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Abstract

Though overlapping in distribution through the hypothalamus, orexin (Orx) and melanin concentrating hormone (MCH) neurons play opposite roles in the regulation of sleep-wake states. Orx neurons discharge during waking, whereas MCH neurons discharge during sleep. In the present study, we examined in mice whether $GABA_A$ and $GABA_B$ receptors (Rs) are present on Orx and MCH neurons and might undergo differential changes as a function of their different activities following sleep deprivation (SD) and sleep recovery (SR). Applying quantitative stereological image analysis to dual-immunofluorescent stained sections, we determined that the proportion of Orx neurons positively immunostained for $GABA_A$Rs was significantly higher following SD (~48%) as compared to sleep control (SC, ~24%) and SR (~27%) and that the luminance of the $GABA_A$Rs was significantly greater. In contrast, the average proportion of the MCH neurons immunostained for $GABA_A$Rs was insignificantly lower following SD (~43%) in comparison to SC (~54%) and SR (56%), and the luminance of the $GABA_A$Rs was significantly less. Although, $GABA_B$Rs were observed in all Orx and MCH neurons (100%), the luminance of these receptors was differentially altered following SD. The intensity of $GABA_B$Rs in the Orx neurons was significantly greater after SD than after SC and SR, whereas that in the MCH neurons was significantly less. The present results indicate that GABA receptors undergo dynamic and differential changes in the wake-active, Orx neurons and sleep-active, MCH neurons as a function of and homeostatic adjustment to their preceding activity and sleep-wake state.

Significance Statement

The activity of single neurons is regulated in a homeostatic manner such that prolonged activity results in decreased excitability. Orexin neurons discharge during waking, whereas MCH neurons do so during sleep. Here, we examined whether the inhibitory GABA receptors (Rs) on Orexin and MCH neurons would change differentially as a function of their different activities following sleep deprivation and sleep recovery. Whereas $GABA_AR$ and $GABA_BR$ immunostaining appeared to increase on Orexin neurons, it appeared to decrease on MCH neurons after sleep deprivation relative to sleep control and sleep recovery. GABA receptors thus undergo differential changes on Orx and MCH neurons as a function of and homeostatic adaptation to their different activities during waking and sleep.

Introduction

Orexin (Orx) and melanin concentrating hormone (MCH) peptides are contained in distinct though co-distributed neurons in the hypothalamus (Bittencourt et al., 1992; Broberger et al., 1998; de Lecea et al., 1998). From multiple lines of evidence, they appear to play opposite roles in the regulation of waking and sleep. Pre-pro Orx knockout mice present with a syndrome of narcolepsy with cataplexy, marked by the sudden passage from waking to REM sleep with muscle atonia (Chemelli et al., 1999). Humans having narcolepsy with cataplexy have a reduced number of Orx neurons or an absence of its peptide in cerebrospinal fluid (Peyron et al., 2000; Thannickal et al., 2000). In rats, Orx neurons fire maximally during waking and become virtually silent during sleep (Lee et al., 2005), and they express c-Fos, a marker for neuronal activity, following sleep deprivation (SD) and not sleep recovery (SR) (Modirrousta et al., 2005). In contrast, MCH neurons do not fire during waking, but fire sparsely during slow wave sleep (SWS) and maximally during REM or paradoxical sleep (PS) (Hassani et al., 2009), and they do not express c-Fos after SD but do so after SR (Verret et al., 2003; Modirrousta et al., 2005). We queried whether the different discharge profiles of the Orx and MCH neurons would be associated with different homeostatic responses of those neurons to SD.

Neuronal activity is regulated in a homeostatic manner such that increases in activity are compensated for by decreases in excitability and decreases in activity by increases in excitability (Turrigiano, 1999). These changes are mediated in part by changes in receptors to the inhibitory neurotransmitter GABA, as well as by reciprocal changes in those to the excitatory neurotransmitter, glutamate (Turrigiano et al., 1998; Kilman et al., 2002; Marty et al., 2004). With the knowledge that Orx neurons are active whereas MCH neurons are silent during continuous waking with SD, we thus examined whether the changes in activity that occur in

95 those neurons would be associated with differential changes in the receptors to GABA. Through
96 *in vitro* studies, it is known that Orx neurons are hyperpolarized and inhibited by both $GABA_A$
97 (e.g. muscimol) and $GABA_B$ (e.g. baclofen) receptor agonists and that MCH neurons are
98 inhibited by $GABA_A$R agonists (Eggermann et al., 2003; van den Pol et al., 2004; Xie et al.,
99 2006). We thus investigated whether homeostatic changes in response to state specific
100 prolonged activity or absence thereof would be evident in $GABA_A$R and $GABA_B$R
101 immunostaining following SD and SR in the Orx and MCH neurons.
102
103
104

105 **Materials and methods**

106 All procedures were approved by the [Authors' university's] animal care committee.

107

108 **Animals**

109 Male adult mice (n = 25) (C57BL/6, 20-25g) were received from the supplier (Charles River)
110 and housed individually in cages, which were maintained at an ambient temperature of 22°C, in a
111 12/12 h light/dark cycle (lights on from 7 A.M. to 7 P.M.) and they were given free access to
112 water and food. Animals were maintained in their home cages for the duration of the experiment
113 and therein recorded by video alone (VM, n = 13) or by video plus telemetry (VTM, n = 12).
114 For telemetric recording of the electroencephalogram (EEG), a transmitter (F20-EET, Data
115 Sciences International, DSI) was implanted subcutaneously along the flank and connected to two
116 EEG electrodes placed symmetrically over parietal cortex and two reference electrodes placed
117 over the cerebellum. Following surgery, the mice were allowed one week to recover.

118

119 **Sleep deprivation and recovery experimental procedures**

120 As described in another manuscript by the Authors, four experimental groups of mice were
121 processed: 1) sleep control (SC), having undisturbed sleep and waking for 2 h from ~2 P.M. to
122 ~4 P.M. (~ZT 7-9) (n = 7), 2) sleep deprivation (SD), being submitted to 2 h of SD from ~2 P.M.
123 to ~4 P.M. (~ZT 7-9) (n = 6), 3) sleep deprivation (SD), being submitted to 4 h of SD from ~12
124 P.M. to ~4 P.M. (~ZT 5-9) (n = 5) and 4) sleep recovery (SR), being subjected to 4 h of SD from
125 ~10 A.M. to ~2 P.M. followed by 2 h SR from ~2 P.M. to ~4 P.M. (~ZT 7-9) (n = 7). SD was
126 performed by preventing mice from going to sleep by stimulation of the whiskers with a soft
127 paint brush. For scoring of sleep and waking, mice were recorded by video alone for behavior

(VM, n = 13) or by video plus telemetry for behavior with EEG (VTM, n = 12) using HomeCageScan software (HCS, 3.0; Clever Systems, CleverSys). At the end of the experimental period ~4 P.M. (~ZT 9), the mice were immediately anesthetized with sodium pentobarbital (Euthanyl, 100 mg/kg; Bimeda-MTC Pharmaceutical). Brains were fixed by transcardial perfusion with 30 mL saline followed by 200 mL of 3% paraformaldehyde. The brains were removed and placed for 1 h in 3% paraformaldehyde for post-fixation at 4° C, transferred to 30% sucrose solution for cryoprotection at 4° C for 2 days, then frozen and stored at -80° C.

Immunohistochemical processing

Brains were cut and processed in batches of 2-4 that included mice from SC, SD and/or SR groups of the same experimental session or period. Coronal sections were cut on a freezing microtome at 20 µm thickness through the diencephalon. Adjacent series of sections were collected at 200 µm intervals for immunohistochemical staining. Free floating sections were rinsed in 0.1M trizma saline buffer (pH 7.4), then incubated in 6% normal donkey serum buffer for 30 min and subsequently incubated overnight at room temperature in a buffer containing 1% normal donkey serum with combinations of two primary antibodies: goat anti-MCH (1:250, Santa Cruz Biotechnology, CAT# sc-14507, RRID: AB_2166711) or goat anti-Orx (1:500, Santa Cruz Biotechnology, CAT# sc-8070, RRID: AB_653610) with mouse anti-$GABA_AR$ β-chain (clone BD17, 1:100, Millipore (Chemicon), CAT# MAB 341, RRID: AB_2109419) or guinea pig anti-$GABA_BR1$ (1:2500, Millipore (Chemicon) CAT# AB1531, RRID: AB_2314472). Both the $GABA_AR$ β-chain and $GABA_BR1$ antibodies were produced and characterized years ago and have since been in use over many years (Fritschy and Mohler, 1995;

Wan et al., 1997; Nusser et al., 1998; Bonino et al., 1999; Margeta-Mitrovic et al., 1999; Filippov et al., 2000; Straessle et al., 2003). Subsequently, sections were incubated at room temperature for 2 h in appropriate combinations of Cyanine-conjugated (Cy3 or Cy5) secondary antibodies from donkey (Jackson ImmunoResearch Laboratories): Cy5-conjugated anti-goat (1:800, CAT# 705-175-147, RRID: AB_2340415) with Cy3-conjugated anti-mouse (1:1000, CAT# 715-165-150, RRID: AB_2340813) or Cy3-conjugated anti-guinea pig (1:1000, CAT# 706-165-148, RRID: AB_2340460). After rinsing the sections with trizma saline, sections were stained with green fluorescent Nissl stain (FNS) (1:2000, Molecular Probes, CAT# N-21480) for 20 minutes. Finally, sections were rinsed, mounted and coverslipped with glycerol.

Image analysis

Triple-stained sections were viewed with a Leica DMLB microscope equipped with fluorescence filters for excitation and emission of Cy2, Cy3 and Cy5 dyes, a digital camera (Orca-R^2, C10600-10B, Hamamatsu photonics K.K) and an x/y/z movement sensitive stage. Images were acquired from 3 sections in each series (with 200 μm intervals between sections) through the Orx and MCH neurons in the tuberal hypothalamus using StereoInvestigator software (MicroBrightField, MBF). With the Optical Fractionator Probe for unbiased sampling and counting, contours were first traced with a 5x objective around all the Orx or MCH neurons in each section within the lateral hypothalamus, perifornical area, dorsomedial nucleus and/or zona incerta (Modirrousta et al., 2003). For sampling, a grid size of 250x150 μm^2 was employed over each contour, and for cell counting and measurements, a counting frame of 120x120 μm^2 was used and placed within each rectangular space by the program. In these, multi-channel image stacks were acquired under a 40x objective and were comprised by optical sections of 0.5 μm

thickness through the mounted histological section of approximately 15 µm thickness. Within these images, the tops of all cells located below 1 µm from the surface of the section were counted, thus through 14 µm of the section within the counting frame. Across the three sections, approximately 38 counting frames for Orx neurons and 59 for MCH neurons were acquired and analyzed per series. With this sampling, the average number of Orx+ cells counted across series on one side was 56.6 ± 0.70 (mean ± SEM), corresponding to an estimated total number of 1559 ± 70 Orx+ neurons within one side of the tuberal hypothalamus of the mouse. The average number of MCH cells counted was 83.96 ± 0.80, corresponding to an estimated total number of 2364 ± 86 MCH+ neurons. By moving through the z plane, the double labeling of the cells for the $GABA_A$Rs on the membrane or $GABA_B$Rs in the cytoplasm was determined. Estimated total numbers of double-labeled cells were computed for each series ($GABA_A$R-Orx or $GABA_A$R-MCH in 12 VTM and $GABA_B$R-Orx or $GABA_B$R-MCH in 13 VM) and expressed as % of Orx+ or MCH+ cell populations per series.

Luminance measurements were performed on the Orx+ and MCH+ cells that had been counted as positively stained for $GABA_A$R or $GABA_B$R in the images randomly acquired and counted using Optical Fractionator (above). So as to analyze similar numbers across groups, 8-10 double-labeled Orx+ or MCH+ cells, which were present in all animals, were analyzed per animal. The images had been acquired under the same gain and exposure for each series using an 8-bit setting of the digital camera to yield arbitrary units between 0 and 256 in the converted gray scale of the fluorescent images. To measure the luminance of the receptors, different approaches were used for the $GABA_A$Rs concentrated over the plasma membrane vs. the $GABA_B$Rs located in the cytoplasm as well as over the membrane. For membrane $GABA_A$Rs, a box of 1.5x0.3 μm^2 was placed over the membrane and another over the nucleus to measure

and subtract background staining in each cell. For membrane plus cytoplasmic $GABA_BRs$, a donut-shaped contour was drawn around the cytoplasm and plasma membrane, and another traced around the nucleus to measure and substract background staining in each cell.

Cell counts and luminance measurements were analyzed between experimental groups for each cell type (Orx or MCH) and receptor ($GABA_A$ or $GABA_B$) using one-way analysis of variance (ANOVA) for main effect of group followed by post-hoc paired comparisons with Tukey's HSD correction for differences between groups (SYSTAT Software Inc., version 13, Table 1). Given that there was no significant difference between the two SD2 and SD4 h groups, they were combined into one SD group.

Sections were also viewed and images acquired for this publication with an LSM 710 confocal laser scanning microscope equipped with Ar 488-nm, He-Ne 543-nm and He-Ne 633-nm lasers for excitation and emission of Cy2, Cy3 and Cy5 dyes. Image stacks were acquired under 63x oil objective (1.4 numerical aperture, 0.5 μm thickness for each optical section) with a 1.0 airy unit pinhole size for each channel. All figures were prepared and composed in a consistent manner for brightness and contrast across groups using Adobe Creative Suite (CS4, Adobe System).

Results

Sleep-wake states across groups

Mice were prevented from falling asleep in the SD group (n = 11) and were thus continuously awake, whereas those in the SC group (n = 7) and SR group (n = 7) were awake for only a small percentage of the time during the 2 h prior to termination at ~4 P.M. (Figure 1A and Table 1). After having been previously sleep deprived, mice in the SR group were awake less or reciprocally asleep significantly more of the time (92.61 ± 2.21 %, Mean ± SEM) than the SC mice (76.77 ± 2.56 %), indicating a homeostatic response to SD. Mice in SC and SR groups spent the majority of time in NREM sleep (66.93 ± 1.71 %, n = 3 and 82.29 ± 4.07 %, n = 3, respectively) and minimal time in REM sleep (9.28 ± 0.89 %, n = 3 and 12.03 ± 0.87 %, n = 3, respectively). Both NREM and REM sleep were significantly increased during SR relative to SC (another manuscript by the Authors).

$GABA_A$Rs on Orx and MCH neurons after SD and SR

Triple-stained sections for $GABA_A$R/FNS with either Orx or MCH were analyzed to assess the presence and intensity of $GABA_A$Rs on Orx and MCH neurons across the three groups (SC, SD and SR). $GABA_A$R immunostaining appeared to be located on the plasma membrane of the Orx and MCH neurons, as well as that of other surrounding neurons (Figures 2 and 3).

$GABA_A$R immunostaining was minimal and patch-like on the plasma membrane of the Orx-positive (+) neurons, whereas it was often moderate and continuous on the membrane of surrounding Orx-negative neurons in the same sections (Figure 2). Though minimal on the Orx+ neurons, the $GABA_A$R immunostaining appeared to be more intense after SD as compared to that after SC or SR (Figure 2A-C). The average proportion of the Orx+ neurons which appeared

positively immunostained (+) for $GABA_A$Rs on the membrane was significantly greater in the SD group (48.45 ± 4.09%, n = 6 mice) compared to that in the SC and SR groups (23.93 ± 3.74%, n = 3 and 26.93 ± 4.37%, n = 3, respectively; Figure 1B1 and Table 1). The average luminance of the $GABA_A$R immunostaining on the Orx+ neurons was also significantly higher in SD (58.71 ± 2.90, n = 60 cells) than in SC and SR groups (46.4 ± 3.42, n = 30 and 43.17 ± 2.56, n = 28, respectively; Figure 1C1 and Table 1). The luminance measures did not differ between SC and SR, indicating that the $GABA_A$R returned to control or baseline levels during SR.

$GABA_A$R immunostaining appeared to be relatively continuous around the plasma membrane of the MCH+ neurons and somewhat more intense as compared to that on Orx+ neurons (Figure 3). Moreover, the $GABA_A$R immunostaining on the MCH+ neurons appeared to be moderate in the SC and SR groups (Figure 3A-C). In contrast, it appeared minimal following SD, even though it was prominent on surrounding MCH-negative neurons (Figure 3B). The average proportion of MCH+ neurons which appeared $GABA_A$R+ decreased, though not significantly so, following SD (42.86 ± 6.40%, n = 6 mice) as compared to SC and SR (54.38 ± 3.74%, n = 3 and 55.7 ± 2.55%, n = 3 respectively; Figure 1B2 and Table 1). The average luminance of the $GABA_A$R on the MCH+ neurons decreased significantly after SD (34.43 ± 2.45, n = 60 cells) as compared to SC and SR (45.95 ± 3.31, n = 30 and 44.95 ± 2.59, n = 30, respectively; Figure 1C2 and Table 1). The measures did not differ between SC and SR, indicating that the $GABA_A$R returned to control or baseline levels during SR.

$GABA_B$Rs on Orx and MCH neurons after SD and SR

Triple-staining for $GABA_B$R/FNS and either Orx or MCH was performed to examine the incidence of $GABA_B$Rs on Orx or MCH neurons across the three groups (SC, SD and SR).

GABA$_B$R immunostaining appeared to be predominantly located over the cytoplasm of the cells while only minimally located on the membrane of both the Orx and MCH neurons (Figures 4 and 5).

In the Orx+ neurons, GABA$_B$R immunostaining was prominent in the soma and proximal dendrites and appeared to be more dense and intense after SD than after SC and SR (Figure 4A-C). Nonetheless, all Orx+ neurons (100%) were judged to be positively immunostained for the GABA$_B$R in all mice of all groups (n = 4 in SC and SR groups, n = 5 in SD group, Figure 1D1 and Table 1). On the other hand, the luminance of the GABA$_B$R in the Orx neurons was significantly higher following SD (22.84 ± 1.35, n = 50 cells) as compared to SC and SR (14.36 ± 1.21, n = 40 and 14.76 ± 1.03, n = 40, respectively; Figure 1E1 and Table 1). The luminance did not differ between SC and SR, indicating that the GABA$_B$R returned to control or baseline levels during SR.

In the MCH+ neurons, GABA$_B$R immunostaining was prominent in the soma and appeared to be more dense following SC and SR than after SD (Figure 5A-C). As for the Orx+ neurons, GABA$_B$R immunostaining was nonetheless judged to be positive in all MCH+ neurons (100%) and in every group (n = 4 in SC and SR groups, n = 5 in SD group, Figure 1D2 and Table 1). On the other hand, the luminance of GABA$_B$R immunostaining on the MCH+ neurons was significantly lower after SD (24.53 ± 1.59, n = 50 cells) compared to SC and SR (43.24 ± 2.87, n = 40 and 44.92 ± 2.33, n = 40, respectively; Figure 1E2 and Table 1). The luminance did not differ between SC and SR, indicating that the GABA$_B$R returned to control or baseline levels during SR.

Discussion

The present results indicate that $GABA_A$ and $GABA_B$ receptors undergo dynamic and differential changes on Orx, wake-active and MCH, sleep-active neurons as a function of SD and thus their homeostatic response to different activity changes.

$GABA_A$Rs differentially expressed as a function of sleep-wake activity

SD during the day, when mice normally sleep the majority of the time, resulted in increased $GABA_A$R labeling on the membrane of the Orx neurons presumably due to prolonged activity by the Orx neurons during enforced waking, as indicated by previous c-Fos and recording studies (Lee et al., 2005; Modirrousta et al., 2005). The Orx neurons show changes in $GABA_A$Rs that are parallel to those for cholinergic basal forebrain neurons following SD, when those neurons are also active, as indicated by c-Fos expression (Modirrousta et al., 2007). And in the whole hypothalamus, mRNA expression for $GABA_A$R (ß-subunits) is also increased after SD and high activity periods (Volgin et al., 2014). In contrast, however, SD resulted in decreased $GABA_A$R labeling on the membrane of MCH neurons here, presumably due to silence of the MCH neurons during waking, as indicated in previous c-Fos and recording studies (Verret et al., 2003; Modirrousta et al., 2005; Hassani et al., 2009). The changes in $GABA_A$R density on the membrane seen here in the Orx and MCH neurons are similar to those described in cultured hippocampal neurons following pharmacologically induced firing and silencing, respectively (Kilman et al., 2002; Marty et al., 2004). These changes in the density of $GABA_A$R clusters in the cultured neurons were moreover associated with increased vs. decreased amplitude of miniature inhibitory postsynaptic currents (mIPSCs). An increase in membrane $GABA_A$Rs was also shown to occur in hippocampal neurons *in vivo* after increased activity induced by seizures

and was associated with an increase in inhibitory postsynaptic currents (IPSCs) (Nusser et al., 1998). This increase in postsynaptic receptors appears to be the most effective way by which the magnitude of inhibitory transmission is increased (Mody et al., 1994). Somewhat similar to ours, another study in mice showed that $GABA_AR$ immunostaining (for the α1 subunit) was enhanced and that the sensitivity to a $GABA_AR$ agonist was increased along with the amplitude of IPSCs in Orx neurons following 6 h SD (Matsuki et al., 2015). The latter along with our results for the Orx neurons would appear to differ from those in rats showing increased amplitude of miniature excitatory postsynaptic currents (mEPSCs) in Orx neurons following 4 h SD (Rao et al., 2007). However, the latter *in vitro* or *ex vivo* study was done in the presence of a $GABA_AR$ blocker (bicuculline) which did not allow assessment of changes in $GABA_AR$ currents and their potential influence on the mEPSCs. We can only assume that the increased activity by the Orx neurons during prolonged enforced waking stimulates homeostatic down-scaling through increases in membrane $GABA_ARs$, which would render the neurons more susceptible and responsive to inhibition by GABA. Reciprocally, the prolonged absence of activity by the MCH neurons during prolonged waking stimulates homeostatic up-scaling through decreases in membrane $GABA_ARs$, which would render them less susceptible and responsive to inhibition by GABA. The $GABA_ARs$ returned to baseline levels with SR, indicating a return to normal levels of excitability and activity in both Orx and MCH cells.

$GABA_BRs$ differentially expressed as a function of sleep-wake activity

With regard to the metabotropic $GABA_BR$ receptor, we found that all of the Orx and MCH neurons showed positive immunostaining for that receptor across all groups. On the other hand, the density of the $GABA_BR$ immunostaining appeared to differ according to cell type and group.

By measurement of luminance, it was found that the intensity of $GABA_BRs$ in Orx neurons increased with SD presumably due to enhanced and prolonged activity with enforced waking, whereas that in MCH neurons decreased with SD, presumably due to prolonged silence. These results thus paralleled those of the $GABA_AR$. In the case of the $GABA_BR$, however, the immunostaining was most prominent in the cytoplasm and less evident on the plasma membrane. Although we did see staining along the membrane in some cases, we did not have adequate resolution for differentiation and systematic assessment of the membrane staining across groups. We can only assume that the different densities of $GABA_BRs$ with SD reflect different expression of the receptor in homeostatic response to different activities of the Orx and MCH neurons under the abnormal conditions of sustained waking during the day when mice normally sleep the majority of the time. As with the $GABA_ARs$, the density of $GABA_BRs$ returned to SC levels with SR, presumably reflecting the re-establishment of stable levels of excitability and activity during recovery sleep for both the Orx and MCH cells. Evidence from cultured hippocampus has indicated that the $GABA_BR$ is essential for homeostatic regulation of firing within hippocampal circuits through both pre- and postsynaptic mechanisms (Vertkin et al., 2015). Indeed, it has been known that genetic deletion of the $GABA_BR$ results in runaway excitation within these and cortical circuits resulting in seizure activity (Schuler et al., 2001), and that seizure activity is followed by increases in $GABA_BRs$ in hippocampal neurons (Straessle et al., 2003). Deletion of the $GABA_BR$ also leads to disruption of the sleep-wake cycle in mice (Vienne et al., 2010). Fragmentation of the cycle also occurred in mice lacking $GABA_BRs$ specifically on Orx neurons (Matsuki et al., 2009).

Role of GABA receptors in neuronal homeostasis and sleep-wake regulation

GABA receptors, particularly $GABA_A$Rs, have been shown to play an important role in the homeostatic regulation of neuronal excitability as a function of activity (Turrigiano, 1999). Here, we present evidence that dynamic and differential changes in both $GABA_A$ and $GABA_B$ receptors after SD reflect homeostatic down-scaling following prolonged activity by the wake-active, Orx neurons and up-scaling following inactivity by the sleep-active, MCH neurons.

Both $GABA_A$ and $GABA_B$ receptors, along with GABA, are known to play an important role in sleep. Most hypnotic drugs act upon the benzodiazepine binding site of the $GABA_A$R to enhance GABA-mediated currents (Wafford and Ebert, 2008; Winsky-Sommerer, 2009; Feren et al., 2011). Some, like anesthetic agents (e.g. barbiturates), act directly upon the $GABA_A$R ion channel (Franks, 2008). Interestingly, anesthesia with $GABA_A$R agonists (e.g. propofol) can actually serve in the homeostatic response to SD in place of natural sleep recovery (Tung and Mendelson, 2004). Reciprocally, SD lowers the threshold to anesthesia induction, likely due to homeostatic changes in the $GABA_A$R. Gamma hydroxybutyrate (GHB) used in the treatment of narcolepsy with cataplexy acts upon the $GABA_B$R to consolidate sleep with low muscle tone during sleeping periods, such as to reduce narcoleptic attacks during the following waking period in humans and rodents (Xie et al., 2006; Vienne et al., 2010; Boscolo-Berto et al., 2012; Black et al., 2014). Moreover, GHB or its metabolite can alleviate the behavioral and physiological effects of sleep deprivation (Walsh et al., 2010). These results would also suggest that pharmacological effects upon the $GABA_B$R, as upon the $GABA_A$R, can mimic the homeostatic effects of sleep. However, such pharmacological effects are rarely cell specific and thus can affect both wake- and sleep-active cell groups, which as we show here would normally undergo differential homeostatic changes in their GABA receptors depending upon their state selective activity.

376 Sleep is regulated in a homeostatic manner (Borbely and Achermann, 1999), whereby SD
377 is compensated for by enhanced NREM or slow wave sleep and delta EEG activity along with
378 increased REM sleep. Whereas Orx neurons normally promote waking and prevent sleep
379 including importantly REM sleep with muscle atonia (Adamantidis et al., 2007), MCH neurons
380 normally enhance sleep including importantly REM sleep with muscle atonia (Verret et al.,
381 2003; Jego et al., 2013; Konadhode et al., 2013). The reciprocal changes in the inhibitory
382 GABA receptors and presumed excitability and activity of the Orx and MCH neurons seen here
383 with SD could thus underlie the homeostatic response of decreased arousal and increased
384 sleepiness during deprivation and increased sleep, including REM sleep during recovery.
385
386 We conclude that expression and density of both $GABA_A$ and $GABA_B$ receptors increase
387 on Orx neurons because of prolonged activity and reciprocally decrease on MCH neurons
388 because of prolonged inactivity during SD. These reciprocal changes in excitability of the Orx
389 and MCH neurons could decrease arousal and increase sleepiness along with sleep pressure
390 during SD. During SR, the GABA receptors return to baseline presumably returning the
391 excitability and activity of the Orx and MCH neurons to stable levels and thus restoring normal
392 arousal while removing sleep pressure.
393
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Figure legends

Figure 1. Sleep-wake states and GABA receptors in Orx and MCH neurons across groups. ***A,*** Percentage of time spent in wake during the 2 h preceding termination in SC, SD and SR groups. % Wake is significantly higher in SD as compared to SC and SR and significantly lower in SR as compared to SC. ***B,*** Proportion of Orx+ or MCH+ neurons bearing $GABA_AR$s across groups. The % Orx+/$GABA_AR$+ was significantly greater in SD as compared to SC and SR (***B1***), whereas the % MCH+/$GABA_AR$+ neurons was insignificantly less in SD as compared to SC and SR (***B2***). ***C,*** Luminance of the $GABA_AR$ immunofluorescence on Orx and MCH neurons across groups, which was significantly increased on the Orx+ neurons (***C1***) and decreased on the MCH+ neurons (***C2***) in SD as compared to SC and SR. ***D,*** Proportion of the Orx (***D1***) and MCH (***D2***) neurons expressing $GABA_BR$s, which did not change across groups. ***E,*** Luminance of the $GABA_BR$ which was significantly higher in Orx+ neurons (***E1***), and significantly lower in MCH+ neurons (***E2***) following SD as compared to SC and SR. Note that the changes in GABARs on Orx neurons parallel the % Wake, whereas those on MCH+ neurons parallel the % Sleep across groups. * indicates significant difference of SD relative to SC and SR ($p < 0.05$). § indicates significant difference of SR relative to SC ($p < 0.05$), according to post hoc paired comparisons following one-way ANOVA; see Table 1.

Figure 2. $GABA_AR$s in Orx neurons across groups. Confocal images of immunostained sections indicate that the $GABA_AR$ (red) was minimal on Orx+ neurons (blue, indicated by filled arrowheads) as compared to that on adjacent Orx-negative neurons (stained with FNS in green, indicated by carets). ***A,*** The $GABA_AR$ immunofluorescence was minimally visible as small clusters along a portion of the plasma membrane of an Orx+ cell body in an SC mouse, in which

it was readily visible along the full membrane of an Orx-negative cell body. ***B,*** The $GABA_AR$ staining was more visible as larger clusters along a larger portion of the membrane of an Orx+ cell in an SD mouse. ***C,*** The $GABA_AR$ staining was similar in an SR mouse to that in SC. Scale bars: 10μm. Image thickness: 1500 nm in panels ***A*** and ***B***; 2000 nm in panel ***C***.

Figure 3. $GABA_ARs$ in MCH neurons across groups. Confocal images indicate that $GABA_AR$ immunostaining (red) was moderate in MCH neurons (blue, indicated by filled arrowheads) though less than that in some adjacent MCH-negative neurons (stained with FNS, green, indicated by carets). ***A,*** $GABA_AR$ immunostaining was present as clusters visible along the full plasma membrane of the cell body in an SC mouse. ***B,*** $GABA_AR$ immunostaining was barely visible on MCH+ neurons, whereas it was prominent on adjacent MCH-negative neuron in an SD mouse. ***C,*** $GABA_AR$ immunostaining appeared to be moderate in an SR mouse, similar to that in SC. Scale bars: 10μm. Image thickness: 500 nm in all panels.

Figure 4. $GABA_BRs$ in Orx neurons across groups. Confocal images of the $GABA_BR$ immunostaining (red) in Orx neurons (blue, indicated by filled arrowheads). ***A,*** The $GABA_BR$ immunofluorescence was minimally visible as clusters over the cytoplasm of an Orx+ cell body in an SC mouse. ***B,*** The $GABA_BR$ staining was more visible as larger clusters over the cytoplasm and partially on the plasma membrane of an Orx+ cell in an SD mouse. ***C,*** The $GABA_BR$ staining was similar in an SR mouse to that in SC. Scale bars: 10μm. Image thickness: 1500 nm in all panels.

Figure 5. $GABA_BRs$ in MCH neurons across groups. Confocal images of the $GABA_BR$

immunostaining (red) in MCH neurons (blue, indicated by filled arrowheads). ***A,*** $GABA_BR$ immunostaining was present as prominent large clusters over the cytoplasm of an MCH+ neuron in an SC mouse. ***B,*** The $GABA_BR$ immunostaining was minimally visible over the cytoplasm of an MCH+ neuron in an SD mouse. ***C,*** The $GABA_BR$ immunostaining appeared to be prominent over the cytoplasm and near the plasma membrane of an SR mouse, similar to that in SC. Scale bars: 10μm. Image thickness: 2000 nm in panel ***A***; 1500 nm in panels ***B*** and ***C.***

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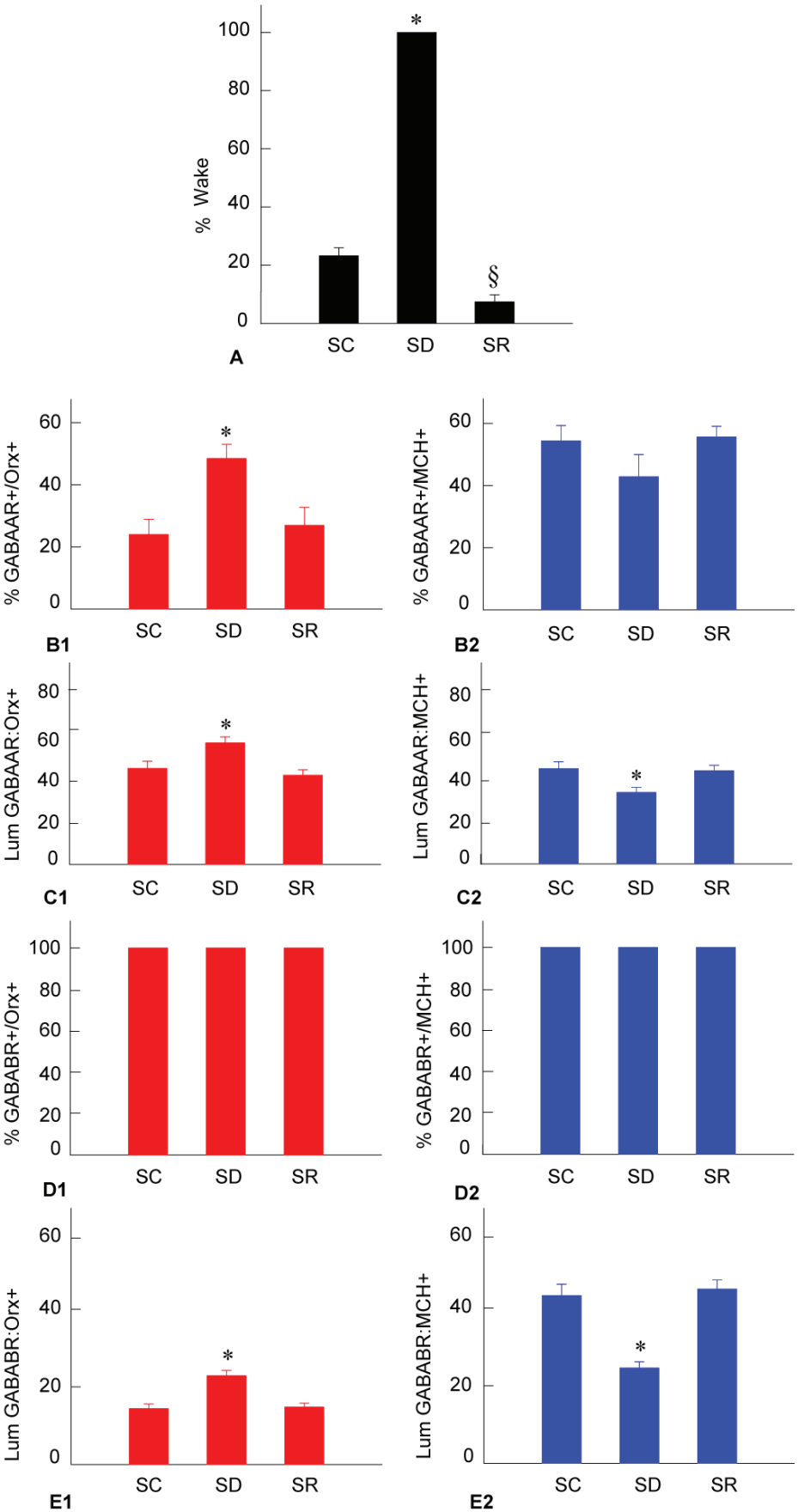

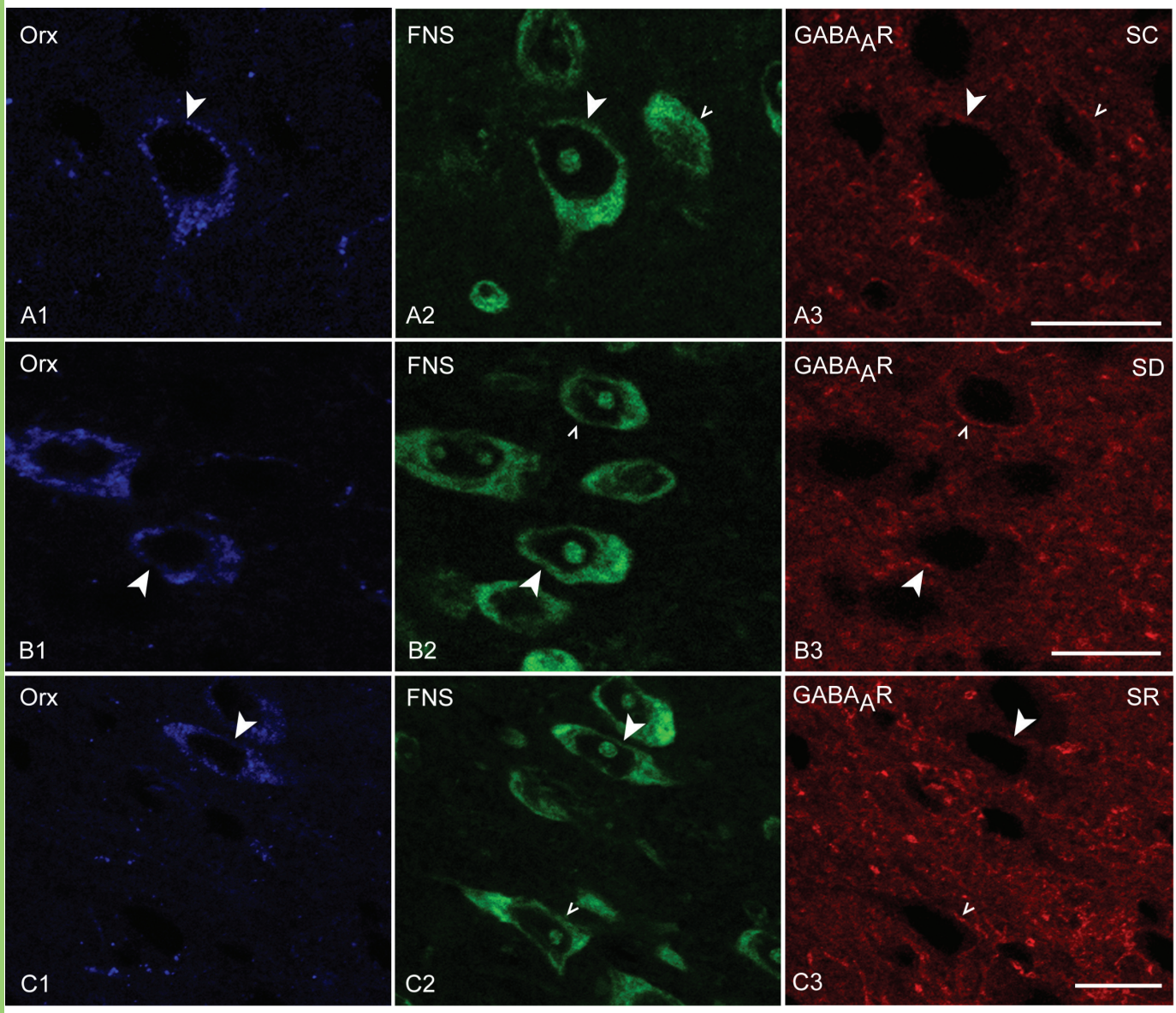

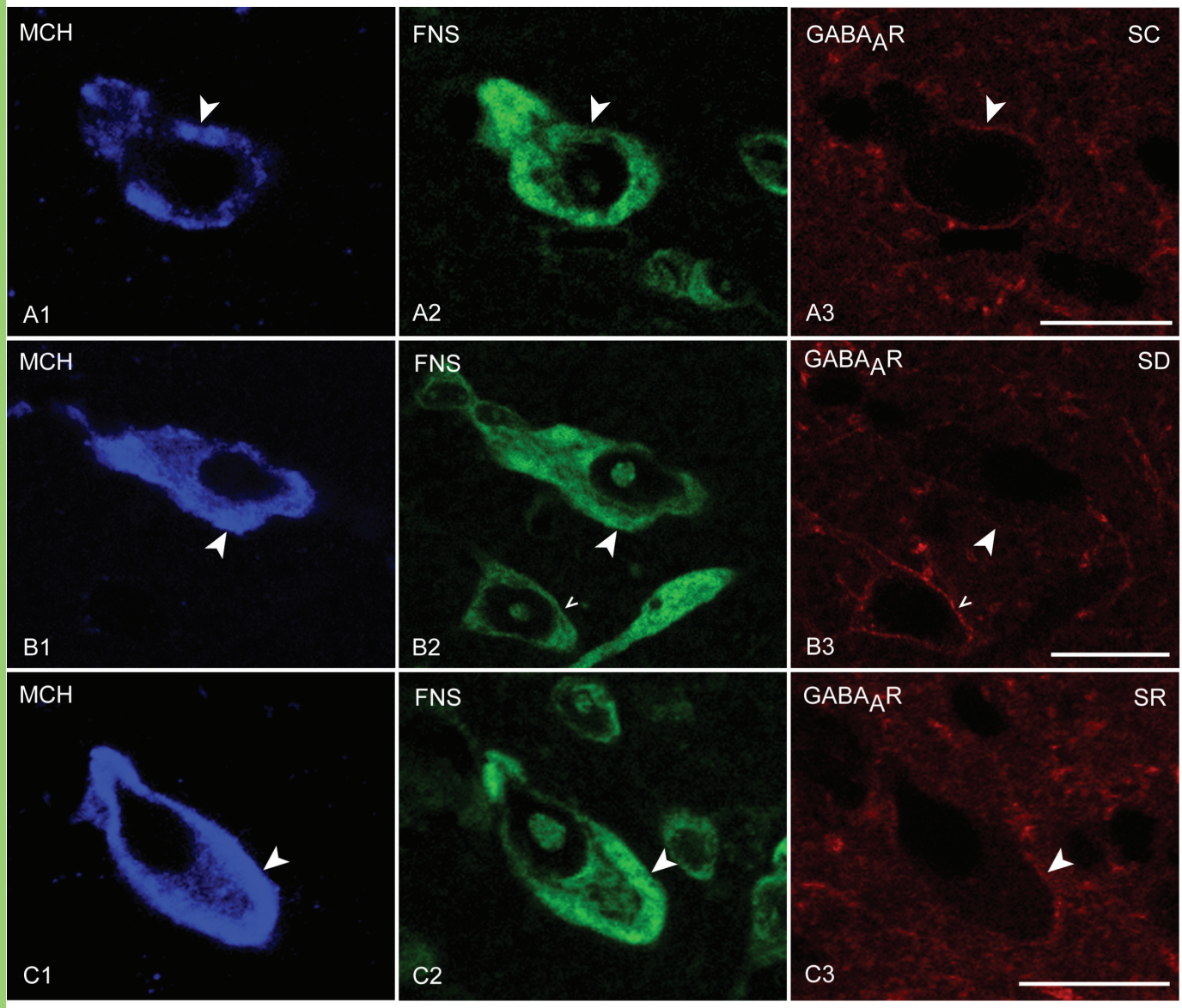

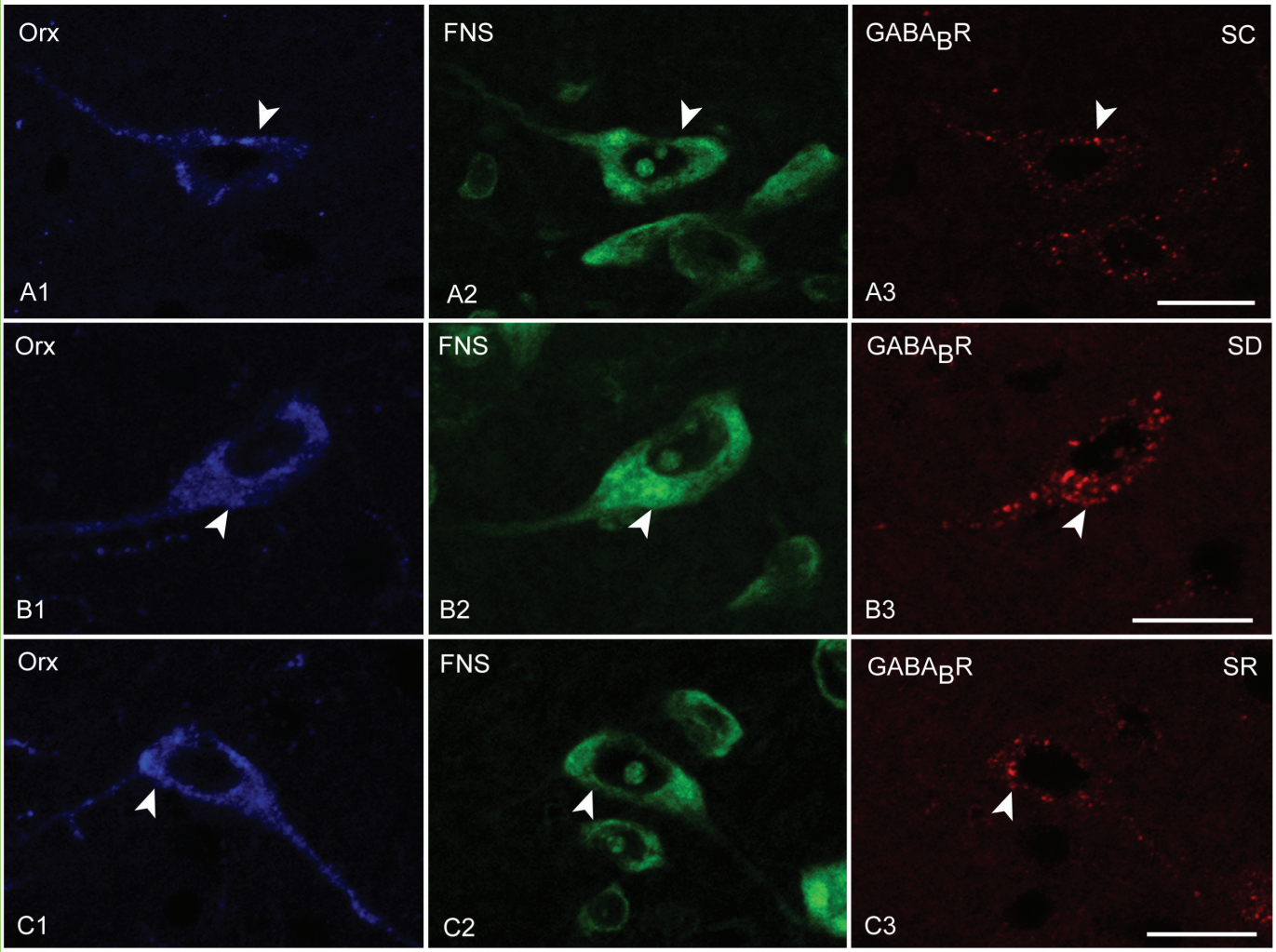

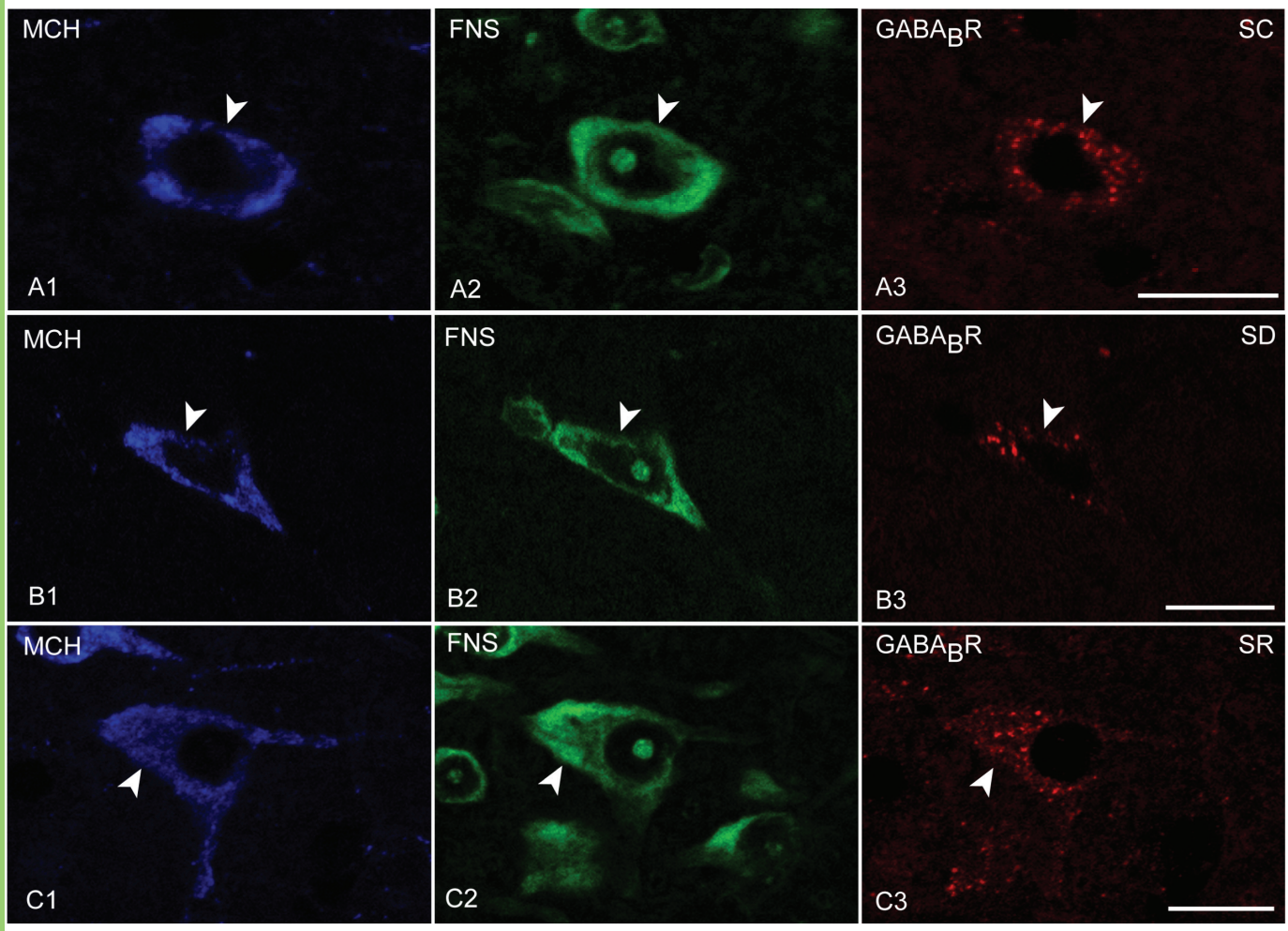

Table 1. Summary of statistics

Data Set	Figure	One-way ANOVA (Group = 3 levels) F value	df (group, error)	p value	Tukey's HSD Paired Comparisons p value		
					SC-SD	SC-SR	SD-SR
% Wake	1A	1032.336	2, 22	<0.001	<0.001*	<0.001§	<0.001*
% $GABA_AR$+/Orx+	1B1	10.27	2, 9	0.005	0.009*	0.910	0.010*
% $GABA_AR$+/MCH+	1B2	1.48	2, 9	0.270	n/a	n/a	n/a
Lum $GABA_AR$:Orx+	1C1	7.43	2, 115	0.001	0.010*	0.800	0.002*
Lum $GABA_AR$:MCH+	1C2	5.88	2, 117	0.004	0.010*	0.970	0.020*
% $GABA_BR$+/Orx+	1D1	n/a	n/a	n/a	n/a	n/a	n/a
% $GABA_BR$+/MCH+	1D2	n/a	n/a	n/a	n/a	n/a	n/a
Lum $GABA_BR$:Orx+	1E1	15.93	2, 127	<0.001	<0.001*	0.970	<0.001*
Lum $GABA_BR$:MCH+	1E2	27.16	2, 127	<0.001	<0.001*	0.860	<0.001*