BIOL 5312 final project report part I

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The data used for part 1 of this report come from a behavioral study of stress-induced relapse to opiates conducted by the Kirby lab in the Center for Substance Abuse Research on Temple's Health Sciences Campus. The serotonin (5-hydroxytryptamine, 5-HT) system is implicated in substance abuse as well as stress-related psychiatric disorders. Previous studies have demonstrated that stressors stimulate the release of corticotropin-releasing factor (CRF), which inhibits serotonin activity in the dorsal raphe nucleus (DRN), the main serotonin hub in the central nervous system located in the brain stem (1). Drug addiction is characterized by repeated relapse, of which stress is a major trigger (2). Stress in animal models of addiction (e.g. foot shock or forced swim) can reinstate drug-seeking behavior in self-administration and conditioned place preference (CPP) paradigms (3,4).

The CPP paradigm uses associative learning to pair one side of a bipartite chamber with drug-induced euphoria, while the other is yoked to (non)effects of injection of vehicle (saline). Preference for the drug-paired side following the training period can be measured by tracking the animal over 15 minutes, and subtracting the time spent in the drug-paired side from that spent in the unpaired side. This difference indicates drug-seeking behavior in the model, because before the training, the animals demonstrated no preference for either side (the pre-conditioning phase). A difference of 100 seconds is set as the threshold to indicate CPP, and those animals that demonstrate a preference then undergo an extinction phase, in which they receive saline injections in either side of the chamber. No longer experiencing a morphine high in the drug-paired side, most subjects discontinue spending more time there when tested. A difference of less than 100 seconds between time spent in the drug-paired side and time spent in the unpaired side indicates successful extinction. Reinstatement occurs when a preference (>100sec difference) is observed in animals that have previously extinguished their morphine CPP. It can be induced with stressors such as forced swim stress.

The preceding study demonstrated that GABAergic DRN inhibition induces reinstatement in morphine CPP, whereas DRN disinhibition attenuates swim-stress induced reinstatement (5). The current study investigated the effect of CRF activity in the DRN with two hypotheses: that intra-DRN CRF administration would induce reinstatement in animals with extinguished morphine CPP (H1), and that intra-DRN CRF receptor subtype 1 (R1) antagonism would attenuate swim-stress induced reinstatement of extinguished morphine CPP (H2). Animals received stereotaxic surgery implanting a cannula into the DRN, which allowed infusion of either ovine CRF (oCRF) 15 minutes prior to the reinstatement test for H1, or the CRF-R1 antagonist NBI 35965 15 minutes prior to the forced swim stress for H2 (the animals are then dried and undergo a reinstatement test 20 minutes later). In the forced swim stress procedure, the animal is placed in a tank of cold water where it cannot stand and is therefore forced to swim. Rats are perfectly capable of this and not at all prone to drown, but it is stressful for them.

Animals that failed to condition or extinguish could not even be tested for reinstatement, so they were excluded from data analysis. Also excluded were those subjects whose cannulation surgery missed the target site (DRN), as verified by dye infusion and histology at the end of the behavioral tests. The data from this 2021 CPP study contain a total of 40 animals that met behavioral and histological criteria. The counts for each treatment group are tabulated below. The columns CT, ET and RT correspond to the conditioning, extinction, and reinstatement scores. The R code for all statistical analyses is below, containing some figures, followed by discussion.

Set up session

```
library(tidyverse)
library(car)
library(ggplot2)
library(ggpubr)
library(rstatix)
```

Read in tabular data

```
cpp<-read.csv("2021CPPstudy.csv")
dim(cpp)
```

[1] 43 8

```
headcpp<-head(cpp)
```

(View() could be used, but clicking in the upper right window of Rstudio was more convenient for viewing tables)

	ID	Drug	Treatment	PRE	CT	ET	RT	Injection
1	071916-1	Morphine	oCRF	n/a	200	-206	502	hit
2	071916-2	Morphine	oCRF	-40	472	-102	384	hit
3	071916-5	Morphine	oCRF	-42	180	-260	292	hit
4	071916-8	Morphine	oCRF	-366	584	370	584	hit
5	071916-9	Morphine	oCRF	160	528	126	-100	hit
6	071916-10	Morphine	oCRF	232	406	-90	608	hit

Filter based on inclusion criteria discussed above and select relevant columns

```
cpp<-filter(cpp,CT>=100,ET<=100,startsWith(Injection,"hit"))[,c(1,3,5:7)]
dim(cpp)
```

[1] 40 5

Number treatments for ordering in graphs

```
cpp$Treatment[cpp$Treatment=="oCRF"]<-"1. oCRF"
cpp$Treatment[cpp$Treatment=="vehicle"]<-"2. vehicle"
cpp$Treatment[cpp$Treatment=="NBI+swim"]<-"3. NBI+swim"
cpp$Treatment[cpp$Treatment=="vehicle+swim"]<-"4. vehicle+swim"
```

Reformat ID

```
for (i in c(1:nrow(cpp))){
  cpp$ID[i]<-toString(cpp$ID[i])
}
cpp$ID[37]<-"9.10"
```

Convert to long-format dataframe

```
cpp<-cpp %>%
gather(key="Phase",value="Score",CT,ET,RT) %>%
convert_as_factor(Treatment,Phase)
write.csv(cpp,"lfcpp.csv")
```

Sample rows

```
> cpp[sample(c(1:nrow(cpp)),6),]
```

	ID	Treatment	Phase	Score
98	7.5	2. vehicle	RT	220
47	3.4	1. oCRF	ET	-172
30	9.1	3. NBI+swim	CT	288
84	071916-10	1. oCRF	RT	608
54	4.9	2. vehicle	ET	-100
12	4.1	2. vehicle	CT	358

Check for outliers

(cpp %>% group_by(Phase,Treatment) %>% identify_outliers(Score) -> ols)

	Treatment	Phase	ID	Score	is.outlier	is.extreme
1	3. NBI+swim	ET	9.9	-308	TRUE	FALSE
2	3. NBI+swim	RT	9.11	-792	TRUE	FALSE

The outliers are not classified as extreme, and it is not suspected that they resulted from measurement or sampling error, so they will not be excluded from analysis.

Counts per treatment group

cnt<-count(group_by(cpp,Treatment))

cnt$n<-cnt$n/3cnt

	Treatment	n
1	1. oCRF	11
2	2. vehicle	11
3	3. NBI+swim	9
4	4. vehicle+swim	9

Summary statistics

cpp %>% group_by(Phase,Treatment) %>% get_summary_stats(Score) -> sumstats

	Treatment	Phase	variable	n	min	max	median	q1	q3	iqr	mad	mean	sd	se	ci
1	1. oCRF	CT	Score	11	180	488	352	242	419	177	124.538	336.545	109.545	33.029	73.593
2	2. vehicle	CT	Score	11	128	598	284	204	366	162	130.469	305.273	139.152	41.956	93.484
3	3. NBI+swim	CT	Score	9	140	392	274	180	358	178	139.364	267.333	98.351	32.784	75.600
4	4. vehicle+swim	CT	Score	9	236	352	292	256	296	40	11.861	284.667	35.270	11.757	27.111
5	1. oCRF	ET	Score	11	-324	42	-90	-189	-49	140	121.573	-116.545	113.293	34.159	76.111
6	2. vehicle	ET	Score	11	-186	60	-84	-110	-10	100	53.374	-67.818	85.564	25.798	57.483
7	3. NBI+swim	ET	Score	9	-308	64	-20	-96	40	136	106.747	-54.667	117.490	39.163	90.311
8	4. vehicle+swim	ET	Score	9	-272	92	-94	-178	-22	156	124.538	-98.000	125.312	41.771	96.323
9	1. oCRF	RT	Score	11	-36	608	288	160	430	270	260.938	276.182	208.945	62.999	140.371
10	2. vehicle	RT	Score	11	-218	344	56	-108	99	207	222.390	21.455	173.115	52.196	116.300
11	3. NBI+swim	RT	Score	9	-792	452	-112	-196	96	292	308.381	-137.778	374.514	124.838	287.877
12	4. vehicle+swim	RT	Score	9	-236	894	624	152	876	724	400.302	463.333	416.166	138.722	319.893

Visualize mean scores for each treatment group and phase

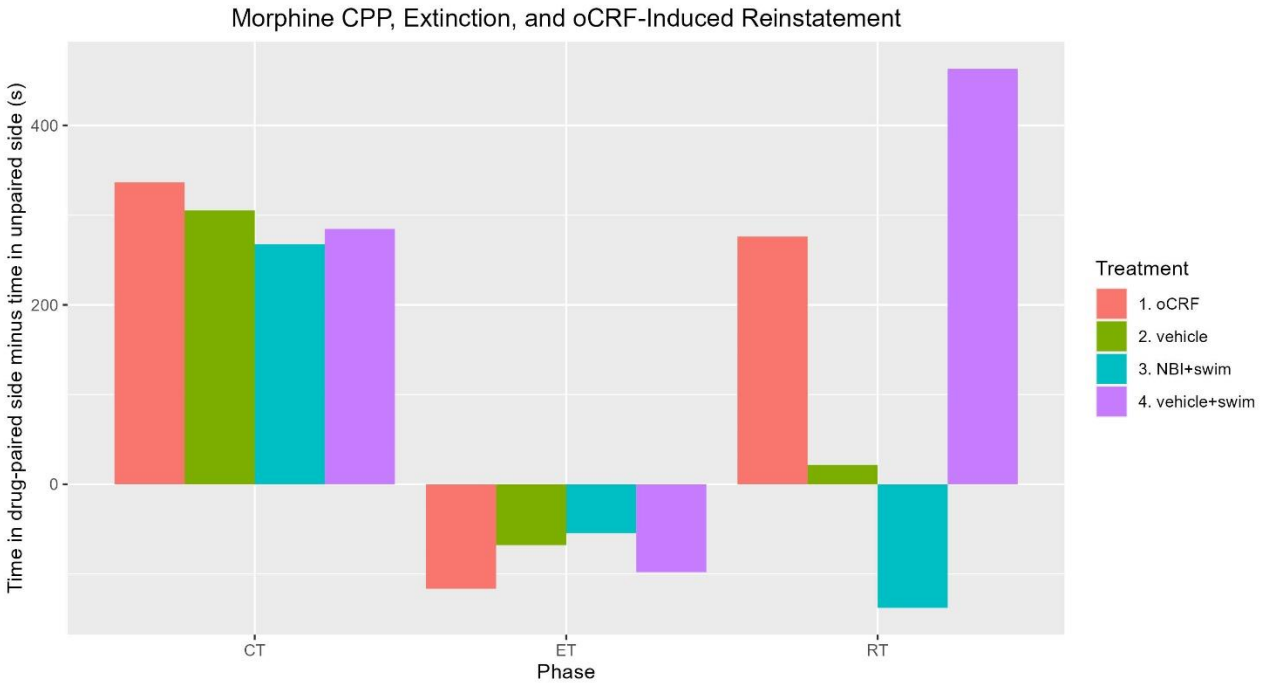
gcpp<-cpp %>% group_by(Treatment,Phase) %>% summarise(mean(Score))

gcpp<-rbind(gcpp[4:9,],gcpp[c(1:3,10:12),])

ggplot(gcpp,aes(x=Phase,y=gcpp$`mean(Score)`,fill=Treatment))+geom_bar(stat="identity",position="dodge")+ggtitle("Morphine CPP, Extinction, and oCRF-Induced Reinstatement")+theme(plot.title = element_text(hjust=0.5))+ylab("Time in drug-paired side minus time in unpaired side (s)")

ggsave("mean_bar.jpeg")

Graph 1: comparison of mean scores across treatment group and phase



Test for normality

cpp %>% group_by(Phase,Treatment) %>% shapiro_test(Score) -> testshapiro

	Treatment	Phase	variable	statistic	p
1	1. oCRF	CT	Score	0.9391870	0.5109876
2	2. vehicle	CT	Score	0.9375497	0.4921348
3	3. NBI+swim	CT	Score	0.9125711	0.3342867
4	4. vehicle+swim	CT	Score	0.9057460	0.2872255
5	1. oCRF	ET	Score	0.9605583	0.7786374
6	2. vehicle	ET	Score	0.9058068	0.2173582
7	3. NBI+swim	ET	Score	0.8801125	0.1574488
8	4. vehicle+swim	ET	Score	0.9489714	0.6787708
9	1. oCRF	RT	Score	0.9618742	0.7948392
10	2. vehicle	RT	Score	0.9639859	0.8202198
11	3. NBI+swim	RT	Score	0.9486062	0.6747649
12	4. vehicle+swim	RT	Score	0.8885345	0.1927117

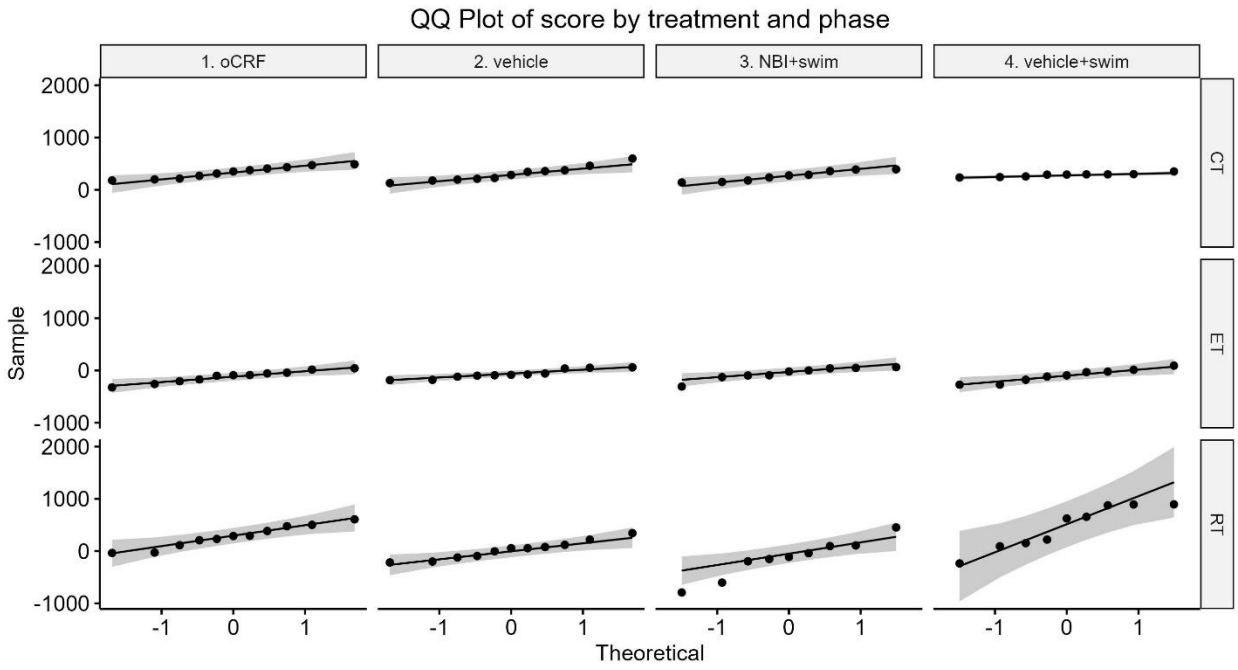
min(testshapiro$p)

[1] 0.1574488 > 0.01 so the data appear to be normally distributed

QQ plot

ggqqplot(cpp,"Score",)+facet_grid(Phase~Treatment)+ggtitle("QQ Plot of score by treatment and phase")+theme(plot.title = element_text(hjust=0.5))

ggsave("qq_plot.jpeg")



Test for homoscedasticity

cpp %>% group_by(Phase) %>% levene_test(Score~Treatment) -> testlevene

	Phase	df1	df2	statistic	p
1	CT	3	36	3.6168848	0.02217542
2	ET	3	36	0.4431528	0.72359157
3	RT	3	36	2.4658329	0.07790636

cpp %>% group_by(Treatment) %>% levene_test(Score~Phase) -> testlevene2

	Treatment	df1	df2	statistic	p
1	1. oCRF	2	30	2.472012	0.1014436870
2	2. vehicle	2	30	1.846199	0.1753234298
3	3. NBI+swim	2	24	3.960488	0.0326302933
4	4. vehicle+swim	2	24	10.029399	0.0006825635

Test for homogeneity of covariance

box_m(cpp[,"Score",drop=F],cpp$Treatment)

```
# A tibble: 1 x 4
  statistic p.value parameter method
      <dbl>   <dbl>     <dbl> <chr>
1      7.15  0.0672         3 Box's M-test for Homogeneity of Covariance Matrices
```

Two-way mixed ANOVA test for CRF experiment

(raovcrf<-anova_test(data=cpp[cpp$Treatment=='1. oCRF'|cpp$Treatment=='2. vehicle',],dv=Score,wid=ID,between=Treatment,within=Phase))

ANOVA Table (type II tests)

$ANOVA
```
            Effect DFn DFd     F       p p<.05   ges
1        Treatment   1  20 3.626 7.10e-02       0.076
2            Phase   2  40 55.538 2.87e-12     * 0.602
3 Treatment:Phase   2  40  7.977 1.00e-03     * 0.179
```

$`Mauchly's Test for Sphericity`
```
            Effect     W       p p<.05
1            Phase 0.482 0.000973     *
2 Treatment:Phase 0.482 0.000973     *
```

$`Sphericity Corrections`
```
            Effect   GGe      DF[GG]    p[GG] p[GG]<.05   HFe      DF[HF]    p[HF] p[HF]<.05
1            Phase 0.659 1.32, 26.35 8.25e-09        * 0.687 1.37, 27.48 4.26e-09        *
2 Treatment:Phase 0.659 1.32, 26.35 5.00e-03        * 0.687 1.37, 27.48 5.00e-03        *
```

raovcrft<-get_anova_table(raov,correction=c("GG"))

	Effect	DFn	DFd	F	p	p<.05	ges
1	Treatment	1.00	20.00	3.626	7.10e-02		0.076
2	Phase	1.32	26.35	55.538	8.25e-09	*	0.602
3	Treatment:Phase	1.32	26.35	7.977	5.00e-03	*	0.179

Assumption of sphericity not met, so Greenhouse-Geisser correction applied.

Two-way mixed ANOVA test for NBI experiment

(raovnbi<-anova_test(data=cpp[cpp$Treatment=='3. NBI+swim'|cpp$Treatment=='4. vehicle+swim',],dv=Score,wid=ID,between=Treatment,within=Phase))

ANOVA Table (type II tests)

$ANOVA
```
            Effect DFn DFd     F       p p<.05   ges
1        Treatment   1  16 8.894 0.009000     * 0.149
2            Phase   2  32 9.614 0.000537     * 0.292
3 Treatment:Phase   2  32 9.407 0.000612     * 0.287
```

$`Mauchly's Test for Sphericity`
```
            Effect     W       p p<.05
```

```
1         Phase 0.106 5.06e-08     *
2 Treatment:Phase 0.106 5.06e-08     *

$`Sphericity Corrections`
        Effect   GGe     DF[GG] p[GG] p[GG]<.05   HFe      DF[HF] p[HF] p[HF]<.05
1         Phase 0.528 1.06, 16.9 0.006        * 0.534 1.07, 17.08 0.006        *
2 Treatment:Phase 0.528 1.06, 16.9 0.006        * 0.534 1.07, 17.08 0.006        *
```

```
raovnbi<-get_anova_table(raovnbi,correction=c("GG"))
```

	Effect	DFn	DFd	F	p	p<.05	ges
1	Treatment	1.00	16.0	8.894	0.009	*	0.149
2	Phase	1.06	16.9	9.614	0.006	*	0.292
3	Treatment:Phase	1.06	16.9	9.407	0.006	*	0.287

Post-hoc tests

Pairwise comparison between phases

```
pwcp<-cpp %>% group_by(Treatment) %>% pairwise_t_test(Score~Phase)
```

Pairwise comparison between treatments

```
pwct<-cpp %>% group_by(Phase) %>% pairwise_t_test(Score~Treatment)
```

FDR correction

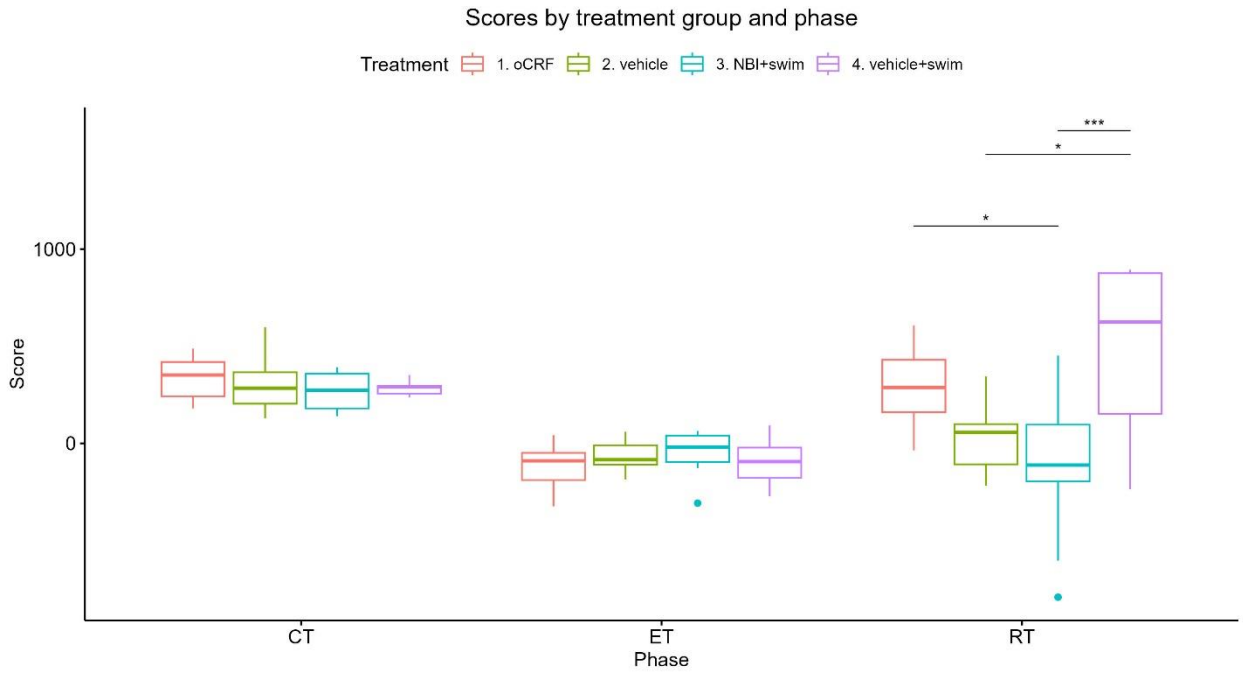
```
allp<-c(pwcp$p,pwct$p)
newp<-p.adjust(allp,method="fdr")
newpsig<-matrix(rep("ns",length(newp)),nrow=length(newp),ncol=1)
for (i in c(1:length(newp))){
 if (newp[i] < 0.05){
  newpsig[i]<-'*'
 }
 if (newp[i] < 0.01){
  newpsig[i]<-'**'
 }
 if (newp[i] < 0.001){
  newpsig[i]<-'***'
 }
}
npwc<-data.frame(bind_rows(pwcp[,1:8],pwct[,1:8]))
npwc<-data.frame(npwc[,1],npwc[,9],npwc[,2:8],newp,newpsig)
colnames(npwc)<-c("Treatment","Phase",colnames(npwc)[3:11])
```

	Treatment	Phase	.y.	group1	group2	n1	n2	p	p.signif	adj.p	adj.p.signif
1	1. oCRF	*NA*	Score	CT	ET	11	11	8.13e-08	****	0.000002439	***
2	1. oCRF	*NA*	Score	CT	RT	11	11	3.56e-01	ns	0.508571429	ns
3	1. oCRF	*NA*	Score	ET	RT	11	11	1.07e-06	****	0.000010700	***
4	2. vehicle	*NA*	Score	CT	ET	11	11	5.02e-07	****	0.000007530	***
5	2. vehicle	*NA*	Score	CT	RT	11	11	3.62e-05	****	0.000271500	***
6	2. vehicle	*NA*	Score	ET	RT	11	11	1.38e-01	ns	0.306000000	ns
7	3. NBI+swim	*NA*	Score	CT	ET	9	9	7.43e-03	**	0.020263636	*
8	3. NBI+swim	*NA*	Score	CT	RT	9	9	1.18e-03	**	0.005057143	**
9	3. NBI+swim	*NA*	Score	ET	RT	9	9	4.58e-01	ns	0.572500000	ns
10	4. vehicle+swim	*NA*	Score	CT	ET	9	9	3.62e-03	**	0.012066667	*
11	4. vehicle+swim	*NA*	Score	CT	RT	9	9	1.45e-01	ns	0.306000000	ns
12	4. vehicle+swim	*NA*	Score	ET	RT	9	9	8.25e-05	****	0.000495000	***
13	*NA*	CT	Score	1. oCRF	2. vehicle	11	11	4.92e-01	ns	0.590400000	ns
14	*NA*	CT	Score	1. oCRF	3. NBI+swim	11	9	1.53e-01	ns	0.306000000	ns
15	*NA*	CT	Score	2. vehicle	3. NBI+swim	11	9	4.29e-01	ns	0.559565217	ns
16	*NA*	CT	Score	1. oCRF	4. vehicle+swim	11	9	2.81e-01	ns	0.443684211	ns
17	*NA*	CT	Score	2. vehicle	4. vehicle+swim	11	9	6.67e-01	ns	0.741111111	ns
18	*NA*	CT	Score	3. NBI+swim	4. vehicle+swim	9	9	7.30e-01	ns	0.755172414	ns
19	*NA*	ET	Score	1. oCRF	2. vehicle	11	11	3.07e-01	ns	0.460500000	ns
20	*NA*	ET	Score	1. oCRF	3. NBI+swim	11	9	2.20e-01	ns	0.388235294	ns
21	*NA*	ET	Score	2. vehicle	3. NBI+swim	11	9	7.92e-01	ns	0.792000000	ns
22	*NA*	ET	Score	1. oCRF	4. vehicle+swim	11	9	7.10e-01	ns	0.755172414	ns
23	*NA*	ET	Score	2. vehicle	4. vehicle+swim	11	9	5.46e-01	ns	0.630000000	ns
24	*NA*	ET	Score	3. NBI+swim	4. vehicle+swim	9	9	4.10e-01	ns	0.559090909	ns
25	*NA*	RT	Score	1. oCRF	2. vehicle	11	11	5.42e-02	ns	0.135500000	ns
26	*NA*	RT	Score	1. oCRF	3. NBI+swim	11	9	4.08e-03	**	0.012240000	*
27	*NA*	RT	Score	2. vehicle	3. NBI+swim	11	9	2.46e-01	ns	0.410000000	ns
28	*NA*	RT	Score	1. oCRF	4. vehicle+swim	11	9	1.74e-01	ns	0.326250000	ns
29	*NA*	RT	Score	2. vehicle	4. vehicle+swim	11	9	2.34e-03	**	0.008775000	**
30	*NA*	RT	Score	3. NBI+swim	4. vehicle+swim	9	9	1.45e-04	***	0.000725000	***

Pairwise comparison grouping by phase to visualize significant differences

```
pwc<-pwct %>% add_xy_position(x="Phase")
ggboxplot(cpp,x="Phase",y="Score",color="Treatment") +
stat_pvalue_manual(pwc,tip.length=0,hide.ns=T)+ggtitle("Scores by treatment group and phase")+theme(plot.title = element_text(hjust=0.5))
ggsave("boxplot.jpeg")
```

Graph 2: boxplots of distributions across treatment group and phase



Relative Risk and Odds Ratio of Reinstatement across oCRF and vehicle groups

The difference between the means of these groups is not significant, so these measures, based on the proportion of animals that reinstated in either group, are conducted to observe an effect of treatment in this experiment.

```
cpp<-read.csv("2021CPPstudy.csv")
crf<-filter(cpp,Treatment=="oCRF")
crfctrl<-filter(cpp,Treatment=="vehicle")
PPcrfR<-(sum(crf$RT>=100)/(nrow(crf)))
PPcrfctrlR<-(sum(crfctrl$RT>=100)/(nrow(crfctrl)))
(RRcrfR<-PPcrfR/PPcrfctrlR)
```

[1] 2.880952

```
OcrfR<-PPcrfR/(1-PPcrfR)
OcrfctrlR<-PPcrfctrlR/(1-PPcrfctrlR)
(ORcrfR<-OcrfR/OcrfctrlR)
```

[1] 9.777778

A two-way mixed measures ANOVA test was selected for each experiment to detect if there are any significant differences between the means across groups grouped by the within- and between-subject factors to evaluate the effects of drug treatment, CPP phase and their interaction. The ANOVA test was two-way, because there were two factors; and it was mixed, because treatment was a between-subjects factor, while phase was a within-subjects factor. This test assumes normality, homoscedasticity, homogeneity of covariance, and sphericity – all of which are addressed below. Significance level (alpha) is set to 0.01. The null hypotheses for the CRF experiment are (1) that there is no significant difference between the mean scores grouped by pre-reinstatement test intraDRN treatment of oCRF or vehicle – any observed differences are the result of sampling or experimental error, or coincidence; and (2) that there is no significant difference between the mean scores grouped by CPP phase – any observed differences are the result of sampling or experimental error, or coincidence. There is a third null hypothesis that posits that there is no significant interaction effect of these two factors (treatment and phase), but this hypothesis is not for testing differences between means. The null hypotheses for the NBI experiment are very similar: (1) that there is no significant difference between the mean scores grouped by pre-swim stress intraDRN treatment of NBI or vehicle – any observed differences are the result of sampling or experimental error, or coincidence; and (2) that there is no significant difference between the mean scores grouped by CPP phase – any observed differences are the result of sampling or experimental error, or coincidence. Again, there is a third null hypothesis of no significant interaction effect between these factors.

To verify the assumption of normality, a Shapiro test was conducted and confirmed that the distributions of scores from each phase within each treatment group are normally distributed. The null hypothesis of this test states that the data are normally distributed, and any observed deviation from normality is due to error. The fact that all p-values are above 0.01 indicates that the null hypothesis cannot be rejected, and normality can be assumed. Graphical confirmation comes from the QQ plot, which compares the quantiles of two distributions, whose similarity is evidenced by the x=y linearity of the plot (6). The NBI+swim and vehicle+swim groups show the most deviation from x=y linearity, which corresponds to their lower p-values in the Shapiro test table, but they are sufficiently normally distributed to meet the ANOVA assumptions.

Levene's test was conducted to evaluate homoscedasticity between all groups, and its null hypothesis is that there is no significant difference between the variances of the distributions being compared – any differences observed are due to error. For the distributions of scores grouped by phase, this test was not passed with flying colors: the p-values for the conditioning and reinstatement phases were about 0.02 and 0.08 respectively, which are not much larger than alpha=0.01. Homogeneity of variance is assumed, but it is acknowledged that Type 1 errors are more likely when assumptions are violated, and this one is very close to violation. The Levene's test for the distributions of scores grouped by treatment revealed that the NBI+swim group at $p=0.0326$ is close to violating the assumption, and its control the vehicle+swim group does violate it at $p<0.01$. This violation and the near-violations will be tolerated, with an important acknowledgement that Type 1 error is more likely in comparisons involving groups that (almost) fail the test of homogeneity of variance.

Box's M-test was conducted to test for homogeneity of covariance. Its null hypothesis states that there is no significant difference between the covariances from different groups, and any observed differences are due to error. The resulting p-value is 0.0672, which is even larger than a more tolerant alpha of 0.05, so homogeneity of covariance is assumed, but this value is still close enough to the threshold that it is appropriate to acknowledge almost violating the assumption and therefore possibly

incurring more Type 1 error. Lastly, the ANOVA test assumes sphericity, the property of equality of variances of the differences between all related groups (7). The R function performing the ANOVA test automatically tested for sphericity with Mauchly's test and determined that for the phase factor and the interaction of treatment and phase, the null hypothesis of sphericity must be rejected for both experiments. A Greenhouse Geisser correction was applied in both ANOVAs to correct for the failure to meet this assumption.

The two-way mixed measures ANOVA for the CRF experiment revealed a main effect of phase ($F(1.32,26.35) = 55.538$, $p<0.01$) and a significant interaction between treatment and phase ($F(1.32,26.35) = 7.977$, $p<0.01$). This interaction effect indicates that the factors had different influences at their different levels. The main effect of treatment (predicted by H1) was not significant ($F(1,20) = 3.626$, $p=0.071$). Inspecting the effect visually in Graph 1, however, shows a difference of almost 300 seconds between the means of reinstatement scores of the oCRF-treated group and its control. Given that the total time during which animals are tested is 900 seconds, this effect is large, and consistent with H1 that intraDRN oCRF induces reinstatement. The two-way mixed measures ANOVA for the NBI experiment revealed a main effect of treatment ($F(1,16)=8.894$, $p<0.01$), phase ($F(1.06,16.9)=9.614$, $p<0.01$) with significant interaction ($F(1.06,16.9)=9.407$, $p<0.01$). In Graph 1, the difference between the means of reinstatement scores across treatment groups in this experiment is over 500 seconds – a large effect consistent with H2 that intraDRN NBI attenuates swim stress-induced reinstatement in morphine CPP.

Because the ANOVA test did not reveal which pairs of means in each factor group specifically differ significantly, post-hoc pairwise t-tests with false discovery rate correction (to correct for the increased likelihood of type 1 errors upon calculating many p-values) were conducted to detect significant differences between specific pairs of mean scores across phase within treatment groups and across treatment groups within phase. It must be noted, however, that the vehicle+swim group failed Levene's test across phase, so these results may falsely present some differences as significant. It is expected that for each treatment group, the conditioning score differs from the extinction score. Indeed, this is required to proceed to the reinstatement test, so the statistical significance of the difference between the mean scores across these phases in each treatment group is not surprising. Two groups were expected to reinstate: the oCRF and the vehicle+swim group, so their RT mean should differ from their ET mean significantly, while this should not be the case for the other two groups. This is what is observed in the pairwise comparison table. It was expected that the means of RT scores across treatment groups in each experiment would be significantly different, but this is the case only for the NBI experiment ($p<0.001$). For the CRF experiment, the p-value for the difference between the mean RT scores across groups treated with intraDRN oCRF and vehicle is 0.0542. While this is not statistically significant, the effect can be observed in Graphs 1 and 2, as well as in the relative risk (2.88) and the odds ratio (9.78) of reinstatement across the oCRF and vehicle groups. A relative risk above 2.00 and an odds ratio of 10 can be interpreted to represent meaningful differences (8,9).

A redundant subset of post-hoc pairwise t-tests of phase within treatment groups was conducted only to generate Graph 2. Inspecting the boxplots shows that conditioning and extinction did not vary appreciably between treatment groups, which is expected because at these stages the animals were all treated the same. The reinstatement phase aligns with the above expectations about which groups reinstate. The oCRF group RT score mean was labeled as significantly different from that of the control of the NBI experiment, but not of its control by pairwise comparison testing. The mean of the RT score of the vehile+swim group shows a significant difference between the means of its experimental

group and the CRF control group. These differences are expected, as only the oCRF and vehicle+swim groups were hypothesized to reinstate. (Significance is also observed in the differences between the mean scores of these groups in the FDR-corrected t-tests, though at different levels – again, these tests were run just for visualization purposes.)

This study aimed to elucidate the neurochemical mechanisms underlying relapse to opiates by induction of drug-seeking behavior in animals with extinguished morphine CPP. The phases of conditioning and extinction are necessary in this behavioral model, but the effects were to be found in the reinstatement scores, in which phase the treatment groups received different intraDRN infusions and (across experiments) underwent different reinstatement procedures. There was no significant difference found between the means of reinstatement scores between animals pretreated with intraDRN oCRF vs vehicle. The observation of a fairly substantial effect in visualization, relative risk, and odds ratio, however, suggests that CRF inhibition of serotonergic neurons in the DRN may induce drug-seeking behavior (H1), as the animals in the experimental group demonstrated more reinstatement (a previously extinguished preference for the drug-paired side of their chamber). An effect in the opposite direction was observed in the complementary experiment, and the significant ($p<0.01$) difference between the mean reinstatement scores of animals pretreated with intraDRN NBI 35965 vs vehicle prior to forced-swim stress supports the hypothesis (H2) that inhibition of the serotonin inhibitor CRF can attenuate swim-stress induced reinstatement to previously extinguished morphine CPP. Although not all ANOVA assumptions were met, and not all experimental effects were statistically significant, the observed effects in this analysis nonetheless appear to be consistent with the hypotheses H1 and H2, which might be preliminarily accepted as reasonable explanations, contingent on validation from further experimentation.

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