Homeostatic regulation through GABA and acetylcholine muscarinic receptors of motor trigeminal neurons following sleep deprivation

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Received: 8 August 2016 / Accepted: 20 February 2017 / Published online: 15 March 2017
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Abstract Muscle tone is regulated across sleep-wake states, being maximal in waking, reduced in slow wave sleep (SWS) and absent in paradoxical or REM sleep (PS or REMS). Such changes in tone have been recorded in the masseter muscles and shown to correspond to changes in activity and polarization of the trigeminal motor 5 (Mo5) neurons. The muscle hypotonia and atonia during sleep depend in part on GABA acting upon both GABA_A and GABA_B receptors (Rs) and acetylcholine (ACh) acting upon muscarinic 2 (AChM2) Rs. Here, we examined whether Mo5 neurons undergo homeostatic regulation through changes in these inhibitory receptors following prolonged activity with enforced waking. By immunofluorescence, we assessed that the proportion of Mo5 neurons positively stained for GABA_A Rs was significantly higher after sleep deprivation (SD, ~65%) than sleep control (SC, ~32%) and that the luminance of the GABA_A fluorescence was significantly higher after SD than SC and sleep recovery (SR). Although, all Mo5 neurons were positively stained for GABA_BRs and AChM2Rs (100%) in all groups, the luminance of these receptors was significantly higher following SD as compared to SC and SR. We conclude that the density of GABA_A, GABA_B and AChM2 receptors increases on Mo5 neurons during SD. The increase in these receptors would be associated with increased inhibition in the presence of GABA and ACh and thus a homeostatic down-scaling in the excitability of the Mo5 neurons after prolonged waking and resulting increased susceptibility to muscle hypotonia or atonia along with sleep.

Keywords GABA_A · GABA_B · AChM2 · Mice · Muscle atonia · Waking

Introduction

Motor activity and muscle tone are regulated across the sleep-waking cycle. Muscle tone is maximal in alert waking, reduced in slow wave sleep (SWS) and absent in paradoxical or REM sleep (PS or REMS) (Jouvet 1967; Chase 1983). During this cycle, motor neurons in the brainstem and spinal cord fire during waking, decrease their firing during SWS and cease firing during REMS. Intracellular recordings showed that the motor trigeminal, 5th nerve (Mo5) neurons are slightly hyperpolarized during SWS and strongly hyperpolarized during REMS relative to active waking (Chandler et al. 1980; Chase et al. 1980), as are also motor neurons of the motor hypoglossal, 12th nerve (Mo12) and spinal cord (Morales and Chase 1978; Fung and Chase 2015). The post-synaptic inhibition of the Mo5 and motor spinal neurons was shown to be blocked by strychnine and thus dependent upon the inhibitory neurotransmitter, glycine and its postsynaptic receptors (Soja et al. 1987; Chase et al. 1989). Yet, studies also indicated that the atonia of the masseter muscles could not be fully blocked by antagonism of glycine receptors with strychnine alone but only by the additional antagonism of GABA receptors, and not solely of GABA_ARs with bicuculline but only additionally of GABA_BRs with baclofen (Soja et al. 1987; Brooks and Peever 2008, 2012). It was also found that glycine and GABA_A receptor antagonism increased tone during waking and SWS as well as REMS.
in both masseter and genioglossus muscles (Soja et al. 1987; Brooks and Peever 2008; Morrison et al. 2003). In the genioglossus muscles, it was discovered that the muscle atonia during REMS could be selectively prevented by antagonism of acetylcholine (ACh) muscarinic (M) receptors using scopolamine or by blocking the inhibitory pathway through the G-protein coupled inwardly rectifying potassium channel (GIRK) to which the AChM2R is linked (Grace et al. 2013a, b). Given the demonstrated post-synaptic inhibitory action of the AChM2R through GIRK channels in motor neurons (Chevallier et al. 2006; Miles et al. 2007; Zhu et al. 2016), the demonstrated presence of AChM2Rs on Mo5 neurons (Hellstrom et al. 2003; Brischoux et al. 2008) and the established role of ACh in PS or REMS (Jones 1991), it would appear that such AChM2R-mediated inhibition could well occur during REMS. These results indicate that motor neurons in the brainstem, including the Mo5 neurons, are inhibited during sleep by both ionotropic glycine/GABA receptors and metabotropic GABAB and AChM2 receptors.

The sleep-waking cycle is known to be regulated in a homeostatic manner, since sleep deprivation (SD) with enforced waking leads to a subsequent decrease in waking and increase in sleep, including both SWS and REMS (Borbely et al. 1984). The homeostatic drive is evident following SD in both cortical activity, as an increase in slow wave activity, and peripheral muscle tone, as an increase in muscle atonia (Werth et al. 2002; Borbely and Achermann 1999; Tobler and Borbely 1986), which suggest homeostatic changes in central neurons, including motor neurons.

The activity of individual neurons is also known to be regulated in a homeostatic manner, such that prolonged increases in activity can lead to decreases in activity associated with decreases in excitability of the individual neurons (Turrigiano 1999). These cell autonomous adjustments have been referred to as homeostatic synaptic scaling that is triggered and sensed by altered levels of neuronal discharge and/or membrane polarization and is mediated by global changes in excitatory and/or inhibitory receptors in individual neurons (Turrigiano et al. 1998; Kilman et al. 2002; Marty et al. 2004). In both in vitro and in vivo studies, prolonged activity has been shown to lead to increases in GABA_A receptors that are associated with increases in post-synaptic inhibitory currents (Nusser et al. 1998; Marty et al. 2004). We thus hypothesized that prolonged activity of motor neurons during enforced waking with SD could lead to homeostatic increases in inhibitory receptors, including GABA_A, GABA_B and AChM2 receptors. We investigated this possibility by quantitative assessment of the receptors in immunofluorescent-stained sections from brains of mice following enforced waking with SD compared to those following normal or enhanced sleep with sleep control (SC) and sleep recovery (SR) conditions. The work has now been published in abstract form (Toossi et al. 2016a).

Materials and methods

All procedures were performed according to the guidelines of the Canadian Council on Animal Care and approved by the animal care committee of McGill University.

Sleep deprivation and recovery experimental procedures

A total number of 12 adult male mice (C57BL/6, 20-25g) were received from the supplier (Charles River) and housed individually under 12-h light : 12-h dark schedule (lights on from 7:00 to 19:00) at 22 °C ambient temperature and with unlimited access to food and water at all time. Animals were maintained in their home cages for the duration of the experiment and recorded by video and telemetric electroencephalogram (EEG) using HomeCageScan software (HomeCageScan™ 3.0; Clever Systems) (del Cid-Pellitero, Plavski and Jones, unpublished results). For telemetric recording of the EEG, two electrodes were placed symmetrically over parietal cortex along with two for reference over cerebellum and were connected by wires to a transmitter (F20-EET, Data Sciences International, DSI) implanted subcutaneously along the flank. Following surgery, the mice were allowed 1 week to recover.

The three experimental groups were composed of: (1) sleep control (SC) mice allowed to sleep undisturbed for 2 h from ~14:00 to ~16:00 (~ZT 7–9) (n = 3), (2) sleep deprived mice (SD) maintained awake for 2 h (n = 3) or 4 h (n = 3) from ~12:00 to ~16:00 (~ZT 5–9) and (3) sleep recovery (SR) mice allowed to sleep for 2 h from ~14:00 to ~16:00 (~ZT 7–9) after being maintained awake for 4 h prior to euthanasia (n = 3). The mice were maintained awake by gentle stimulation with a soft paintbrush of the whiskers each time the mouse appeared to be preparing to sleep. Mice were immediately anaesthetized after the experimental period at ~16:00 (~ZT 9) with sodium pentobarbital (Euthanyl, 100 mg/kg; Bimeda-MTC) and perfused transcardially with 30 ml of cold saline followed by 200 ml of 3% paraformaldehyde solution. Brains were removed, post-fixed in 3% paraformaldehyde for 1 h at 4°C, then placed in 30% sucrose solution at 4°C for 2 days, frozen to −50°C and stored at −80°C.

Sleep and waking were scored by behavior and EEG using HomeCageScan software.
Immunohistochemistry

Brains were cut and processed for fluorescent staining 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 through the brainstem on a freezing microtome at a 20 µm thickness and collected in 5 adjacent series, such that sections were separated by 100 µm intervals in each series. Free floating sections were rinsed in 0.1 M 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 one of the primary antibodies. The following antibodies were employed: mouse anti-GABAAR β2-3-chain (clone BD17, 1:100, Millipore (Chemicon), CAT# MAB 341, RRID: AB_2109419), guinea pig anti-GABAAR1 (1:2500, Millipore (Chemicon), CAT# AB1531, RRID: AB_2314472) or rabbit anti-AChM2R (1:600, Sigma, CAT# M9558, RRID: AB_260727). Subsequently, sections were incubated at room temperature for 2 h in Cyanine-conjugated (Cy3) secondary antibodies from donkey (Jackson ImmunoResearch Laboratories): Cy3-conjugated anti-mouse (1:1000, CAT# 715-165-150, RRID: AB_2340813), Cy3-conjugated anti-guinea pig (1:1000, CAT# 706-165-148, RRID: AB_2340460) or Cy3-conjugated anti-rabbit (1:1000, CAT# 711-165-152, RRID: AB_2307443). Sections were subsequently stained with green fluorescent Nissl stain (FNS) (1:2000, CAT # N-21,480, Molecular Probes) for 20 min. Finally, sections were rinsed, mounted and coverslipped with glycerol (Fisher).

All the receptor antibodies employed were produced and characterized years ago and have since been in use over many years (as cited herewith). For the GABAAR, the antibody against the β2-3-chain was employed because it stains the most prevalent types of GABAAR heterodimeric receptors on neurons in the brain and stains clusters of the GABAAR-R on the plasma membrane, which are associated with functional inhibitory post-synaptic currents (IPSCs) (Fritschy and Mohler 1995; Wan et al. 1999; Nusser et al. 1998). For the GABAAR, the antibody against the R1 subunit was employed because it also stains vast populations of neurons in the brain and is visible over cytoplasmic organelles and the plasma membrane, where it forms together with the R2 subunit the heteromeric functional receptor (Margeta-Mitrovic et al. 1999; Filippov et al. 2000; Straessle et al. 2003). For the AChM2R, the antibody employed was shown to be highly specific and to stain most prominently the surface membranes of all cholinergic as well as diverse noncholinergic neurons in the brain (Levey et al. 1991).

Immunohistochemical image analysis

Stained sections were viewed using a Leica DMLB microscope equipped with x/y/z motorized stage, a digital camera (Orca-R2, C10600-10B, Hamamatsu photonics K.K) and fluorescence filters for excitation and emission of Cy2 and Cy3 dyes. Images were acquired and analyzed using the Optical Fractionator Probe of StereoInvestigator (MicroBrightField, MBF), which allows unbiased, systematic random sampling of a region of interest for cell number estimation or measurement of specific parameters, including luminance. Given application of systematic random sampling for examining and marking cells at high magnification and the subsequent measurement of fluorescence intensity of the receptor staining in the marked receptor + cells employed here, double blind procedures were not applied in this process. In each series, three sections (at 100 µm intervals) were taken through the Mo5 nucleus. In each section, a contour was traced around the Mo5 nucleus under a 5x objective. Multi-channel image stacks with 0.5 µm thickness of optical sections were then acquired under a 40x objective through the mounted histological section of approximately 15 µm thickness. For the image acquisition, the exposure time and contrast were set for each receptor series according to the parameters which provided suitable images of the brightest to the dimmest fluorescence. These parameters were maintained for all sections and mice across all groups for each receptor series. In the Optical Fractionator Probe, a grid size of 200×200 µm² and a counting frame of 120×120 µm² were used in the image acquisition and assessment with marking of positively labeled cells. Across the three sections, approximately 21 counting frames for Mo5 neurons were acquired and analyzed per series. Within these images, cell somata with visible nuclei located >1 µm below the surface of the section were marked for counting, thus through 14 µm of the section. The motor neurons were identified in the FNS stained sections by their distinct morphology and thus referred to as MoFNS-positive (+). The average number of MoFNS+ cells counted across series on one side was 32.86 ± 1.16 (mean ± SEM). For marking of cells positively stained for receptors, the immunostaining for the GABAAR, GABAβ and AChM2 receptors on the membrane or over the cytoplasm of the MoFNS + soma was assessed by moving through the z stack of 0.5 µm thick optical sections of each MoFNS + cell soma. Estimated total numbers of double-labeled cells were computed for each series (GABAAR-FNS, GABAβ-FNS, and AChM2R-FNS) and expressed as % of MoFNS+ cell population per series through the Mo5 nucleus.

Luminance measurements of the receptor immunofluorescence were performed in the marked double-labeled cells, comprising 5–10 cells per animal for GABAAR+...
and 10 cells per animal for GABA<sub>B</sub>R+ and AChM2R+. The images had been acquired with the 8-bit setting of the digital camera, which thus provided converted gray scale images of the fluorescence with arbitrary units of 0–256 for luminance measures. Image acquisition was made as rapidly as possible for each cell so as to avoid bleaching of the fluorescence. As it was previously described in (Toossi et al. 2016b), two approaches were used to measure the intensity of receptor immunofluorescence over the membrane vs. that over the cytoplasm plus membrane of the soma. A rectangular box sized at 1.5×0.3 μm<sup>2</sup> was placed over the plasma membrane for luminance measurement of the membrane fluorescence and another box over the nucleus for luminance measurement and subsequent subtraction of the background fluorescence in each cell. A donut-shaped contour was drawn around the perikaryon to include the cytoplasm and plasma membrane for luminance measurement of the cytoplasm plus membrane and another spherical contour drawn around the nucleus for measurement and subtraction of background fluorescence in each cell.

Cell counts and luminance measurements were analyzed across experimental groups for each receptor series (GABA<sub>A</sub>, GABA<sub>B</sub> or AChM2R) using one-way analysis of variance (ANOVA) followed by post-hoc paired comparisons with Fisher’s LSD using SYSTAT (SYSTAT Software Inc., version13). In an initial analysis, the proportion of GABA<sub>B</sub>R+/MoFNS+ neurons was found to differ significantly across the original 4 groups (F<sub>(3,8) = 4.27, p = 0.045</sub>), however not significantly between the SD2 and SD4 groups (post-hoc paired comparisons, <i>p</i> = 0.387), for which reason they were subsequently combined into one SD group for subsequent analysis and presentation of results. In addition, the proportion of GABA<sub>B</sub>R+/ and AChM2R+ of the MoFNS+ neurons was 100% in all groups.

For higher resolution, images were also acquired using an LSM 710 confocal laser scanning microscope equipped with Ar 488-nm and He–Ne 543-nm lasers for excitation and emission of Cy2 and Cy3 dyes. Images were acquired under 63× oil objectives with a 1.0 airy unit pinhole size for each channel and 0.5 μm thick optical section. Image plates were prepared and composed using Adobe Creative Suite (CS4, Adobe System) from fluorescent microscopic images which were used for quantitative measurements and from confocal images which were used for qualitative assessment with higher resolution of the receptor immunostaining. In all cases as stated above, the parameters of acquisition including exposure time and contrast, were set at the beginning for each receptor series and maintained the same across sections, mice and groups. In no case were brightness or contrast adjusted on individual images. However, for the fluorescent microscopic images of the GABA<sub>A</sub>R immunofluorescence, which was quite dim in the SC brains, the brightness and contrast were enhanced uniformly across the three images of the three mice for better visibility of the receptor immunostaining in the image plate.

**Results**

**Sleep-wake states across groups**

Whereas mice are normally awake a small percentage of the time during the day, as evident in the SC (<i>n</i> = 3) group, the mice in the SD group (<i>n</i> = 6) were maintained awake ~100% of the time according to behavioral and EEG criteria during the 2 h prior to termination at 16:00 (F<sub>(2, 9) = 1381, p < 0.001</sub>; <i>n = 12</i>) (Fig. 1A). During this time, the SD mice engaged in walking, rearing, eating and grooming behaviors, which were all significantly increased relative to the SC mice, or remained still while awake with eyes open. Mice in the SC group were awake ~24% of the time (23.79 ± 1.02%, Mean±SEM) and mice in the SR group ~6% of the time (5.67 ± 2.63%, <i>n = 3</i>), which was significantly less than in the SC group (post hoc paired comparison, <i>p</i> < 0.001). Being undisturbed, the mice in the SC group, thus slept ~76% of the time and mice in the SR group, which were allowed 2 h recovery sleep after 4 h SD, slept ~94% of the time, indicating a homeostatic response to SD. The major proportion of time for the SC and SR groups was spent in NREMS (66.93 ± 1.71, and 82.29 ± 4.07%, respectively), and a minor proportion in REMS (9.28 ± 0.89 and 12.03 ± 0.87%, respectively). Both NREM and REMS were significantly increased during SR relative to SC.

**GABA<sub>A</sub>Rs on Mo5 neurons after SD and SR**

Immunostaining for the GABA<sub>A</sub>R was examined on the Mo5 neurons, which were positively (+) stained for Nissl substance with FNS and morphologically identified as motor neurons (MoFNS+) in brains of mice from SC, SD and SR groups (Fig. 2A–C). As evident in the fluorescent microscopic images examined for determining GABA<sub>A</sub>R positive (+) immunostaining and measuring its intensity (Fig. 2A–C) and in confocal microscopic images examined for its localization with higher resolution (Fig. 3A–C), the GABA<sub>A</sub>R immunostaining was located over the plasma membrane of the soma and dendrites of the MoFNS+ neurons. As evident in these images, the immunofluorescent staining varied in intensity among neurons and mice yet appeared to be consistently most intense in neurons of the SD mice.
As determined in the fluorescent microscopic image stacks of optical sections (Fig. 2A–C), a proportion of the MoFNS+ neurons appeared to be positively immunostained for the GABA_A R over the plasma membrane in all mice (Fig. 1B1). The proportion of GABA_A R+/MoFNS+ neurons, however, differed significantly between groups (F(2,9) = 6.1, p = 0.021; n = 3–6 mice per group) and was significantly greater in the SD group (65.34 ± 6.57%).
as compared to the SC group (32.34 ± 7.38%, post hoc paired comparison \( p = 0.008 \)) and in a trend as compared to the SR group (47.38 ± 3.27%, \( p = 0.096 \)). As measured in the same fluorescent microscopic image stacks (Fig. 2A–C), the average luminance measures of the GABA\(_A\)R immunostaining on the plasma membrane of GABA\(_A\)R+/MoFNS+ neurons was also significantly different between groups (\( F_{(2,110)} = 10.9, p < 0.001; n = 23–60 \)).

**Fig. 2** Fluorescent microscopic images of GABA\(_A\)Rs in Mo5 neurons across groups. Images of single optical sections show several large motor neurons stained for Nissl with FNS (green, A1, B1, C1) and immunostained for the GABA\(_A\)R (red, A2, B2, C2) within the Mo5 nucleus of each mouse. Through the one optical section shown, GABA\(_A\)R immunofluorescence is visible over the plasma membrane of one Mo5 neuron (arrowhead) which is magnified on the right (~3×, A3, B3, C3) for each mouse. In the SC mouse (MST23), the GABA\(_A\)R immunofluorescence is very dim, whereas in the SD mouse (MST20), it is very bright and in the SR mouse (MST22), less bright than in the SD mouse. For measurement of the GABA\(_A\)R fluorescence intensity, a small rectangular box was placed over the plasma membrane and for that of the background fluorescence of the same cell, another over the nucleus to be subtracted from that of the plasma membrane (A3, B3, C3, see Methods). Scale bars 20 μm. Optical image thickness: 0.5 μm.
1 cells per condition, Fig. 1B2) and was significantly higher in SD (45.63 ± 2.61) as compared to SC (36.26 ± 2.53, post hoc paired comparison, \( p = 0.023 \)) and SR groups (28.67 ± 1.84, \( p < 0.001 \)).

**GABA\(_B\)Rs on Mo5 neurons after SD and SR**

Immunostaining for the GABA\(_B\)R was examined on the Mo5FNS+ neurons in brains from mice of the SC, SD and SR groups (Fig. 4A–C). As evident in the fluorescent microscopic images and in the higher resolution confocal
microscopic images of the same material (Fig. 5A–C), GABA₉R immunostaining appeared over granules or vesicles of different sizes which were evident through the cytoplasm extending from the nucleus out to the periphery and in some cases near the plasma membrane of the soma and proximal dendrites. The specific association

Fig. 4 Fluorescent microscopic images of GABA₉Rs in Mo5 neurons across groups. Images of single optical sections show several large Mo5 neurons stained for Nissl with FNS (green, A1, B1, C1) and immunostained for the GABA₉R (red, A2, B2, C2) within the Mo5 nucleus of each mouse. Through the one optical section shown, GABA₉R immunofluorescence is clearly visible over the cytoplasm of all Mo5 neurons of which one representative one is magnified for each mouse (A3, B3, C3, indicated by arrowheads). In the SC mouse (MST21), the GABA₉R immunofluorescence is moderately bright, whereas in the SD mouse (MST20), it is very bright, and in the SR mouse (MST8), it is also moderately bright and similar to that in the SC mouse. For measurement of the intensity of the GABA₉R immunofluorescence, a donut-shaped contour was traced around the perikaryon to include all the cytoplasm and plasma membrane, and for that of the background fluorescence for subtraction, a spherical contour to contain the nucleus of each cell (A3, B3, C3, see Methods). Scale bars 20 μm. Optical image thickness: 0.5 μm
of these granules with the plasma membrane, however, could not be adequately resolved to be distinguished from the granules distributed through the cytoplasm. The density and intensity of the granular immunostaining through the cytoplasm however appeared to vary across neurons and mice and to be consistently greatest in the SD group of mice.

As determined in the fluorescent microscopic image stacks of optical sections (Fig. 4A–C), all MoFNS+ neurons were judged to be positively immunostained for the GABA$_B$R through their cytoplasm in all groups (100%, n=3–6 mice per group, Fig. 1C1). On the other hand, as measured in the same fluorescent microscopic image stacks (Fig. 4A–C), the luminance of the GABA$_B$R
immunofluorescence in the perikarya of the GABA$_A$R+/MoFNS+ somata was significantly different between groups (F$(_{2,117})$=9.35, $p<0.001$; $n=30$ to 60 cells per group) (Fig. 1C2) being significantly higher in the SD (46.97±2.11) as compared to the SC (32.26±2.99, post hoc paired comparison $p<0.001$) and SR groups (36.45±3.07, $p=0.005$).

AChM2Rs on Mo5 neurons after SD and SR

Immunostaining for the AChM2R was examined on the MoFNS+neurons across the three groups of mice (Fig. 6A–C). As evident in the fluorescent microscopic images and in the higher resolution confocal microscopic images of the same material (Fig. 7A–C), the AChM2R immunostaining was observed over the plasma membrane of the soma and dendrites of the Mo5 neurons. It appeared to extend along the entire membrane of most cells though to vary in intensity, appearing most intense in the brains of the SD group of mice.

As determined in the fluorescent microscopic image stacks of optical sections (Fig. 6A–C), all MoFNS+neurons were judged to be positively immunostained for the AChM2R on the plasma membrane of the soma and proximal dendrites (100%, $n=3$ to 6 mice per group, Fig. 1D1). On the other hand, as measured in the same fluorescent microscopic image stacks (Fig. 6A–C), the luminance of the AChM2R immunofluorescence over the membrane of the AChM2R+/MoFNS+ cells was significantly different between groups (F$(_{2,117})$=16.34, $p<0.001$; $n=30$ to 60 cells per group, Fig. 1D2) and was significantly greater in the SD (96.41±3.90) as compared to SC (58.71±4.27, post hoc paired comparison $p<0.001$) and SR groups (75.47±6.56, $p<0.001$).

Discussion

The present results indicate that GABA$_A$, GABA$_B$ and AChM2 receptors increase in Mo5 neurons with enforced waking during the day when mice normally sleep the majority of the time. The results suggest that the Mo5 neurons undergo homeostatic down-scaling in their excitability through increases in inhibitory receptors following prolonged activity during SD.

Homeostatic regulation through GABA$_A$Rs

In immunofluorescent stained sections, GABA$_A$Rs were apparent on the plasma membrane in a proportion of the Mo5 neurons, which was much higher in the SD group than in the SC group, and for which the luminance or density was also higher, indicating increases in membrane GABA$_A$Rs following enforced waking and presumed prolonged activity during SD. These increases following SD are similar to those seen on orexin neurons, which are also active during waking and silent during sleep. They are opposite to decreases in GABA$_A$Rs seen on MCH neurons, which are silent during waking and active during sleep (Toossi et al. 2016b). The increases in GABA$_A$Rs following SD are also similar to the increases in GABA$_A$Rs seen in vitro and in vivo on cortical neurons pharmacologically or electrophysiologically stimulated to fire continuously at high rates (Marty et al. 2004; Nusser et al. 1998). Moreover, increases in the density of the GABA$_A$Rs in these experiments were shown to be associated with increases in inhibitory postsynaptic currents (IPSCs). The increased clusters of the GABA$_A$R on the membrane seen here thus likely correspond to increases in functional receptors. Reciprocally, pharmacologically induced cessation of firing in vitro led to a decrease of GABA$_A$Rs on the membrane associated with a decrease in miniature IPSCs (Marty et al. 2004; Kilman et al. 2002). Similarly here, the density of GABA$_A$Rs was reduced to return to SC levels following SR, when the Mo5 neurons would be mostly silent during increased SWS and REMS.

Pharmacological evidence indicates that the GABA$_A$R together with the glycine receptor on motor neurons is implicated in the muscle hypotonia and atonia that respectively occur during SWS and REMS (Brooks and Peever 2012; Morrison et al. 2003; Soja et al. 1987). Indeed, mutant mice with deficient GABA$_A$ and glycine receptors present symptoms of incomplete muscle atonia and motor inhibition with aberrant twitches and movements during SWS and REMS that resemble the symptoms of REMS behavior disorder in humans (Brooks and Peever 2011). Hypertonic mutant mice were also shown to harbor deficient GABA$_A$Rs in the CNS and on motor neurons, such as to suggest that dysregulation of GABA$_A$R homeostasis can cause hypertonia in multiple clinical disorders (Gilbert et al. 2006). Here, we present evidence that GABA$_A$Rs are normally homeostatically regulated on motor neurons according to activity and state.

Homeostatic regulation through GABA$_B$Rs

GABA$_B$Rs were apparent through the cytoplasm of all Mo5 neurons; however the luminance of the GABA$_B$R was higher in SD than in the SC and SR groups. Given a lack of resolution to distinguish receptor labeling over the plasma membrane, the increased immunostaining for the R1 subunit cannot be inferred to represent an increase in functional GABA$_B$Rs but only an increased expression or trafficking of these receptors within intracellular organelles. Nonetheless, the increased expression during SD suggests a contribution of the GABA$_B$R to homeostatic down-scaling.
in the excitability of the motor neurons with enforced waking. We recently found that SD induced a similar increase of GABABRs in orexin neurons which fire during waking, whereas it induced a decrease in GABABRs on MCH neurons, which are silent during waking (Toossi et al. 2016b).

Pharmacological evidence has shown that GABABRs in addition to the glycine and GABA_A receptors are implicated in muscle hypotonia and atonia of sleep and that only with antagonism of all three receptors can complete muscle atonia be prevented (Brooks and Peever 2016).
In vitro evidence has indicated that the GABABR is essential for homeostatic regulation of firing within hippocampal circuits (Vertkin et al. 2015). Compensatory increases in GABABR immunostaining have been reported in dentate gyrus of mice in response to recurrent seizures (Straessle et al. 2003). Genetic deletion of the GABABR results in a disrupted sleep-waking cycle (Vienne et al. 2010) and hyper locomotor activity along with seizures (Schuler et al. 2001). The present results suggest that homeostatic regulation of the GABABR in motor neurons may be important in down-scaling their excitability during prolonged activity with SD.

**Fig. 7** Confocal microscopic images of AChM2Rs in Mo5 neurons across groups. Images of single optical sections show large Mo5 neurons stained for Nissl with FNS (green, A1, B1, C1) and immunostained for the AChM2R (red, indicated by arrowheads) in single (A2, B2, C2) and merged images (A3, B3, C3) for each mouse. The AChM2R immunofluorescence is evident over the plasma membrane in all three mice, though just barely visible in the SC mouse (MST21). In the SD mouse (MST2), the AChM2R staining is very bright and clearly visible along the full membrane of the soma and proximal dendrites. In the SD mouse, it is also prominent on the plasma membrane of the large dendrites which are cut in cross section. In the SR mouse (MST8), the staining is less bright than in the SD mouse. Scale bars 20 μm. Optical image thickness: 0.5 μm
**Role of GABA in regulating muscle tone across sleep-wake states**

GABA and GABAergic neurons have been shown to play roles in sleep including SWS, PS or REMS and associated muscle hypotonia or atonia (Boissard et al. 2002; Holmes and Jones 1994; Maloney et al. 1999, 2000; Xi et al. 1999; Jones 1991; Weber et al. 2015; Krenzer et al. 2011). Juxtaglottic and labeling of neurons located in the region of the laterodorsal and sublaterodorsal tegmental nuclei (LDT and SubLDT) identified GABAergic neurons that fire at increasingly higher rates during SWS and PS relative to waking and in negative correlation with neck muscle tone (Boucetta et al. 2014). Motor neurons receive GABAergic and glycineergic input from local interneurons in the brainstem and spinal cord. They also receive such input from projecting neurons as was demonstrated in the Mo5 neurons as monosynaptic inhibitory input from neurons in the medullary reticular formation (Chase et al. 1984), where both GABAergic and glycineergic neurons are located (Holmes et al. 1994; Jones et al. 1991; Rampon et al. 1996). They discharged at progressively higher rates during SWS and REM as compared to waking in a manner reciprocally related to the increasing inhibition of the Mo5 neurons during sleep (Chase et al. 1984) and thus in a manner similar to identified GABAergic neurons in the pons (Boucetta et al. 2014). Such identified GABAergic pontine and presumed GABAergic (or glycineergic) medullary neurons could thus be responsible for the progressive inhibition of motor neurons during sleep, culminating in that during PS or REMS.

Here, the increase in both GABA_A and GABA_B receptors following prolonged waking would suggest that motor neurons would become progressively more sensitive to GABA released during waking and sleep and accordingly more sensitive to inhibition and resulting muscle hypotonia and atonia with SD. Such homeostatic changes could underlie the increases in muscle atonia that occur during SWS following SD (Werth et al. 2002) and the increased propensity to cataplectic attacks and paralysis with sleep deficiency or disruption in patients having narcolepsy with cataplexy (Nishino and Mignot 1997). Moreover, daytime cataplectic attacks can be prevented in these patients by administration during the night of gamma hydroxybutyrate (GHB), a GABA_B agonist, which allows consolidation of sleep during the night (Boscolo-Berto et al. 2012), presumably associated as suggested here, with decreases of GABA_BRs to normal levels on motor neurons.

**Homeostatic regulation through AChM2Rs**

Although AChM2Rs were apparent on the plasma membrane of all Mo5 neurons, the lumiance of the AChM2Rs markedly increased following SD, presumably due to prolonged activity of the Mo5 neurons during enforced waking, and then returned to SC levels following SR, presumably due to quiescence of the Mo5 neurons during sleep. These changes are interpreted as a homeostatic response to enhanced neuronal activity, which to our knowledge, have not previously been described for AchMRs.

Pharmacological evidence has indicated that ACh participates in the muscle atonia of REMS through M2 receptors on Mo12 neurons (Grace et al. 2013a). Acting upon GIRK channels, ACh inhibits spinal motor neurons through M2Rs (Chevallier et al. 2006; Miles et al. 2007), which have also been visualized here and in previous studies upon the postsynaptic membrane of Mo5 neurons opposite cholinergic terminals (Hellstrom et al. 2003; Brischoux et al. 2008). The Mo5 neurons could thus be homeostatically regulated following enforced waking by down-scaling through increases in inhibitory AChM2Rs. Such increases in these receptors would render the motor neurons more susceptible to inhibition by ACh and thus the muscles to atonia.

**Role of ACh in regulating muscle tone across sleep-wake states**

ACh has long been known to play an important role in waking and PS or REMS, including muscle atonia as evidenced by severe reduction or elimination of PS following lesions of the LDT/PPT cholinergic neurons (Webster and Jones 1988) and induction of PS or REMS by administration of cholinergic agonists into the pontine reticular formation, an effect which is dependent upon AChM2Rs (Baghdoyan and Lydic 1999; Velazquez-Moctezuma et al. 1989). ACh can apparently play this role in part through direct influence on motor neurons, which for the Mo5 and Mo12, have been shown to receive input from cholinergic neurons of the LDT and pedunculopontine tegmental (PPT) nuclei and of the medullary reticular formation (Jones 1990; Woolf and Butcher 1989; Rukhadze and Kubin 2007; Fort et al. 1990). Cholinergic neurons of LDT/PPT discharge during waking and PS and are silent during SWS (Boucetta et al. 2014). They could thus exert an inhibitory influence through AChM2Rs during PS, but also during waking. According to the pharmacological studies on Mo12 neurons, however, antagonism of muscarinic receptors only affects the muscle atonia of REMS and not the level of muscle tone during waking or SWS (Grace et al. 2013a), suggesting differences that might in part be due to the density of the AChM2Rs. Accordingly, the homeostatic increase in AChM2Rs on motor neurons following SD could contribute to the increased propensity in waking to cataplexy and paralysis following sleep deficits in patients having narcolepsy with cataplexy (Nishino and Mignot 1997). Indeed, the sleep cycle of these patients is greatly disrupted and often
associated with insomnia during the night, which according to our results could result in increased AChM2, along with GABA_A and GABA_B receptors on motor neurons during the day.

We conclude that Mo5 neurons are homeostatically regulated across the sleep-waking cycle through changes in the inhibitory receptors for GABA and ACh. Their prolonged activity during enforced waking with SD results in homeostatic across the sleep-waking cycle through changes in the restorative up-scaling to return the neurons to normal stable levels of excitability and activity. The increases in these inhibitory receptors following SD would render the motor neurons more responsive and thus susceptible to inhibition by GABA and ACh, which would also render the muscles more susceptible to hypotonia or atonia, as can occur with sleep deficits and particularly in association with sleep disorders, such as narcolepsy with cataplexy.

Acknowledgements We thank Lynda Mainville for her technical assistance and Anton Plavski for his participation in the experiments. The research was supported by a research grant (to B.E.I.) from the Canadian Institutes of Health Research (CIHR MOP-130502).

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References

Baghdoyan HA, Lydic R (1999) M2 muscarinic receptor subtype in the feline medial pontine reticular formation modulates the amount of rapid eye movement sleep. Sleep 22(7):835–847
Boissard R, Gervasini D, Schmidt MH, Barbagli B, Fort P, Luppi PH (2002) The rat ponto-medullary network responsible for paradoxical sleep onset and maintenance: a combined micro-injection and functional neuroanatomical study. Eur J Neurosci 16(10):1959–1973
Borbely AA, Achermann P (1999) Sleep homeostasis and models of sleep regulation. J Biol Rhythms 14(6):557–568
Borbely AA, Tobler I, Hanagasiglu M (1984) Effect of sleep deprivation on sleep and EEG power spectra in the rat. Behav Brain Res 14(3):171–182
Boscolo-Berto R, Viel G, Montagnese S, Raduazzo DI, Ferrara SD, Dauvilliers Y (2012) Narcolepsy and effectiveness of gamma-hydroxybutyrate (GHB): a systematic review and meta-analysis of randomized controlled trials. Sleep Med Rev 16(5):431–443. doi:10.1016/j.smrv.2011.09.001
Boucetta S, Cisse Y, Mainville L, Morales M, Jones BE (2014) Discharge profiles across the sleep-waking cycle of identified cholinergic, GABAergic, and glutamatergic neurons in the pontomesencephalic tegmentum of the rat. J Neurosci 34(13):4708–4727. doi:10.1523/JNEUROSCI.2617-13.2014
Brischoux F, Mainville L, Jones BE (2008) Muscarinic-2 and orexin-2 receptors on GABAergic and other neurons in the rat mesopontine tegmentum and their potential role in sleep-wake state control. J Comp Neurol 510(6):607–630. doi:10.1002/cne.21803
Brooks PL, Peejer JH (2008) Glycerinergic and GABA(A)-mediated inhibition of somatic motoneurons does not mediate rapid eye movement sleep motor atonia. J Neurosci 28(14):3535–3545. doi:10.1523/JNEUROSCI.5023-07.2008
Brooks PL, Peejer JH (2011) Impaired GABA and glycinergic transmission triggers cardinal features of rapid eye movement sleep behavior disorder in mice. J Neurosci 31(19):7111–7121. doi:10.1523/JNEUROSCI.0347-11.2011
Brooks PL, Peejer JH (2012) Identification of the transmitter and receptor mechanisms responsible for REM sleep paralysis. J Neurosci 32(29):9785–9795. doi:10.1523/JNEUROSCI0482-12.2012
Chandler SH, Nakamura Y, Chase MH (1980) Intracellular analysis of synaptic potentials induced in trigeminal jaw-closer motor neurons by pontomesencephalic reticular stimulation during sleep and wakefulness. J Neurophysiol 44(2):372–382
Chase MH (1983) Synaptic mechanisms and circuitry involved in motoneuron control during sleep. Int Rev Neurobiol 28:213–258
Chase MH, Chandler SH, Nakamura Y (1980) Intracellular determination of membrane potential of trigeminal motoneurons during sleep and wakefulness. J Neurophysiol 44(2):349–358
Chase MH, Enomoto S, Hiraba K, Katoh M, Nakamura Y, Sahara Y, Taira M (1984) Role of medullary reticular neurons in the inhibition of trigeminal motoneurons during active sleep. Exp Neurol 84(2):364–373
Chase MH, Soja PJ, Morales FR (1989) Evidence that glycine mediates the postsynaptic potentials that inhibit lumbar motoneurons during the atonia of active sleep. J Neurosci 9(3):743–751
Chevallier S, Nagy F, Cabelguen JM (2006) Cholinergic control of excitability of spinal motoneurones in the salamander. J Physiol 570 (Pt 3):525–540. doi:10.1113/jphysiol.2005.089970
Filippov AK, Couve A, Pangalos MN, Walsh FS, Brown DA, Moss SJ (2000) Heteromeric assembly of GABA(B)R1 and GABA(B)R2 receptor subunits inhibits Ca(2+)-current in sympathetic neurons. J Neurosci 20(8):2867–2874
Fort P, Luppi PH, Sakai K, Salvert D, Jouvet M (1990) Nuclei of rostral and caudal motor nuclei of the cat trigeminal motor nucleus: a double-labeling study with cholera-toxin as a retrograde tracer. J Comp Neurol 323(3):308–328
Grace KP, Hughes SW, Horner RL (2013a) Identification of the mechanism mediating genioglossus muscle suppression in REM sleep. Am J Respir Crit Care Med 187(3):311–319. doi:10.1164/rccm.201209-1654OC
Grace KP, Hughes SW, Shahabi S, Horner RL (2013b) K + channel modulation causes genioglossus inhibition in REM sleep and is a strategy for reactivation. Respir Physiol Neurobiol 188 (3):277–288. doi:10.1016/j.resp.2013.07.011
Hellstrom J, Oliveira AL, Meister B, Cullheim S (2003) Large cholinergic nerve terminals on subsets of motoneurons and

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their relation to muscarinic receptor type 2. J Comp Neurol 460(4):476–486

Holmes CJ, Jones BE (1994) Importance of cholinergic, GABAergic, serotonergic and other neurons in the medullary reticular formation for sleep-wake states studied by cytotoxic lesions in the cat. Neuroscience 62:1179–1200

Holmes CJ, Mainville LS, Jones BE (1994) Distribution of cholinergic, GABAergic and serotonergic neurons in the medullary reticular formation and their projections studied by cytotoxic lesions in the cat. Neuroscience 62:1155–1178

Jones BE (1990) Immunohistochemical study of choline acetyl transferase-immunoreactive processes and cells innervating the pontomedullary reticular formation. J Comp Neurol 295:485–514

Jones BE (1991) Paradoxical sleep and its chemical/structural substrates in the brain. Neuroscience 40(3):637–656

Jones BE, Holmes CJ, Rodriguez-Veiga E, Mainville L (1991) GABA-synthesizing neurons in the medulla: their relationship to serotonin-containing and spinally projecting neurons in the rat. J Comp Neurol 313(2):349–367. doi:10.1002/cne.903130210

Jouvet M (1967) Neurophysiology of the states of sleep. Physiol Rev 47(2):117–177

Kilman V, van Rossum MC, Turrigiano GG (2002) Activity deprivation reduces IPSC amplitude by increasing the number of postsynaptic GABA(A) receptors clustered at neocortical synapses. J Neurosci 22(4):1328–1337

Krenzer M, Anacet C, Vetrivelan R, Wang N, Vong L, Lowell BB, Fuller PM, Lu J (2011) Brainstem and spinal cord circuitry regulating REM sleep and muscle atonia. PLOS ONE 6(10):e24998. doi:10.1371/journal.pone.0024998

Levey AI, Kitt CA, Simonds WF, Price DL, Brann MR (1991) Immunohistochemical localization of GABA(B) receptor clusters in hippocampal slice cultures. Eur J Neurosci 3(4):476–486

Maloney KJ, Mainville L, Jones BE (1999) Differential c-Fos expression in cholinergic, monoaminergic and GABAergic cell groups of the pontomesencephalic tegmentum after paradoxic sleep deprivation and recovery. J Neurosci 19:3057–3072

Maloney KJ, Mainville L, Jones BE (2000) c-Fos expression in GABAergic, serotonergic and other neurons of the pontomedullary reticular formation and raphe after paradoxic sleep deprivation and recovery. J Neurosci 20:4669–4679

Margata-Mitrovic M, Mitrovic I, Riley RC, Jan LY, Basbaum AI (1999) Immunohistochemical localization of GABA(B) receptors in the rat central nervous system. J Comp Neurol 405(3):299–321

Marty S, Wehrle R, Fritschy JM, Sotelo C (2004) Quantitative effects of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B)1. Neuron 31(1):47–58

Soja PJ, Finch DM, Chase MH (1987) Effect of inhibitory amino acid antagonists on masseteric reflex suppression during active sleep. Exp Neuro 96(1):178–193

Strassels A, Loup F, Arabadzisz D, Ohning GV, Fritschi JM (2003) Rapid and long-term alterations of hippocampal GABAB receptors in a mouse model of temporal lobe epilepsy. Eur J Neurosci 18(8):2213–2226

Tobler I, Borbely AA (1986) Sleep EEG in the rat as a function of prior waking. Electroenceph Clin Neurophysiol 64(1):74–76

Turrigiano GG (1999) Homeostatic plasticity in neuronal networks: the more things change, the more they stay the same. Trends Neurosci 22(5):221–227

Turrigiano GG, Leslie KR, Desai NS, Rutherford LC, Nelson SB (1998) Activity-dependent scaling of quantal amplitude in neocortical neurons. Nature 391(6670):892–896

Velazquez-Moctezuma J, Gillin JC, Shiromani PJ (1989) Effect of specific M1, M2 muscarinic receptor agonists on REM sleep generation. Brain Res 503(1):128–131

Vertkin I, Styr B, Slomowicz E, Ofrir N, Shapiro I, Berner D, Fedorova T, Laviv T, Barak-Broner N, Greiter-Antes D, Gassmann M, Bettler B, Lotan I, Slutsky I (2015) GABAB receptor deficiency causes failure of neuronal homeostasis in hippocampal networks. Proc Natl Acad Sci USA 112(25):E3291–E3299. doi:10.1073/pnas.1424810112

Vienne J, Bettler B, Franken P, Tafti M (2010) Differential effects of GABAB receptor subtypes, (gamma)-hydroxybutyric Acid, and Baclofen on EEG activity and sleep regulation. J Neurosci 30(42):14194–14204. doi:10.1523/JNEUROSCI.3145-10.2010

Webster HH, Jones BE (1988) Neurotoxic lesions of the dorsolateral pontomesencephalic tegmentum-cholinergic cell area in the cat. II. Effects upon sleep-waking states. Brain Res 458:285–302

Webster HH, Jones BE (1988) Neurotoxic lesions of the dorsolateral pontomesencephalic tegmentum-cholinergic cell area in the cat. II. Effects upon sleep-waking states. Brain Res 458:285–302

Weber F, Chung S, Beier KT, Xu M, Luo L, Dan Y (2015) Control of REM sleep by ventral medulla GABAergic neurons. Nature 526(7573):435–438. doi:10.1038/nature14979

Webster HH, Jones BE (1988) Neurotoxic lesions of the dorsolateral pontomesencephalic tegmentum-cholinergic cell area in the cat. II. Effects upon sleep-waking states. Brain Res 458:285–302

Werb E, Achermann P, Borbely AA (2002) Selective REM sleep deprivation during daytime. II. Muscle atonia in non-REM sleep. Am J Physiol Regul Integr Comp Physiol 283(2):R527–R532. doi:10.1152/ajpregu.00466.2001

Woolf NJ, Butcher LL (1989) Cholinergic systems in the rat brain: IV. Descending projections of the pontomesencephalic tegmentum. Brain Res Bull 23(6):519–540

Xi M-C, Morales FR, Chase MH (1999) Evidence that wakefulness and REM sleep are controlled by a GABAergic pontine mechanism. J Neurophysiol 82:2015–2019

Toossi H, Del Cid-Pellitero E, Jones BE (2016a) GABA and muscarinic receptors on motor trigeminal neurons are homeostatically regulated with sleep deprivation. Society for Neuroscience On-line Abstracts:342.313

Rampon C, Luppi PH, Fort P, Peyron C, Jouvet M (1996) Distribution of glycine-immunoreactive cell bodies and fibers in the rat brain. Neuroscience 75(3):737–755

Rukhadze I, Kubin L (2007) Mesopontine cholinergic projections to the hypoglossal motor nucleus. Neurosci Lett 413(2):121–125. doi:10.1016/j.neulet.2006.11.059

Schuler V, Luscher C, Blanchet C, Klix N, Sansig G, Klebs K, Schmutz M, Heid J, Gentry C, Urban L, Fox A, Spooren W, Jaton AL, Vigouret J, Pozza M, Kelly PH, Mosbacher J, Froestl W, Kaslin E, Korn R, Bischoff S, Kaupmann K, van der Putten H, Bettler B (2001) Epilepsy, hyperalgesia, impaired memory, and loss of pre- and postsynaptic GABA(B) responses in mice lacking GABA(B)1. Neuron 31(1):47–58
Toossi H, Del Cid-Pellitero E, Jones BE (2016b) GABA receptors on orexin and melanin-concentrating hormone neurons are differentially homeostatically regulated following sleep deprivation. eNeuro 3 (3). doi:10.1523/ENEURO.0077-16.2016

Zhu L, Ferrari LL, Park D, Chamberlain NL, Arrigoni E (2016) An in vitro study of pre and post synaptic cholinergic control of hypoglossal motor neurons in adult mice. Society for Neuroscience On-line Abstracts:254.204