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")),
  #reintroduce 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)

#checking if the cell counts are normally distributed
shapiro.test(counts$INL_chat)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  counts$INL_chat
## W = 0.89571, p-value = 0.1396
```

```
shapiro.test(counts$INL_bendo)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  counts$INL_bendo
## W = 0.91934, p-value = 0.2805
```

```
shapiro.test(counts$GCL_chat)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  counts$GCL_chat
## W = 0.88355, p-value = 0.09733
```

```
shapiro.test(counts$GCL_bendo)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  counts$GCL_bendo
## W = 0.86341, p-value = 0.05396
```

```
shapiro.test(counts$total_chat)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  counts$total_chat
## W = 0.88056, p-value = 0.08911
```

```
shapiro.test(counts$total_bendo)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  counts$total_bendo
## W = 0.90417, p-value = 0.1795
```

```r
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
```

```r
#checking if the percentages are normally distributed
gc <- percs %>% filter(layer =="GCL")
shapiro.test(gc$perc)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  gc$perc
## W = 0.94973, p-value = 0.633
```

```r
ic <- percs %>% filter(layer =="INL")
shapiro.test(ic$perc)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  ic$perc
## W = 0.94583, p-value = 0.5771
```

```r
tc <- percs %>% filter(layer =="total")
shapiro.test(tc$perc)
```

```
##
##  Shapiro-Wilk normality test
##
## data:  tc$perc
## W = 0.96135, p-value = 0.8029
```

```
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"))
```

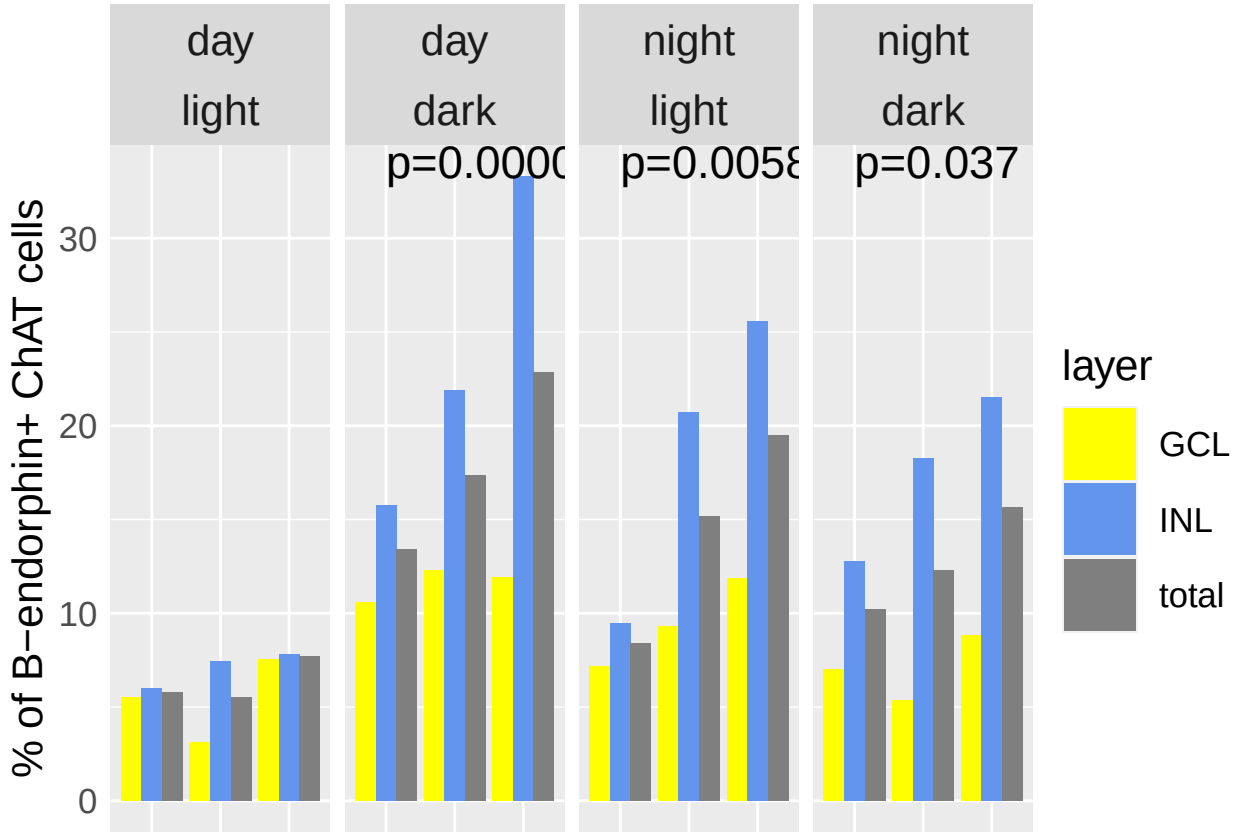
```
#1 row facet wrap, small
bendo_ihc <- percs %>% ggplot(aes(x=mouse, y=perc)) +
  geom_bar(aes(fill=layer), stat="identity", position="dodge") +
  facet_wrap(time ~ factor(light, c("light", "dark")), scales="free_x", nrow=1, as.table=FALSE) +
  labs(y="% of B-endorphin+ ChAT cells",
       col="Light 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)
```