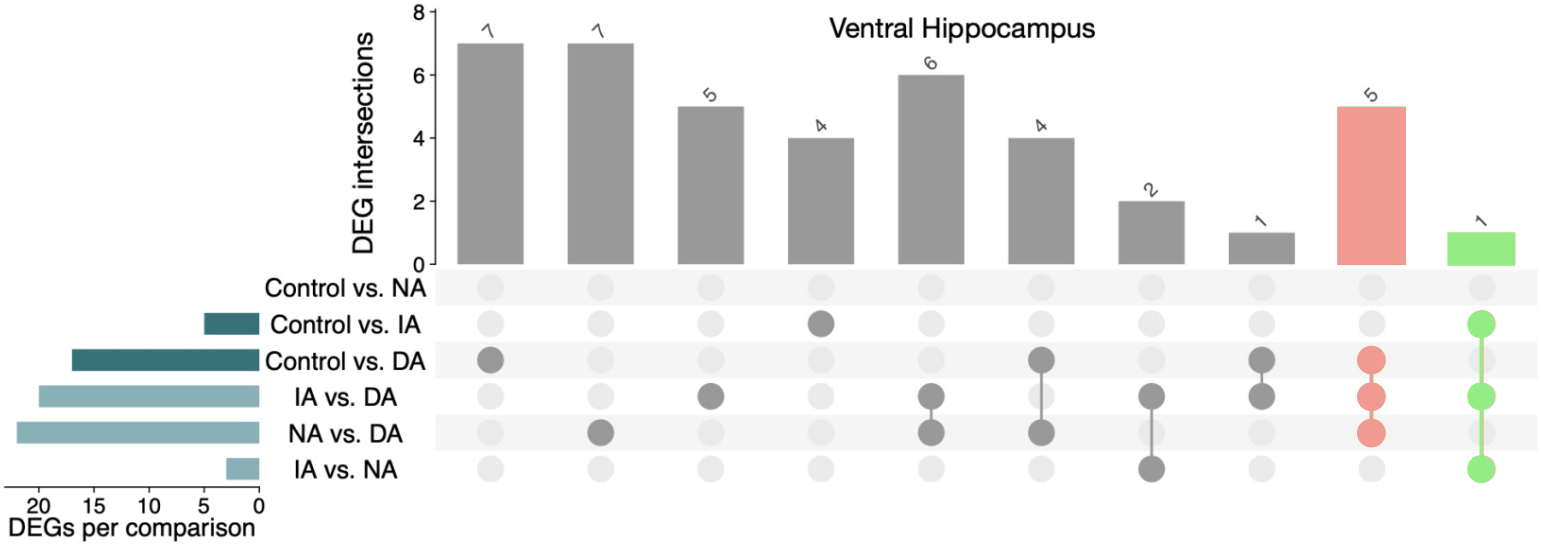
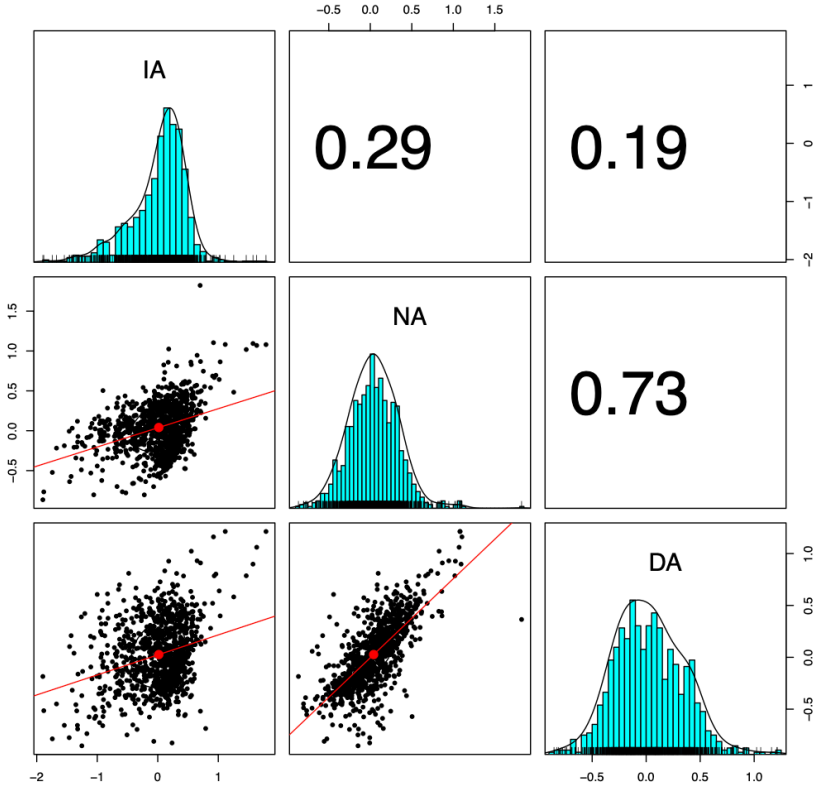
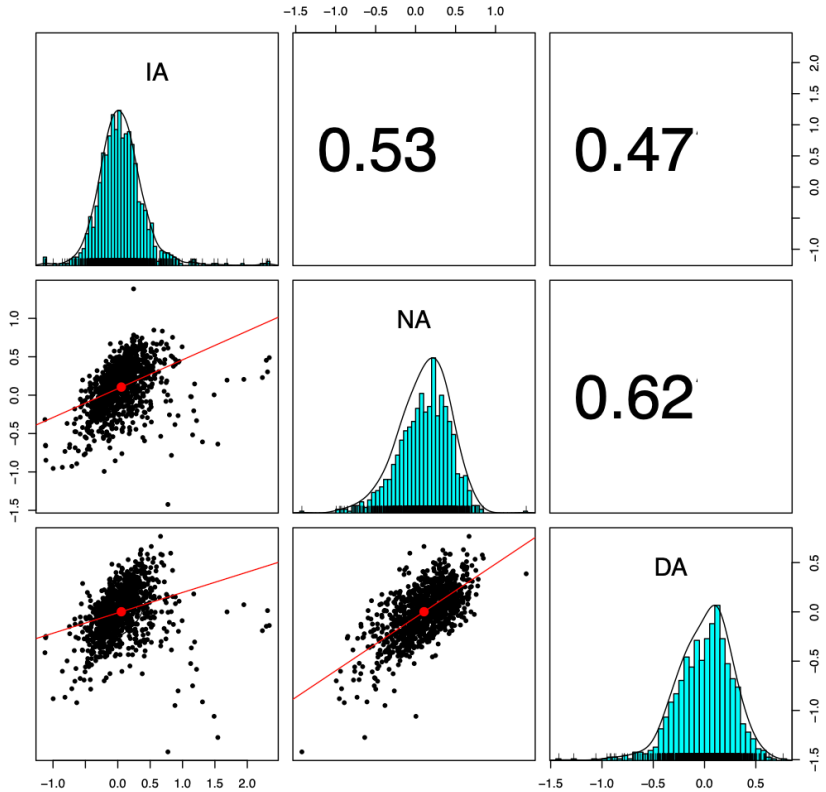

Ventral Hippocampus
DEG intersections
7
7
5
4
6
4
2
1
5
1
Control vs. NA
Control vs. IA
Control vs. DA
IA vs. DA
NA vs. DA
IA vs. NA
20 15 10 5 0
DEGs per comparison

Figure S4: Subgroup and brain region specific DEGs
UpSet plots of the intersections of DEGs across different sub/groups' comparisons. The horizontal bar plots on the left show the total number of DEGs per comparison. The upper bar plots show the number of DEGs in the intersection of specific combinations of comparisons, which are indicated by the black connected circles. Basolateral Amygdala: four DEGs in the *Indiscriminate-avoiders* (IA) subgroup were shared across all comparisons of this subgroup i.e. with the two other *Defeated* subgroups and the *Control* group, rendering them specific to the IA subgroup. Medial Prefrontal Cortex: 14 DEGs were specific to the IA subgroup. Meanwhile, 12 DEGs were specific to the *Non-avoiders* (NA) subgroup. Ventral Hippocampus: one DEG was specific to the IA subgroup while five DEGs were specific to the *Discriminating-avoiders* (DA) subgroup. Criteria for significance: ≥2-fold change compared to the respective anatomical sub/group at p-adjusted ≤0.05*, n=4-7 per sub/group.

Basolateral Amygdala



Medial Prefrontal Cortex



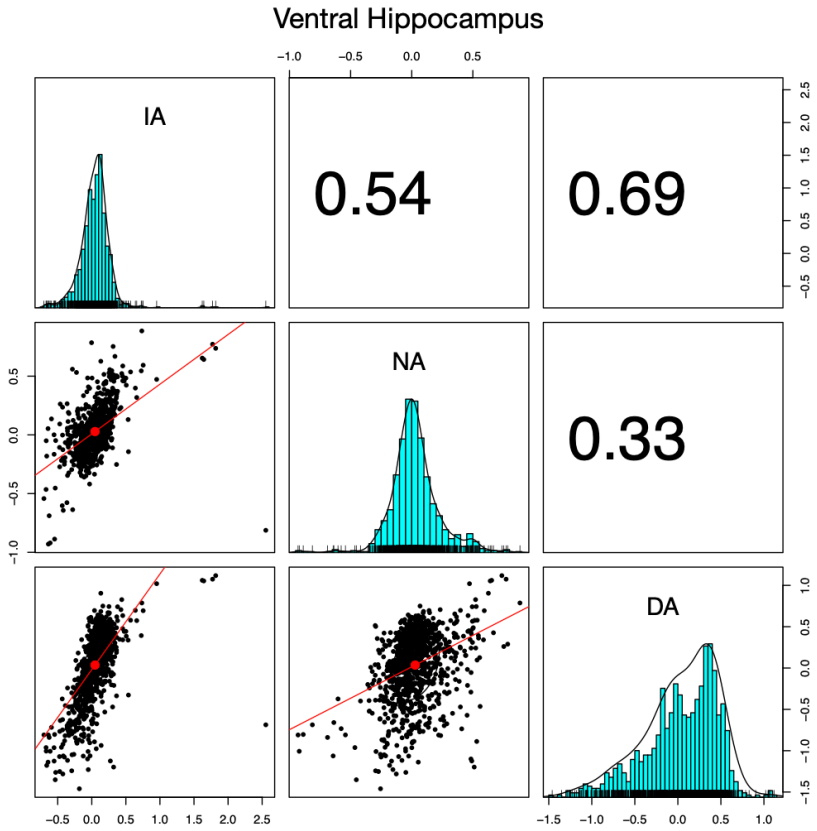

Ventral Hippocampus
IA
0.54
0.69
NA
0.33
DA

Figure S5 Correlation analysis on effect sizes for *Control* vs. *Defeated* subgroup comparisons: Diagonal matrix elements show histogram plots of log2-fold-change values for *Control* vs. *Defeated* subgroup comparisons for 1000 most variable genes across all samples. Lower triangle elements show spearman correlation plots for log2-fold-changes of most variable genes for two subgroups (subgroup in column for x-axis, row for y-axis). Upper triangle entries represent spearman correlation coefficient and significance level (***=$p<0.001$) for the corresponding correlation.

Table S1. *Non-avoiders* subgroup show impairment in conditioned learning of aversive cues

	NA	IA	DA
Theoretical mean (chance level)	50.00	50.00	50.00
Actual mean±s.e.m	53.61±6.578	80±3.005	81.88±8.288
Number of values	18	9	8

The *Non-avoiders* (NA) subgroup was the only one not to reach a conditioned avoidance response% above chance level (50%) on the last day, whereas the other two subgroups were significantly above chance level suggesting that this is the only subgroup that failed to learn the task. Results presented as mean±s.e.m. One sample t-test, *Non-avoiders* (n=18): t=0.5490, df=17, p=0.5902, *Indiscriminate-avoiders* (IA; n=9): t=9.985, df=8, p<0.0001***, *Discriminating-avoiders* (DA; n=8): t=3.846, df=7, p=0.0063**.

Dataset S1. Differential gene expression analysis reveals subgroup and brain region-specific genes
The output of the differential gene expression analysis. Titles of the sheets indicate comparisons where group A is the second sub/group in the title. *Non-avoiders*, *Indiscriminate-avoiders*, and *Discriminating-avoiders*, n=4-7 per subgroup.

Dataset S2. Enrichment analysis of the WGCNA-identified co-expression modules identifies significant subgroup-specific enriched terms
Functional enrichment analysis reveals 32 specific (exclusive) terms of the *Control* group, 28 of the *Non-avoiders* subgroup, 48 of the *Discriminating-avoiders* subgroup, and 66 of the *Indiscriminate-avoiders* subgroups. *Control* group-specific terms can be seen as "lost" functionality in the respective *Defeated* subgroup and *Defeated* subgroup-specific terms as “gained”.

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