

# Analysis of B-endorphin/ChAT IHC Count Data

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```
library(knitr)
library(tidyverse)
library(dplyr)
library(WebPower)
```

```
counts <- read_csv("../data/B-endo-totals.csv")
```

```
## Rows: 12 Columns: 12
```

```
## -- Column specification -----
## Delimiter: ","
## chr (3): Light, Time, Sex
## dbl (9): Mouse, ChAT, Bendo, Percentage, Age, GCLchat, GCLbendo, INLchat, IN...
```

```
##
## i Use 'spec()' to retrieve the full column specification for this data.
## i Specify the column types or set 'show_col_types = FALSE' to quiet this message.
```

```
counts <- counts %>%
  dplyr::select(-ChAT, -Bendo, -Percentage) %>%
  #make columns lowercase
  rename(mouse = Mouse,
         light = Light,
         time = Time,
         sex = Sex,
         age = Age,
         GCL_chat = GCLchat,
         GCL_bendo = GCLbendo,
         INL_chat = INLchat,
         INL_bendo = INLbendo) %>%
  #update the classes of some columns
  mutate(mouse = as.character(mouse),
         light = factor(as.factor(light), levels=c("light", "dark")),
  #reinroduce total counts
         total_chat = GCL_chat+INL_chat,
         total_bendo = GCL_bendo+INL_bendo) %>%
  #calculate percentages
  mutate(GCL_perc=GCL_bendo/GCL_chat*100,
         INL_perc=INL_bendo/INL_chat*100,
```

```

total_perc=total_bendo/total_chat*100)

percs <- counts %>%
  #give one row for each GCL, INL, and total
  #retain only the percentages, not the raw counts
  dplyr::select(-c(GCL_chat:total_bendo)) %>%
  pivot_longer(GCL_perc:total_perc,
               names_to="type",
               values_to="perc") %>%
  separate(type, sep="_", into=c("layer", "protein")) %>%
  dplyr::select(-protein) %>%
  mutate(layer = as.factor(layer),
         mouse = fct_reorder(as.factor(mouse), perc,
                             .fun=max, .desc=FALSE),
         time = as.factor(time))
head(percs, 3)

```

```

## # A tibble: 3 x 7
##   mouse light time  sex    age layer  perc
##   <fct> <fct> <fct> <chr> <dbl> <fct> <dbl>
## 1 11    light day   F      95 GCL    3.14
## 2 11    light day   F      95 INL    7.44
## 3 11    light day   F      95 total  5.54

```

```

aov <- aov(perc ~ light * time * layer, percs)
summary(aov)

```

```

##              Df Sum Sq Mean Sq F value    Pr(>F)
## light          1  212.5    212.5   10.418 0.003591 **
## time           1   15.1     15.1    0.742 0.397479
## layer          2  418.1    209.1   10.252 0.000605 ***
## light:time      1  388.4    388.4   19.048 0.000209 ***
## light:layer     2   51.4     25.7    1.261 0.301426
## time:layer      2   12.9      6.5    0.317 0.731103
## light:time:layer 2   30.8     15.4    0.754 0.481324
## Residuals     24  489.4     20.4
## ---
## Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

```

```

TukeyHSD(aov)

```

```

##   Tukey multiple comparisons of means
##     95% family-wise confidence level
##
## Fit: aov(formula = perc ~ light * time * layer, data = percs)
##
## $light
##              diff          lwr          upr          p adj
## dark-light 4.858586 1.751826 7.965347 0.0035915
##
## $time

```

```

##          diff      lwr      upr      p adj
## night-day 1.296833 -1.809928 4.403594 0.3974787
##
## $layer
##          diff      lwr      upr      p adj
## INL-GCL    8.341742  3.7377600 12.945724 0.0003954
## total-GCL  4.448280 -0.1557018 9.052262 0.0596404
## total-INL -3.893462 -8.4974436 0.710520 0.1085536
##
## $'light:time'
##          diff      lwr      upr      p adj
## dark:day-light:day 11.428229  5.5557074 17.3007501 0.0000914
## light:night-light:day 7.866475  1.9939540 13.7389967 0.0058274
## dark:night-light:day 6.155420  0.2828981 12.0279409 0.0375175
## light:night-dark:day -3.561753 -9.4342748 2.3107680 0.3591453
## dark:night-dark:day -5.272809 -11.1453306 0.5997121 0.0894675
## dark:night-light:night -1.711056 -7.5835772 4.1614655 0.8519894
##
## $'light:layer'
##          diff      lwr      upr      p adj
## dark:GCL-light:GCL 1.887984 -6.1734056 9.949374 0.9769840
## light:INL-light:GCL 5.414707 -2.6466830 13.476097 0.3318687
## dark:INL-light:GCL 13.156761  5.0953713 21.218151 0.0004753
## light:total-light:GCL 2.919412 -5.1419781 10.980802 0.8684102
## dark:total-light:GCL 7.865133 -0.1962573 15.926522 0.0587237
## light:INL-dark:GCL 3.526723 -4.5346673 11.588112 0.7534577
## dark:INL-dark:GCL 11.268777  3.2073870 19.330167 0.0028346
## light:total-dark:GCL 1.031428 -7.0299623 9.092817 0.9985819
## dark:total-dark:GCL 5.977148 -2.0842415 14.038538 0.2355895
## dark:INL-light:INL 7.742054 -0.3193355 15.803444 0.0648709
## light:total-light:INL -2.495295 -10.5566849 5.566095 0.9269133
## dark:total-light:INL 2.450426 -5.6109640 10.511816 0.9319400
## light:total-dark:INL -10.237349 -18.2987392 -2.175960 0.0073912
## dark:total-dark:INL -5.291629 -13.3530183 2.769761 0.3557905
## dark:total-light:total 4.945721 -3.1156690 13.007111 0.4278716
##
## $'time:layer'
##          diff      lwr      upr      p adj
## night:GCL-day:GCL -0.2357322 -8.2971220 7.825658 0.9999990
## day:INL-day:GCL 6.8778753 -1.1835145 14.939265 0.1263860
## night:INL-day:GCL 9.5698762 1.5084864 17.631266 0.0135549
## day:total-day:GCL 3.6132987 -4.4480911 11.674688 0.7347357
## night:total-day:GCL 5.0475292 -3.0138606 13.108919 0.4059680
## day:INL-night:GCL 7.1136075 -0.9477823 15.174997 0.1059989
## night:INL-night:GCL 9.8056084 1.7442186 17.866998 0.0109586
## day:total-night:GCL 3.8490309 -4.2123589 11.910421 0.6817713
## night:total-night:GCL 5.2832614 -2.7781284 13.344651 0.3574517
## night:INL-day:INL 2.6920009 -5.3693889 10.753391 0.9022373
## day:total-day:INL -3.2645766 -11.3259664 4.796813 0.8069848
## night:total-day:INL -1.8303461 -9.8917359 6.231044 0.9798955
## day:total-night:INL -5.9565775 -14.0179673 2.104812 0.2387215
## night:total-night:INL -4.5223470 -12.5837368 3.539043 0.5237205
## night:total-day:total 1.4342305 -6.6271593 9.495620 0.9932964
##

```

```

## $'light:time:layer'
##
##               diff               lwr               upr
## dark:day:GCL-light:day:GCL      6.18425591    -7.11037198  19.478884
## light:night:GCL-light:day:GCL    4.06053948    -9.23408842  17.355167
## dark:night:GCL-light:day:GCL     1.65225203   -11.64237587  14.946880
## light:day:INL-light:day:GCL      1.68702656   -11.60760134  14.981654
## dark:day:INL-light:day:GCL     18.25297992     4.95835202  31.547608
## light:night:INL-light:day:GCL    13.20292650    -0.09170139  26.497554
## dark:night:INL-light:day:GCL    12.12108175    -1.17354615  25.415710
## light:day:total-light:day:GCL     0.93818817   -12.35643973  14.232816
## dark:day:total-light:day:GCL    12.47266512    -0.82196278  25.767293
## light:night:total-light:day:GCL   8.96117478    -4.33345311  22.255803
## dark:night:total-light:day:GCL    7.31813947    -5.97648843  20.612767
## light:night:GCL-dark:day:GCL    -2.12371644   -15.41834434  11.170911
## dark:night:GCL-dark:day:GCL     -4.53200388   -17.82663178   8.762624
## light:day:INL-dark:day:GCL      -4.49722936   -17.79185725   8.797399
## dark:day:INL-dark:day:GCL     12.06872400    -1.22590389  25.363352
## light:night:INL-dark:day:GCL     7.01867059    -6.27595731  20.313298
## dark:night:INL-dark:day:GCL     5.93682583    -7.35780207  19.231454
## light:day:total-dark:day:GCL    -5.24606775   -18.54069564   8.048560
## dark:day:total-dark:day:GCL     6.28840921    -7.00621869  19.583037
## light:night:total-dark:day:GCL   2.77691887   -10.51770903  16.071547
## dark:night:total-dark:day:GCL    1.13388355   -12.16074435  14.428511
## dark:night:GCL-light:night:GCL  -2.40828744   -15.70291534  10.886340
## light:day:INL-light:night:GCL   -2.37351292   -15.66814081  10.921115
## dark:day:INL-light:night:GCL    14.19244044     0.89781255  27.487068
## light:night:INL-light:night:GCL   9.14238703    -4.15224087  22.437015
## dark:night:INL-light:night:GCL   8.06054227    -5.23408563  21.355170
## light:day:total-light:night:GCL  -3.12235131   -16.41697921  10.172277
## dark:day:total-light:night:GCL   8.41212565    -4.88250225  21.706754
## light:night:total-light:night:GCL 4.90063531    -8.39399259  18.195263
## dark:night:total-light:night:GCL 3.25759999   -10.03702791  16.552228
## light:day:INL-dark:night:GCL     0.03477453   -13.25985337  13.329402
## dark:day:INL-dark:night:GCL    16.60072789     3.30609999  29.895356
## light:night:INL-dark:night:GCL   11.55067447    -1.74395342  24.845302
## dark:night:INL-dark:night:GCL   10.46882971    -2.82579818  23.763458
## light:day:total-dark:night:GCL   -0.71406387   -14.00869176  12.580564
## dark:day:total-dark:night:GCL   10.82041309    -2.47421481  24.115041
## light:night:total-dark:night:GCL  7.30892275    -5.98570514  20.603551
## dark:night:total-dark:night:GCL  5.66588743    -7.62874046  18.960515
## dark:day:INL-light:day:INL     16.56595336     3.27132546  29.860581
## light:night:INL-light:day:INL    11.51589995    -1.77872795  24.810528
## dark:night:INL-light:day:INL    10.43405519    -2.86057271  23.728683
## light:day:total-light:day:INL    -0.74883839   -14.04346629  12.545790
## dark:day:total-light:day:INL    10.78563856    -2.50898933  24.080266
## light:night:total-light:day:INL   7.27414823    -6.02047967  20.568776
## dark:night:total-light:day:INL   5.63111291    -7.66351499  18.925741
## light:night:INL-dark:day:INL    -5.05005341   -18.34468131   8.244574
## dark:night:INL-dark:day:INL     -6.13189817   -19.42652607   7.162730
## light:day:total-dark:day:INL   -17.31479175   -30.60941965  -4.020164
## dark:day:total-dark:day:INL     -5.78031480   -19.07494269   7.514313
## light:night:total-dark:day:INL   -9.29180513   -22.58643303   4.002823
## dark:night:total-dark:day:INL  -10.93484045   -24.22946835   2.359787
## dark:night:INL-light:night:INL  -1.08184476   -14.37647266  12.212783

```

## light:day:total-light:night:INL	-12.26473834	-25.55936624	1.029890
## dark:day:total-light:night:INL	-0.73026138	-14.02488928	12.564367
## light:night:total-light:night:INL	-4.24175172	-17.53637962	9.052876
## dark:night:total-light:night:INL	-5.88478704	-19.17941494	7.409841
## light:day:total-dark:night:INL	-11.18289358	-24.47752148	2.111734
## dark:day:total-dark:night:INL	0.35158338	-12.94304452	13.646211
## light:night:total-dark:night:INL	-3.15990696	-16.45453486	10.134721
## dark:night:total-dark:night:INL	-4.80294228	-18.09757018	8.491686
## dark:day:total-light:day:total	11.53447695	-1.76015094	24.829105
## light:night:total-light:day:total	8.02298662	-5.27164128	21.317615
## dark:night:total-light:day:total	6.37995130	-6.91467660	19.674579
## light:night:total-dark:day:total	-3.51149034	-16.80611823	9.783138
## dark:night:total-dark:day:total	-5.15452565	-18.44915355	8.140102
## dark:night:total-light:night:total	-1.64303532	-14.93766322	11.651593
##	p adj		
## dark:day:GCL-light:day:GCL	0.8615822		
## light:night:GCL-light:day:GCL	0.9916500		
## dark:night:GCL-light:day:GCL	0.9999983		
## light:day:INL-light:day:GCL	0.9999979		
## dark:day:INL-light:day:GCL	0.0022131		
## light:night:INL-light:day:GCL	0.0527451		
## dark:night:INL-light:day:GCL	0.0971061		
## light:day:total-light:day:GCL	1.0000000		
## dark:day:total-light:day:GCL	0.0799854		
## light:night:total-light:day:GCL	0.4242217		
## dark:night:total-light:day:GCL	0.6985677		
## light:night:GCL-dark:day:GCL	0.9999777		
## dark:night:GCL-dark:day:GCL	0.9807004		
## light:day:INL-dark:day:GCL	0.9817623		
## dark:day:INL-dark:day:GCL	0.0999122		
## light:night:INL-dark:day:GCL	0.7464078		
## dark:night:INL-dark:day:GCL	0.8890279		
## light:day:total-dark:day:GCL	0.9471557		
## dark:day:total-dark:day:GCL	0.8490240		
## light:night:total-dark:day:GCL	0.9996968		
## dark:night:total-dark:day:GCL	1.0000000		
## dark:night:GCL-light:night:GCL	0.9999227		
## light:day:INL-light:night:GCL	0.9999329		
## dark:day:INL-light:night:GCL	0.0292835		
## light:night:INL-light:night:GCL	0.3962974		
## dark:night:INL-light:night:GCL	0.5732166		
## light:day:total-light:night:GCL	0.9991064		
## dark:day:total-light:night:GCL	0.5135873		
## light:night:total-light:night:GCL	0.9663865		
## dark:night:total-light:night:GCL	0.9986938		
## light:day:INL-dark:night:GCL	1.0000000		
## dark:day:INL-dark:night:GCL	0.0064639		
## light:night:INL-dark:night:GCL	0.1316525		
## dark:night:INL-dark:night:GCL	0.2250368		
## light:day:total-dark:night:GCL	1.0000000		
## dark:day:total-dark:night:GCL	0.1902906		
## light:night:total-dark:night:GCL	0.7000753		
## dark:night:total-dark:night:GCL	0.9150848		
## dark:day:INL-light:day:INL	0.0066100		

```
## light:night:INL-light:day:INL      0.1340584
## dark:night:INL-light:day:INL      0.2287165
## light:day:total-light:day:INL      1.0000000
## dark:day:total-light:day:INL      0.1935306
## light:night:total-light:day:INL    0.7057456
## dark:night:total-light:day:INL    0.9181224
## light:night:INL-dark:day:INL      0.9588146
## dark:night:INL-dark:day:INL      0.8676738
## light:day:total-dark:day:INL      0.0040758
## dark:day:total-dark:day:INL      0.9045963
## light:night:total-dark:day:INL    0.3739931
## dark:night:total-dark:day:INL    0.1799276
## dark:night:INL-light:night:INL    1.0000000
## light:day:total-light:night:INL    0.0897569
## dark:day:total-light:night:INL    1.0000000
## light:night:total-light:night:INL  0.9882601
## dark:night:total-light:night:INL  0.8943604
## light:day:total-dark:night:INL    0.1589995
## dark:day:total-dark:night:INL    1.0000000
## light:night:total-dark:night:INL  0.9990048
## dark:night:total-dark:night:INL  0.9707589
## dark:day:total-light:day:total     0.1327686
## light:night:total-light:day:total  0.5796328
## dark:night:total-light:day:total  0.8375150
## light:night:total-dark:day:total   0.9974847
## dark:night:total-dark:day:total   0.9528520
## dark:night:total-light:night:total 0.9999984
```

```
#three way ANOVA power calc
#factors A=light, B=time, C=layer
#number levels in each J=2, K=2, C=3
#ng = J * K * L = 2*2*3 = 12
#n = #/group * ng = 3*12 = 36
#ndf = (J-1)*(K-1)*(L-1) = 1*1*2 = 2
#f=0.5 is default
#no documentation on calculating f for a 3-way ANOVA
wp.kanova(n=36, ndf=2, f=0.5, alph=0.05, ng=12)
```

```
## Multiple way ANOVA analysis
##
##      n ndf ddf   f ng alpha   power
##    36   2  24 0.5 12  0.05 0.7133372
##
## NOTE: Sample size is the total sample size
## URL: http://psychstat.org/kanova
```

```
#set up a table of p-values for labeling the graphs below
label <- tibble(mouse = c("97", "93", "57"),
                perc = c(Inf, Inf, Inf),
                time = c("day", "night", "night"),
                light = c("dark", "light", "dark"),
                label = c("p=0.00009", "p=0.0058", "p=0.037"))
```

```

#1 row facet wrap, small
bendo_ihc <- percs %>% ggplot(aes(x=mouse, y=perc)) +
  geom_bar(aes(fill=layer), stat="identity", position="dodge") +
  facet_wrap(time ~ factor(light, c("light", "dark")), scales="free_x", nrow=1, as.table=FALSE) +
  labs(y="% of B-endorphin+ ChAT cells",
       col="Light Condition", shape="Cell Layer",
       title = "Figure 2") +
  theme(axis.title.x=element_blank(),
        axis.text.x=element_blank(),
        text = element_text(size=4),
        plot.title = element_text(size=4, margin=margin(0,0,0,0), face = "bold"),
        legend.key.size = unit(0.25, 'cm'),
        strip.text.x = element_text(size = 3, margin = margin(0, 0, 0.02, 0, "cm")),
        axis.ticks = element_blank(),
        legend.margin=margin(0,0,0,0)) +
  scale_fill_manual(values = c("yellow", "cornflowerblue", "grey50")) +
  geom_text(aes(label=label), data=label, vjust="top", hjust="left", size=1)

ggsave(filename="../figures/bendo_ihc.png", plot=bendo_ihc, height=1, width=2.5)

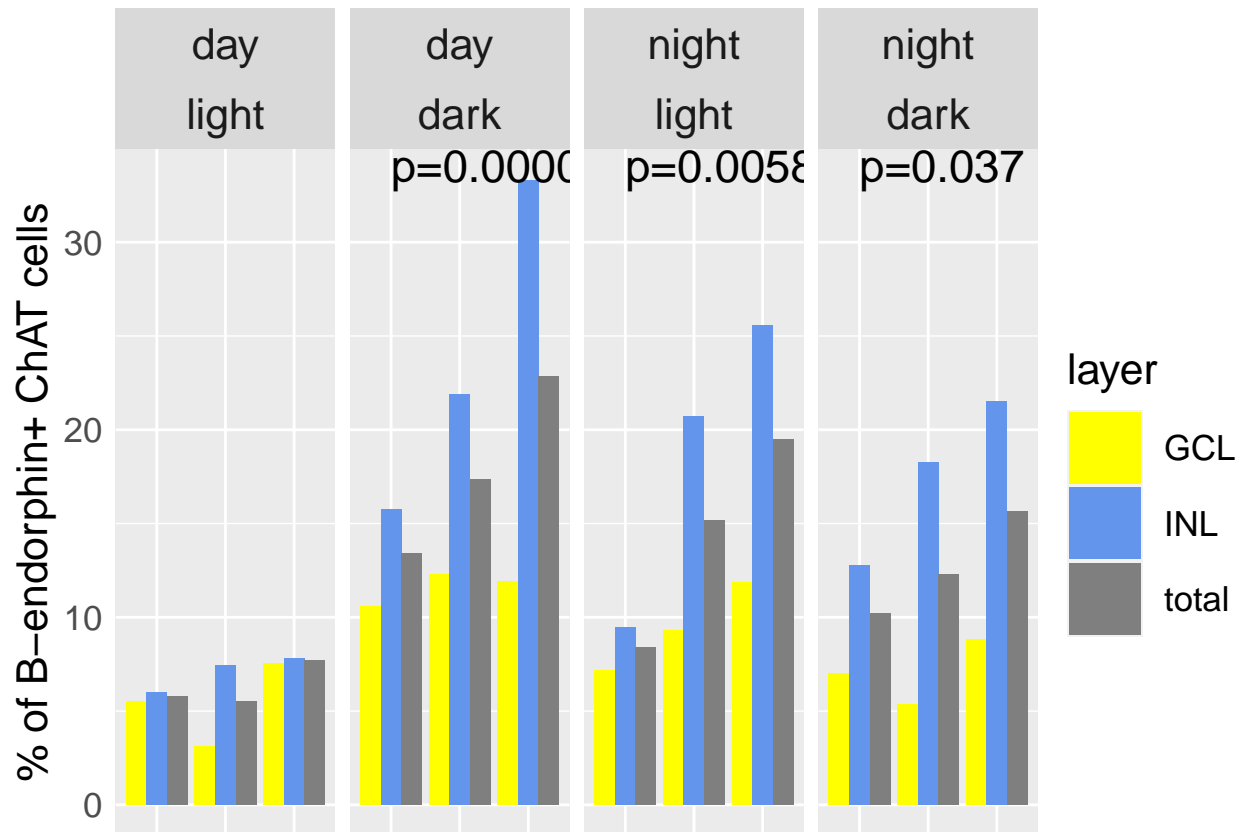
```

```

#1 row facet wrap, large
bendo_ihc_large <- percs %>% ggplot(aes(x=mouse, y=perc)) +
  geom_bar(aes(fill=layer), stat="identity", position="dodge") +
  facet_wrap(time ~ factor(light, c("light", "dark")), scales="free_x", nrow=1, as.table=FALSE) +
  labs(y="% of B-endorphin+ ChAT cells",
       col="Light Condition", shape="Cell Layer") +
  theme(axis.title.x=element_blank(),
        axis.text.x=element_blank(),
        text = element_text(size=16),
        legend.key.size = unit(1, 'cm'),
        strip.text.x = element_text(size = 16, margin = margin(0.2, 0, 0.2, 0, "cm")),
        axis.ticks = element_blank(),
        legend.margin=margin(0,0,0,0)) +
  scale_fill_manual(values = c("yellow", "cornflowerblue", "grey50")) +
  geom_text(aes(label=label), data=label, vjust="top", hjust="left", size=6)

bendo_ihc_large

```



```
ggsave(filename="../figures/bendo_ihc_large.png", plot=bendo_ihc_large, height=5, width=12)
```

```
#2 BY 2 FACET WRAP
bendo_ihc_boxy <- percs %>% ggplot(aes(x=mouse, y=perc)) +
  geom_bar(aes(fill=layer), stat="identity", position="dodge") +
  facet_wrap(time ~ factor(light, c("light", "dark")), scales="free_x", nrow=2, as.table=TRUE) +
  labs(y="% of B-endorphin+ ChAT cells",
       col="Light Condition", shape="Cell Layer") +
  theme(axis.title.x=element_blank(),
        axis.text.x=element_blank(),
        text = element_text(size=4),
        plot.title = element_text(size=4, margin=margin(0,0,0,0), face = "bold"),
        legend.key.size = unit(0.25, 'cm'),
        strip.text.x = element_text(size = 3, margin = margin(0, 0, 0.02, 0, "cm")),
        axis.ticks = element_blank(),
        legend.margin=margin(0,0,0,0)) +
  scale_fill_manual(values = c("yellow", "cornflowerblue", "grey50")) +
  geom_text(aes(label=label), data=label, vjust="top", hjust="left", size=1)

ggsave(filename="../figures/bendo_ihc_boxy.png", plot=bendo_ihc_boxy, height=4, width=4)
```