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5-$HT_{2A/B}$ receptor expression in the phrenic motor nucleus in a rat model of ALS ($SOD1^{G93A}$)

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Abstract

Despite respiratory motor neuron death, ventilation is preserved in $SOD1^{G93A}$ rats. Compensatory respiratory plasticity may counterbalance the loss of these neurons. Phrenic long-term facilitation (pLTF; a form of respiratory plasticity) in naïve rats is 5-HT_2 and NADPH oxidase-dependent. Furthermore, 5-HT_{2A}, not 5-HT_{2B}, receptor-induced phrenic motor facilitation is NADPH oxidase-independent in naïve rats. pLTF is NADPH oxidase-dependent in pre-symptomatic, but not end-stage, $SOD1^{G93A}$ rats. Here, we hypothesized that in the putative phrenic motor nucleus (PMN) of $SOD1^{G93A}$ rats vs. wild-type littermates: 1) pre-symptomatic rats would have greater 5-HT_{2B} receptor expression that decreases at end-stage; and 2) 5-HT_{2A} receptor expression would increase from pre-symptomatic to end-stage. Putative PMN 5-HT_{2A} receptor expression was reduced when comparing across (but not within) pre-symptomatic vs. end-stage groups ($p<0.05$). In contrast, putative PMN 5-HT_{2B} receptor expression was increased when comparing across pre-symptomatic vs. end-stage groups, and within end-stage groups ($p<0.05$). These data suggest a potential role for 5-HT_2 receptors in pLTF and breathing in $SOD1^{G93A}$ rats.

Keywords

Serotonin; plasticity; breathing; respiratory motor neuron; amyotrophic lateral sclerosis

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects both upper and lower motor neurons. Patients with ALS survive only 3-5 years after the disease has been diagnosed and death most commonly occurs due to respiratory failure (Lechtzin et al., 2002; Bourke et al., 2001; Lyall et al., 2001). However, despite the loss of respiratory motor neurons, patients are able to maintain breathing capacity. Once the disease

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Declaration of Interest

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becomes more severe, there is a turning point at which the patient rapidly succumbs to ventilatory failure leading to ventilator-dependence or death due to a lack of compensation to maintain breathing. The mechanisms by which patients are able to compensate for breathing remain unknown.

Although 90% of ALS cases are sporadic, the other roughly 10% of cases are familial and are a result of genetic mutations. Overexpression of superoxide dismutase 1 (SOD-1) is one cause of familial ALS, although the pathogenesis in transgenic rodent models overexpressing human SOD1 is still up for debate. Regardless of the mechanism of pathogenesis, rodent models including hSOD1^{G93A} (Rosen et al., 1993; Gurney et al., 1994; Howland et al., 2002), hSOD1^{G37R} (Wong et al., 1995), hSOD1^{G85R} (Bruijn et al., 1997), hSOD1^{Quad} (Wang et al., 2003), and hSOD1^{H46R} (Nagai et al., 2001) develop clinical symptoms associated with both familial and sporadic human cases of ALS (Boillée et al., 2006). Interestingly, hSOD1^{G93A} rats experience progressive motor neuron loss but do not develop breathing deficits until late in the disease (Nichols et al., 2013; Dale et al., 2006; Tankersley et al., 2006). This indicates respiratory compensation may be occurring to maintain ventilation despite dramatic phrenic motor neuron loss.

One way that has been speculated to induce respiratory compensation following motor neuron loss is through respiratory plasticity by neighboring surviving motor neurons. Acute intermittent hypoxia (AIH) is one mode to induce respiratory plasticity, which is called phrenic long-term facilitation (pLTF) (Dale-Nagle et al., 2010; Hayashi et al., 2003; Feldman et al., 2003; Bach and Mitchell, 1996). AIH-induced pLTF occurs predominately through the activation of Gq (5-HT$_{2A/B}$) (Bach and Mitchell, 1996; McFarlane et al., 2011) coupled receptor-dependent pathways. Specifically, pLTF is induced through the activation of the serotonin receptor (5-HT$_2$) in the phrenic motor nucleus at cervical spinal cord regions 3-5 of naïve rats (Bach and Mitchell, 1996). When 5-HT$_{2A/B}$ receptors become activated following AIH, protein kinase C theta (PKCΦ) is activated and leads to the new synthesis of brain-derived neurotrophic factor (BDNF) (McGuire and Ling 2004; Baker-Herman et al., 2004, Devinney, et al. 2015). BDNF then binds to the mTrkB receptor (Baker-Herman et al., 2004) and subsequently activates MEK and the phosphorylation of ERK (Hoffman et al., 2012), ultimately resulting in pLTF. pLTF also requires reactive oxygen species (ROS) formation *via* NADPH oxidase activity since ROS disinhibits phosphatase action on PKC (*i.e.*, ROS formation allows pLTF to be evoked; MacFarlane and Mitchell, 2007; MacFarlane et al., 2011).

Pharmacological activation of 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors also leads to respiratory plasticity called phrenic motor facilitation (pMF) in naïve adult rats (MacFarlane and Mitchell 2009; MacFarlane et al., 2011). Furthermore, pMF *via* 5-HT$_{2A}$ activation is NADPH oxidase-independent, while 5-HT$_{2B}$-induced pMF is NADPH oxidase-dependent (MacFarlane et al., 2011). Interestingly, AIH-induced pLTF in pre-symptomatic SOD1^{G93A} rats is NADPH oxidase-dependent, but end-stage SOD1^{G93A} rats exhibit NADPH oxidase-independent pLTF (Nichols, et al. 2015). Thus, in this study, we hypothesized that there would be a shift in the 5-HT$_2$ receptor balance in the putative phrenic motor nucleus of SOD1^{G93A} rats *vs.* age-matched, wild-type littermates. Specifically, we hypothesized that pre-symptomatic SOD1^{G93A} rats would have greater 5-HT$_{2B}$ receptor expression that

decreases over time to end-stage. In contrast, we hypothesized that 5-HT_{2A} receptor expression would increase from pre-symptomatic to end-stage. . However, we found that 5-HT_{2A} receptor expression in the putative phrenic motor nucleus was not different between $SOD1^{G93A}$ rats and wild-type littermates regardless of disease state (*i.e.*, neither pre-symptomatic nor end-stage), while 5-HT_{2B} receptor expression was only increased in the putative phrenic motor nucleus of end-stage $SOD1^{G93A}$ rats compared to wild-type littermates. Interestingly, we also found that 5-HT_{2A} receptor expression is reduced, while 5-HT_{2B} receptor expression is upregulated, over time in the putative phrenic motor nucleus in both wild-type and $SOD1^{G93A}$ rats. This study provides knowledge into how 5-$HT_{2A/B}$ receptor expression changes both within and outside of the putative phrenic motor nucleus following motor neuron death induced by SOD1 overexpression. Furthermore, our results suggest changes in 5-$HT_{2A/B}$ receptor expression, and thus their downstream mechanisms, in response to motor neuron death may contribute to the maintenance of breathing throughout disease progression in $SOD1^{G93A}$ rats.

2. Methods

2.1. Animals and Tissue Samples

Male $SOD1^{G93A}$ Sprague Dawley rats were bred to wild-type female rats (Taconic Laboratories, Germantown, NY). Neurophysiological experiments were performed at the University of Wisconsin-Madison on adult male pre-symptomatic (3-4 months of age) or end-stage (5-6 months of age) $SOD1^{G93A}$ (MT) and age-matched, wild-type (WT) littermates (Nichols et al., 2015). End-stage was defined as a 20% reduction in peak body mass (Nichols et al., 2015). Immediately following neurophysiological protocols for another study (Nichols et al., 2015), rats were transcardially perfused with cold 4% paraformaldehyde in phosphate buffered saline (0.1 M PBS, pH 7.4; Nichols et al., 2015). Spinal cords were immediately removed following perfusion, post-fixed (4% paraformaldehyde in 0.1 M PBS) at 4°C overnight, and then cryoprotected in graded sucrose (20% sucrose for 3 days and 30% sucrose for an additional 3 days) at 4°C until sinking. The spinal cords were transversely sectioned to contain the largest portion of the phrenic motor nucleus (C4; 40 μm) using a freezing-sliding microtome (Leica SM 2000R, Germany), and stored at −20°C in an antifreeze solution (30% glycerol, 30% ethylene glycol, 40% PBS). All experimental procedures were approved by the Animal Care and Use Committee at the University of Wisconsin-Madison. Tissue was then transferred to the University of Missouri-Columbia where the immunohistochemistry protocols, imaging, and analyses stated below were performed.

2.2. Immunohistochemistry

Six sections from the cervical region housing the phrenic motor nucleus (C4) were selected for each animal (pre-symptomatic WT: n=21 for 5-HT_{2A} and n=25 for 5-HT_{2B}; pre-symptomatic MT: n=19 for 5-HT_{2A} and n=23 for 5-HT_{2B}; end-stage WT: n=24 for 5-HT_{2A} and n=17 for 5-HT_{2B}; end-stage MT: n=25 for 5-HT_{2A} and n=22 for 5-HT_{2B}) and were washed with 1X PBS three times for five minutes on a shaker at room temperature. Antigen retrieval was performed by adding a 0.01 M citrate in DDI water solution to the tissue wells and shaken in a Labent Hybaid Maxi 14 Hybridization Oven Incubator (National Labnet

Company) at 60°C for 30 minutes. Three, five minute washes were repeated in 1X PBS at room temperature on a shaker. Sections were then incubated at room temperature in a blocker solution (1X PBS + 0.2% Triton + 5% normal donkey serum) on a shaker for one hour. Sections were then incubated in primary antibody solution (1X PBS + 0.1% Triton + 5% normal donkey serum + antibodies against NeuN (mouse polyclonal, 1:500, Millipore) and either 5-HT_{2A} (rabbit polyclonal, 1:200; Neuromics, Edina, MN) or 5-HT_{2B} (rabbit polyclonal, 1:300; Neuromics, Edina, MN) overnight at 4°C on a shaker. The following day, tissue was washed three times for five minutes at room temperature on a shaker in 1X PBS. The tissue was then incubated for two hours at room temperature on a shaker in the dark in secondary antibody solution (1X PBS + 0.1% Triton + 5% normal donkey serum + donkey anti-mouse Alexa-Fluor 555 (1:1000; Molecular Probes, Eugene, OR) and donkey anti-rabbit Alexa-Fluor 488 (1:1000; Molecular Probes, Eugene, OR)). Tissue was then washed again while covered in 1X PBS three times for 5 minutes on a shaker at room temperature. The tissue was then mounted on positively charged glass slides (Thermo Fisher Scientific, Waltham, MA) and allowed to dry before ProLong™ Gold anti-fade reagent (Thermo Fisher Scientific, Waltham, MA) was applied and a coverslip was put onto the slides. Covered slides were stored at 4°C until quantification of staining was performed.

2.3. Imaging and Analysis

Photomicrographs were taken at the same settings for all images per antibody per group (*i.e.*, gains: pre-symptomatic 5-HT_{2A}=740.01 and NeuN=770.24, end-stage 5-HT_{2A}=765.28 and NeuN=770.22; pre-symptomatic 5-HT_{2B} = 802.25 and NeuN = 765.29, end-stage 5-HT_{2B}=797.28 and NeuN=804.95). The photomicrographs encompassed the putative phrenic motor nucleus (Mantilla et al., 2009; Boulenguez et al., 2007; Watson et al., 2009) and the non-phrenic ventral horn, and were taken using a Leica DM4000 confocal microscope at 20x magnification with Leica Application Suite X (LAS X) software. Densitometry of the 5-$HT_{2A/B}$ receptors was performed on Z-stacked (5μm in z-plane per image) images by creating a region of interest that encompassed the putative phrenic motor nucleus (same region of interest used for all images). Images were taken and analyzed as 8-bit stacks at a resolution of 1024 × 1024 (366.67μm x 366.67μm). Thresholds in ImageJ for analysis of immunopositivity were: pre-symptomatic 5-HT_{2A}: 17-24; pre-symptomatic 5-HT_{2B}: 33-43; end-stage 5-HT_{2A}: 17-34; and end-stage 5-HT_{2B}: 45-55. Immunopositive pixels for 5-$HT_{2A/B}$ receptors were evaluated using ImageJ within the putative phrenic motor nucleus and in the non-phrenic ventral horn in which 5-$HT_{2A/B}$ optical density was expressed as an average density per section/animal. The fractional area occupied by 5-$HT_{2A/B}$ label in raw image files was also computed by Image J (Nichols et al., 2015). Within the putative phrenic motor nucleus, the 5-$HT_{2A/B}$ fractional area was interpreted as the percentage of the total field for the putative phrenic motor nucleus occupied by label positive pixels. However, the fractional area occupied by 5-$HT_{2A/B}$ label in raw image files was calculated manually for the non-phrenic ventral horn. Total label positive pixels were determined for the putative phrenic motor nucleus (fractional area multiplied by total pixels in the putative phrenic motor nucleus) and for the entire ventral horn (fractional area multiplied by total pixels in the ventral horn). Total label positive pixels for the non-phrenic ventral horn were determined by subtracting total label positive pixels in the putative phrenic motor nucleus from those in the area outside of the region of interest in the 20x magnification images. Total

label positive pixels for the non-phrenic ventral horn were then divided by the total pixels of the non-phrenic ventral horn (total pixels of the putative phrenic motor nucleus were subtracted from the total pixels in the area outside of the region of interest in the 20x magnification images).

Photomicrographs were also taken at 4x magnification to provide a visual representation of the location of the putative phrenic motor nucleus (C4) within the ventral horn. In addition, photomicrographs were taken at 40x to enable the visualization of 5-$HT_{2A/B}$ receptor expression on and near neurons using an Olympus BX51 equipped with a three-axis motorized stage (Ludl Electronic Products Ltd., Hawthorne, NY, USA). Filter sets for Cy2 [ex. λ480nm; em. λ510nm] and Cy3 [ex. λ550 nm; λ570 nm] were used to identify positively labeled neurons and 5-$HT_{2A/B}$ receptors. Using the same focal plane, digital images were captured with each filter using a cooled monochrome digital camera (ORCA-AG, Hamamatsu, Bridgewater, NJ, USA) and the software package Neurolucida (version 9, MicroBrightField, Willston, VT, USA).

2.4. Statistical Analysis

A one-way ANOVA was used to compare 5-$HT_{2A/B}$ optical density and fractional area within groups in the putative phrenic motor nucleus and the non-phrenic ventral horn. A student's *t*-test was used to compare 5-$HT_{2A/B}$ optical density and fractional area between groups in the putative phrenic motor nucleus and the non-phrenic ventral horn. A two-way ANOVA was used to compare 5-$HT_{2A/B}$ optical density and fractional area in the putative phrenic motor nucleus and the non-phrenic ventral horn between the different stages of disease progression (pre-symptomatic *vs.* end-stage) in WT and MT rats. The LSD post hoc test was used to detect significantly different individual comparisons, where differences between groups were considered significant if $P < 0.05$ and all values were expressed as means ± 1 SEM.

3. Results

3.1 5-HT_{2A} receptor expression recovers back towards wild-type levels at end-stage in the non-phrenic ventral horn, but is unaffected in the putative phrenic motor nucleus

Representative photomicrographs for 5-HT_{2A} receptors from C4 transverse sections are shown in Figs. 1&2 A–J. 5-HT_{2A} receptor positive staining was evaluated in pre-symptomatic (Fig. 1) and end-stage (Fig. 2) $SOD1^{G93A}$ (MT) and age-matched wild-type (WT) rats at 20x magnification (Figs. 1&2 B,D,G,I). The putative phrenic motor nucleus is predominately located at C4 (indicated by the yellow dotted circle in Figs. 1–4) in the ventral horn. When visualized using immunofluorescence for 5-HT_{2A} receptors (indicated by green fluorescence in Figs. 1&2 A–J), the receptors were localized to the putative phrenic motor nucleus, but were also present in the non-phrenic ventral horn to a lesser degree (area outside of the putative phrenic motor nucleus in the 20x magnification images). Additionally, using NeuN (indicated by red fluorescence in Figs. 1&2 A–J), neurons were visualized both within and outside of the putative phrenic motor nucleus. Interestingly, it appears that 5-HT_{2A} receptors do not solely exist on the membranes of neurons within the putative phrenic motor nucleus and the non-phrenic ventral horn (*e.g.*, on the cell

membranes of cells that are not motor neurons). In pre-symptomatic and end-stage animals, we observed no differences between MT and age-matched WT rats in optical density or fractional area of 5-HT_{2A} receptor expression within the putative phrenic motor nucleus (Fig. 1K & 1L; $p>0.05$). However, 5-HT_{2A} receptor optical density ($p=0.005$) and fractional area ($p<0.001$) were both significantly decreased in pre-symptomatic MT animals in the non-phrenic ventral horn when compared to WT animals. In contrast, this decrease in 5-HT_{2A} optical density and fractional area returns to WT levels in the non-phrenic ventral horn of MT rats at end-stage ($p>0.05$; Figs. 2K & 2L).

3.2. 5-HT_{2B} receptor expression is increased from wild-type levels at end-stage

Figures 3&4 A–J depict representative photomicrographs from C_4 transverse sections for 5-HT_{2B} receptor positive staining. Sections from the same rats as in the previous section were evaluated for 5-HT_{2B} receptor positive staining (Fig. 3A–J, 4A–J). 5-HT_{2B} receptor optical density was quantified at 20x magnification both within the putative phrenic motor nucleus (indicated by the yellow dashed circle) and in the non-phrenic ventral horn in pre-symptomatic (Fig. 3B,D,G,I) and end-stage (Fig. 4B,D,G,I) wild-type (WT) and $SOD1^{G93A}$ mutant (MT) rats. The fractional area of 5-HT_{2B} receptor expression was also quantified within the putative phrenic motor nucleus and the non-phrenic ventral horn. Neurons were visualized both within and outside of the putative phrenic motor nucleus using NeuN (indicated by red fluorescence in Figs. 3&4 A–J). Once again, 5-HT_{2B} receptors (indicated by green immunofluorescence; Fig. 3&4 A–J) were localized to the putative phrenic motor nucleus, but existed in the non-phrenic ventral horn to a much lesser degree (Figs. 3 & 4). Optical density (Fig. 3K) and fractional area (Fig. 3L) for 5-HT_{2B} receptor expression within the putative phrenic motor nucleus and the non-phrenic ventral horn were not different when comparing pre-symptomatic MT and age-matched WT animals ($p>0.05$) However, we did observe that end-stage MT animals had a significant increase in both 5-HT_{2B} optical density (Fig. 4K; $p=0.042$) and fractional area (Fig. 4L; $p=0.045$) within the putative phrenic motor nucleus. There was no difference in 5-HT_{2B} receptor expression at end-stage in the non-phrenic ventral horn observed between MT and age-matched WT animals (Fig. 4K&L; $p>0.05$).

3.3. 5-$HT_{2A/B}$ receptor expression over disease progression

A two-way ANOVA was used to determine if 5-HT_{2A} receptor expression within the putative phrenic motor nucleus and the non-phrenic ventral horn increases over time in MT rats. Surprisingly, we found that optical density and fractional area for 5-HT_{2A} receptor expression within the putative phrenic motor nucleus was significantly decreased in end-stage *vs.* pre-symptomatic rats (both WT and MT; $p<0.05$). In the non-phrenic ventral horn, fractional area for 5-HT_{2A} receptor expression was significantly increased in end-stage *vs.* pre-symptomatic MT rats ($p<0.05$). Lastly, optical density for 5-HT_{2A} receptor expression in the non-phrenic ventral horn was significantly decreased at end-stage in wild-type rats ($p<0.05$ *vs.* pre-symptomatic rats).

A two-way ANOVA was also used to determine if 5-HT_{2B} receptor expression within the putative phrenic motor nucleus and the non-phrenic ventral horn decreases over time in MT rats. However, we found that we found that optical density and fractional area for 5-HT_{2B}

receptor expression within the putative phrenic motor nucleus was significantly increased in end-stage *vs.* pre-symptomatic rats (both WT and MT; $p<0.05$). There were no significant differences in the non-phrenic ventral horn for 5-HT_{2B} receptor expression between end-stage and pre-symptomatic WT or MT rats ($p>0.05$).

4. Discussion

The main findings of this study are that: 1) 5-HT_{2A} receptor expression does not change within the putative phrenic motor nucleus when comparing WT and MT rats within pre-symptomatic and end-stage time-points; and 2) 5-HT_{2B} receptor expression is upregulated at end-stage only within the putative phrenic motor nucleus of MT rats *vs.* pre-symptomatic WT littermates. To our surprise, 5-HT_{2A} receptor expression does not change within the putative phrenic motor nucleus when comparing WT and MT rats within pre-symptomatic and end-stage time-points, but is decreased in pre-symptomatic MT animals in the non-phrenic ventral horn and returns towards WT levels by end-stage (Figs. 1&2). Thus, even though 5-HT_{2A} receptor expression is unaffected in the putative phrenic motor nucleus of end-stage MT rats when compared to WT littermates (Fig. 2), this does not eliminate a role for 5-HT_{2A} receptors for pLTF and breathing. Previous studies using models of spinal cord injury have found that intrathecal administration of a 5-HT_{2A} receptor agonist induced respiratory recovery. Conversely, blocking 5-HT_{2A} receptors resulted in a decrease in phrenic burst amplitude and respiratory rate (Zhou et al., 2001). This study highlights a critical role for the involvement of 5-HT_{2A} receptors in respiration following phrenic motor loss after cervical spinal cord injury (Zhou et al., 2001), which may also be the case in $SOD1^{G93A}$ rats.

Interestingly, 5-HT_{2A} receptor expression was reduced in WT littermates as well as MT rats over time within the putative phrenic motor nucleus, while 5-HT_{2B} receptor expression was increased in WT littermates as well as MT rats over time within the putative phrenic motor nucleus. These receptor changes are observed when comparing end-stage to pre-symptomatic expression, regardless of SOD1 expression. Since the effects are observed in WT littermates, we suggest that 5-HT_{2B} receptors may become more heavily utilized to maintain respiration later in life as 5-HT_{2A} receptors decrease. Other groups have reported an age-related decline in 5-HT_2 receptors (Wang et al., 1995) and serotonin 2A binding sites (Marcusson et al., 1984) in the frontal cortex of healthy human subjects. Thus, our observation of a decline in 5-HT_{2A} receptors in the cervical spinal cord, and more specifically the putative phrenic motor nucleus, is consistent with previous studies in the CNS and to our knowledge the first to describe an age-related decline in putative phrenic 5-HT_{2A} receptors. Additionally, Tadros et al. reported 5-HT_{2B} receptor gene expression was up-regulated in older (~ 28 months of age) CBA/CaJ mice compared to younger (~ 12 months of age) CBA/CaJ mice in the inferior colliculus nucleus of the midbrain. Thus, our observation of an age-related increase in 5-HT_{2B} receptor expression in the putative phrenic motor nucleus in the cervical spinal cord is consistent with previous studies in the CNS, and to our knowledge the first to describe an age-related increase in putative phrenic 5-HT_{2A} receptors.

4.1. 5-HT$_{2A/B}$ receptor expression and pLTF

We found that 5-HT$_{2B}$ receptor expression is upregulated at end-stage only within the putative phrenic motor nucleus of MT *vs.* WT rats (Fig. 4K&L). Similar findings have been reported in other models of motor neuron loss. In spinal cord injury, patients experience spasticity that is thought to be caused by loss of serotonergic axons. Normally serotonin mediates motor neuron excitability through an increase in persistent calcium current, but with loss of serotonergic innervation to lower motor neurons there is a loss of serotonergic transmission and the motor neurons become hypoexcitable (Heckmen et al., 2009). To compensate for this loss of brainstem serotonergic input following spinal cord injury, motor neurons produced 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors that were constitutively active (Murray et al., 2010a; Murray et al., 2010b; Fouad et al., 2010). Although this 5-HT$_2$ receptor compensation was shown to contribute to the recovery of locomotor function, it also resulted in hyperexcitability of the motor neurons and lead to spasticity (Murray et al., 2010a; Murray et al., 2010b). ALS patients also experience a degeneration of serotonergic innervation and a depletion of serotonin, which has been recapitulated and studied in a mouse model of ALS (SOD1^{G86R}) (Dentel et al., 2012). Interestingly, pre-symptomatic SOD1^{G86R} mice had decreased serotonin in the brainstem, spinal cord, and cortex, while serotonin turnover remained unchanged (Dentel et al., 2012). SOD1^{G86R} mice also developed spasticity that was alleviated by the 5-HT$_{2B/C}$ inverse agonist SB206553 (Dentel et al., 2012). These studies suggest that loss of central serotonin due to depletion, not the turnover of serotonin, contributes to early development of ALS and that compensation through constitutively active 5-HT$_{2B}$ and 5-HT$_{2C}$ receptors leads to motor neuron hyperexcitability and spasticity (Heckmen et al., 2009; Dentel et al., 2012; Murray et al., 2010a; Murray et al., 2010b; Fouad et al., 2010). Therefore, it is possible that the increase in 5-HT$_{2B}$ receptor expression we observed in MT rats at end-stage is in response to loss of serotonergic innervation and contributes to plasticity of the surviving motor neurons by making them hyperexcitable.

Previous studies conducted by Nichols et al. (2014) found that blockade of 5-HT receptors with methysergide did not impact breathing at end-stage in MT rats, but that blockade of downstream 5-HT$_{2A}$ and 5-HT$_{2B}$ (Gq) receptor pathway modulators (*e.g.*, new BDNF synthesis and MEK) did impact pLTF (Nichols et al., 2014). Furthermore, ERK/MAPK signaling is required for the induction of AlH-induced pLTF in naïve rats, but not the maintenance of pLTF (Hoffman et al., 2012). Similarly, 5-HT$_{2A}$ receptors are required for the initiation of AlH-induced pLTF but not the maintenance (Fuller et al., 2001). It has also been shown that blocking either 5-HT$_{2A}$ or 5-HT$_{2B}$ prior to AIH attenuates pLTF, indicating the activation of both isoforms during AlH-induced pLTF (Tadjalli and Mitchell, 2019). Recent studies have evaluated pMF in naïve rats by examining MEK/ERK MAPK signaling downstream from 5-HT$_{2A/B}$ receptors and found that inhibition of MEK/ERK MAPK blocks 5-HT$_{2A}$, but not 5-HT$_{2B}$ pMF (Tadjalli and Mitchell, 2019). Taken together with what was previously discussed, we believe that: 1) 5-HT$_{2B}$ receptor expression becomes upregulated in end-stage MT rats following loss of serotonergic input (whether those receptors are constitutively active or not has yet to be determined); 2) 5-HT$_{2A}$ receptor expression is unchanged in the putative phrenic motor nucleus at end-stage in MT rats because initiation of pLTF may have already begun during the pre-symptomatic stage; and 3) that both

receptors are involved in pLTF and breathing because blocking 5-HT_2 receptors or downstream signaling modulators resulted in compromised pLTF or breathing in MT rats at end-stage (Nichols et al., 2014; Nichols et al., 2017). Since 5-HT_{2A} and 5-HT_{2B} receptor expression in the putative phrenic motor nucleus is either not different or upregulated in end-stage MT rats *vs.* WT, age-matched littermates, we suggest both 5-HT_{2A} and 5-HT_{2B} receptors have a role in pLTF. We speculate that there may be compensation by one 5-HT_2 isoform becoming constitutively active to preserve pLTF *via* the Q-pathway as the other 5-HT_2 isoform becomes depleted or desensitized to serotonin throughout disease progression. Given that the overexpression of SOD1 does not initially downregulate reactive oxygen species in MT rats, there may be a switch from 5-HT_{2B} receptor-NADPH oxidase-dependent to 5-HT_{2A} receptor-NADPH oxidase-independent pLTF to preserve plasticity at end-stage through the formation of additional reactive oxygen and nitrogen species, as discussed in later sections. Since we suggest a role for 5-HT_2 receptors in pLTF and breathing, future studies could focus on the intrathecal delivery of the isoform specific antagonists for 5-HT_{2A} and 5-HT_{2B} receptors both before and after AIH to delineate their role in pLTF and breathing in end-stage MT rats (*i.e.*, if isoform antagonism abolishes pLTF or compromises breathing). If combined antagonism of these receptors does not impact pLTF or compromise breathing, then compensatory mechanisms may be occurring through activated Gs-coupled receptors (*i.e.*, 5-HT_7 or A_{2A} receptors). Other possible explanations for the 5-$HT_{2A/B}$ receptor expression observed and compensatory mechanisms *via* 5-$HT_{2A/B}$ receptors contributing to pLTF and breathing are discussed in the following sections.

4.2. 5-HT_2 receptor expression on other cell types

Upon closer investigation of the photomicrographs, it appears that the 5-HT_2 receptors are not solely expressed on surviving motor neurons. Therefore, other cell types such as microglia and/or astrocytes, which both express 5-HT_2 receptors, may express these receptors to maintain receptor expression at wild-type levels (MacFarlane, Vinit, & Mitchell, 2011; Hirst et al., 1998). In congruence with work from MacFarlane et al. 2011, we also report expression of 5-$HT_{2A/B}$ receptors on neighboring neuropil (MacFarlane, Vinit, & Mitchell, 2011). *In vitro* research has shown that serotonin and ATP facilitate microglial migration (Krabbe et al., 2012). Thus, it is possible that motor neuron death itself could lead to the release of ATP and signal microglial migration. In $SOD1^{G93A}$ mice, microglial 5-HT_{2B} receptor expression was found to be upregulated in the lumbar (L_{3-5}) spinal cord tissue and that blocking 5-HT_{2B} receptors on microglia accelerated disease progression (El Oussini et al., 2016), which could also explain why we see an increase in 5-HT_{2B} receptors in end-stage MT rats. Another study evaluating astroglial reactivity in the cervical spinal cord found that astroglial activation occurred in pre-symptomatic MT rats in the ventral horn of the spinal cord before motor neuron degeneration, which was further increased at end-stage when compared to non-transgenic rats (Howland et al., 2002). Therefore, neurons and other cell types, specifically astroglia, outside of the putative phrenic motor nucleus could be upregulating their expression of 5-HT_{2A} receptors by end-stage in the non-phrenic ventral horn, resulting in the return towards WT levels of 5-HT_{2A} expression as our data suggest (Fig. 2). To differentiate these roles, future immunohistochemical studies could be used to further investigate the expression of 5-HT_{2A} and 5-HT_{2B} receptors on microglia and astrocytes.

4.3. The interaction of ROS and 5-HT$_{2A}$ receptors

Lastly, we speculate that other RNS and/or ROS (*e.g.*, nitric oxide or hydrogen peroxide) are formed following 5-HT$_{2A}$ activation independently of NADPH oxidase. This may subsequently lead to the inhibition of phosphatases and activation of PKC, resulting in pLTF at end-stage. When Macfarlane and Mitchell pretreated naïve rats with the nitric oxide synthase (NOS) inhibitor L-NAME, pLTF was attenuated, indicating the requirement for NOS and nitric oxide in pLTF. *In vitro* studies have also shown that serotonin can directly bind to and activate nNOS leading to not only NO production, but ROS production as well (Bréard & Grillon, 2009). Together, these studies may indicate 5-HT$_{2A}$ activation, independently of NADPH oxidase, that leads to the pLTF observed at end-stage in MT rats. Finally, isoform specific antagonism of 5-HT$_{2A}$ and 5-HT$_{2B}$ receptors could not only determine which isoform is required for pLTF in pre-symptomatic and end-stage MT rats, but the underlying mechanisms of pLTF that may include the production of RNS and ROS that are not scavenged by the overexpression of superoxide dismutase I. Future studies would have to utilize RNS and ROS inhibitors (against nitric oxide or hydrogen peroxide) to determine if they are necessary for pLTF. If either RNS and ROS (*e.g.*, nitric oxide or hydrogen peroxide) are required for pLTF, then 5-HT$_{2A}$ or 5-HT$_{2B}$ receptor antagonists should be used to determine if the activation of these receptor isoforms lead to loss of downstream RNS and/or ROS production. These studies would provide insight as to which isoform is responsible for alternative RNS and/or ROS production that could contribute to pLTF despite the presence of superoxide dismutase overexpression and being independent of NADPH oxidase.

4.4. Significance

Overall, this study demonstrates that phrenic motor neuron death in MT rats leads to upregulation of 5-HT$_{2B}$ receptors in the putative phrenic motor nucleus at end-stage, while 5-HT$_{2A}$ remains unaffected in the putative phrenic motor nucleus throughout disease progression. We suspect that 5-HT$_2$ receptor expression is changing on the surviving phrenic motor neurons as a compensatory measure to combat the loss of serotonergic input, as shown previously in other models of motor neuron loss. Because 5-HT$_{2A/B}$ receptors and their downstream modulators have been shown to be required for pMF and pLTF, their expression may be indicative of the mechanism by which respiratory plasticity is being elicited leading to increased output from the surviving motor neurons at end-stage in MT rats. The increase in 5-HT$_{2B}$ receptors may be due to the compensatory production of constitutively active 5-HT$_{2B}$ receptors. Although these receptors may contribute to pLTF and the maintenance of breathing through increased motor neuron excitability, spasticity, particularly of the diaphragm, can occur which may hinder breathing capacity late in disease. Additionally, we speculate that microglia and astrocytes are migrating to the putative phrenic motor nucleus and increasing their expression of 5-HT$_2$ receptors in response to motor neuron death, although this has yet to be discerned. This study highlights a potential role for 5-HT$_2$ receptor-dependent mechanisms for pLTF as well as the maintenance of breathing, especially following motor neuron loss. If the role of each of the 5-HT$_2$ receptor isoforms in pLTF and breathing could be delineated throughout disease progression, then these receptors or their downstream modulators could be

pharmacologically targeted to maintain elevated output of the surviving motor neurons and preserve breathing in those with ALS and similar motor neuron diseases.

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Abbreviations

ALS	amyotrophic lateral sclerosis
SOD1	superoxide dismutase 1
pLTF	phrenic long-term facilitation
MT	mutant
WT	wild-type
pMF	phrenic motor facilitation
NOS	nitric oxide synthase
ROS	reactive oxygen species
RNS	reactive nitrogen species

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Highlights

1. Phrenic 5-HT_{2A} receptor expression is reduced over time in $SOD1^{G93A}$ rats.
2. Phrenic 5-HT_{2A} receptor expression is unchanged in $SOD1^{G93A}$ rats *vs.* wild-type rats.
3. Phrenic 5-HT_{2B} receptor expression is increased over time in $SOD1^{G93A}$ rats.
4. Phrenic 5-HT_{2B} receptor expression is unchanged in pre-symptomatic $SOD1^{G93A}$ rats.
5. Phrenic 5-HT_{2B} receptor expression is upregulated in end-stage $SOD1^{G93A}$ rats.

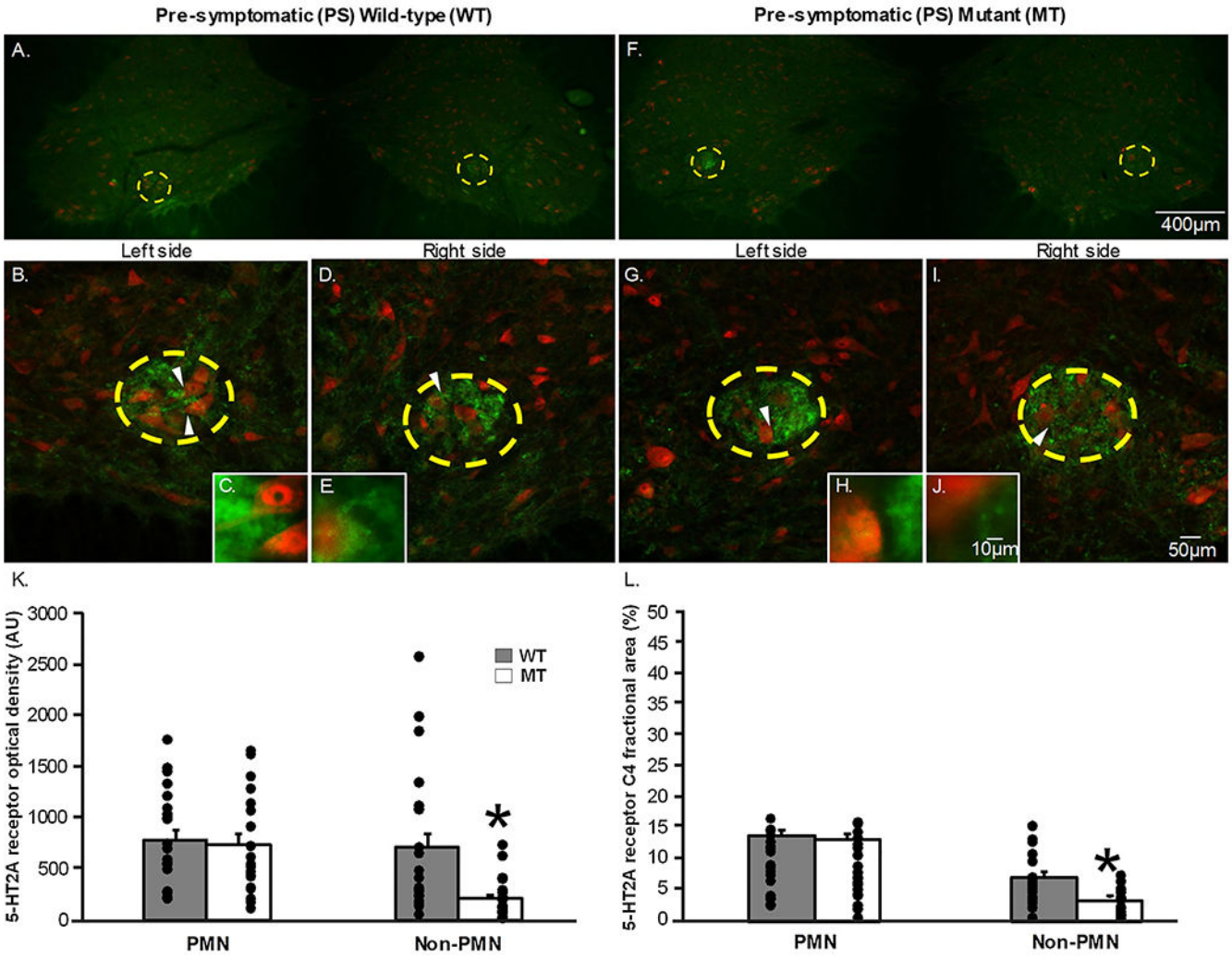

Figure 1: 5-HT$_{2A}$ receptor expression in the putative phrenic motor nucleus and non-phrenic ventral horn in pre-symptomatic wild-type and SOD1^{G93A} mutant in C$_4$ spinal cord sections.

The representative photomicrographs display motor neurons (NeuN; red) and 5-HT$_{2A}$ (green) receptor expression within the putative phrenic motor nucleus (yellow dashed circle) and the non-phrenic ventral horn (area outside of the yellow dashed circle) from C$_4$ spinal cord sections in pre-symptomatic (PS; *1A-J*) wild-type (WT; *1A-E*) and SOD1^{G93A} (Mutant; MT; *1F-J*) rats at 4x (*1A&F*), 20x (*1B,D,G,I*), and 40x (*1C,E,H,J*) magnification. The white arrows in the 20x magnification images indicate what neurons are displayed in the 40x magnification images. 5-HT$_{2A}$ receptor expression is represented as optical density (AU; *1K*) and fractional area in C$_4$ (*1L*) in the putative phrenic motor nucleus and non-phrenic ventral horn). Note, there were no significant differences in 5-HT$_{2A}$ optical density when comparing MT rats to age-matched WT littermates in the putative phrenic motor nucleus within PS and ES animals ($p>0.05$). However, there is a significant decrease in 5HT$_{2A}$ optical density and fractional area in the non-phrenic ventral horn of PS MT rats compared to PS WT rats.

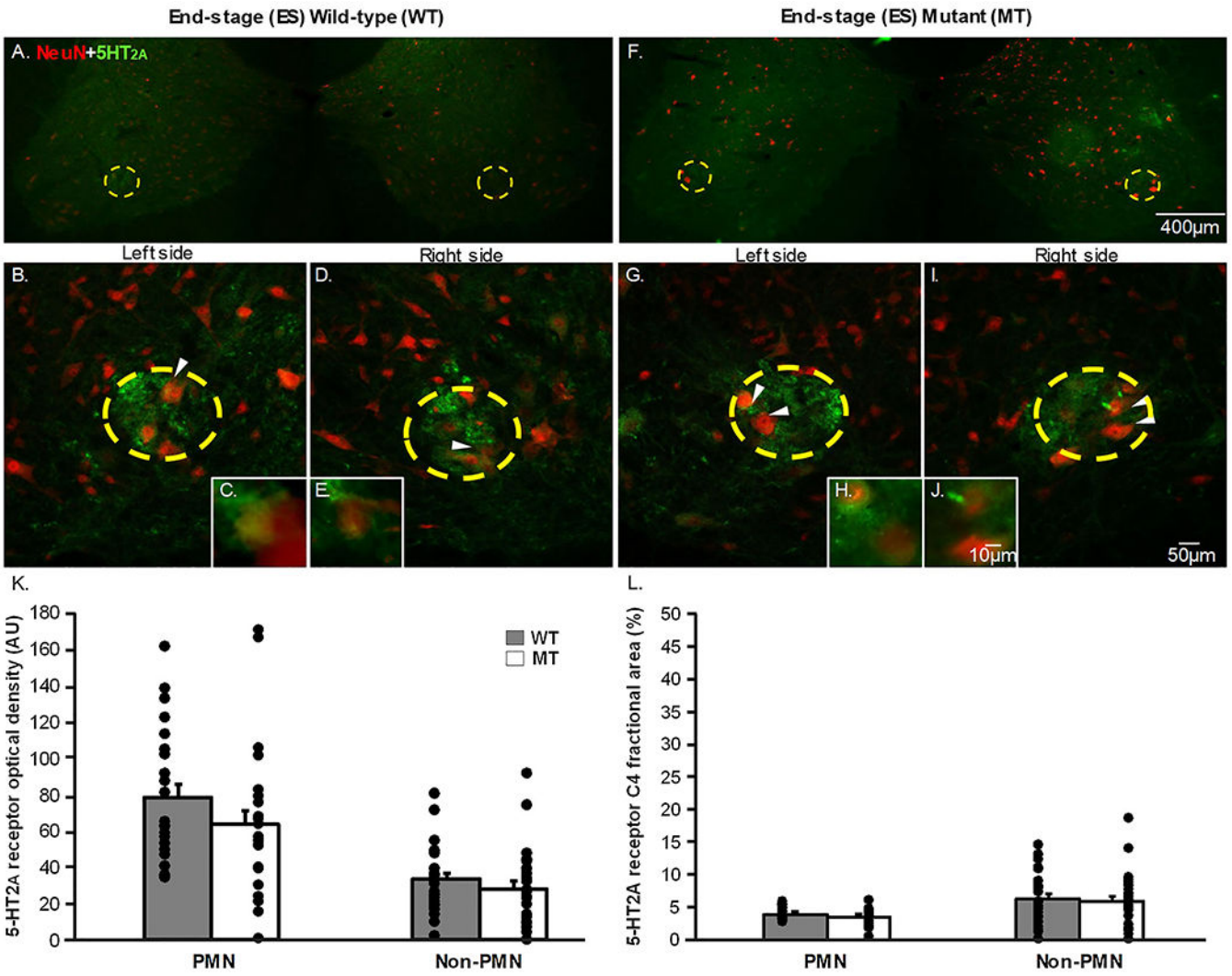

Figure 2: 5-HT$_{2A}$ receptor expression in the putative phrenic motor nucleus and non-phrenic ventral horn in end-stage wild-type and SOD1^{G93A} mutant in C$_4$ spinal cord sections.

The representative photomicrographs display motor neurons (NeuN; red) and 5-HT$_{2A}$ (green) receptor expression within the putative phrenic motor nucleus (yellow dashed circle) and the non-phrenic ventral horn (area outside of the yellow dashed circle) from rat C$_4$ spinal cord sections in end-stage (ES; *2A-J*) wild-type (WT; *2A-E*) and SOD1^{G93A} (Mutant; MT; *2F-I*) rats at 4x (*2A&F*), 20x (*2B,D,G,I*), and 40x (*2C,E,H,J*) magnification. The white arrows in the 20x magnification images indicate what neurons are displayed in the 40x magnification images. 5-HT$_{2A}$ receptor expression is represented as optical density (AU; 2*K*) and fractional area (2*L*) in the putative phrenic motor nucleus and the non-phrenic ventral horn. Note, there were no significant differences in 5-HT$_{2A}$ optical density when comparing MT rats to age-matched WT littermates neither in the putative phrenic motor nucleus nor the non-phrenic ventral horn at ES ($p>0.05$).

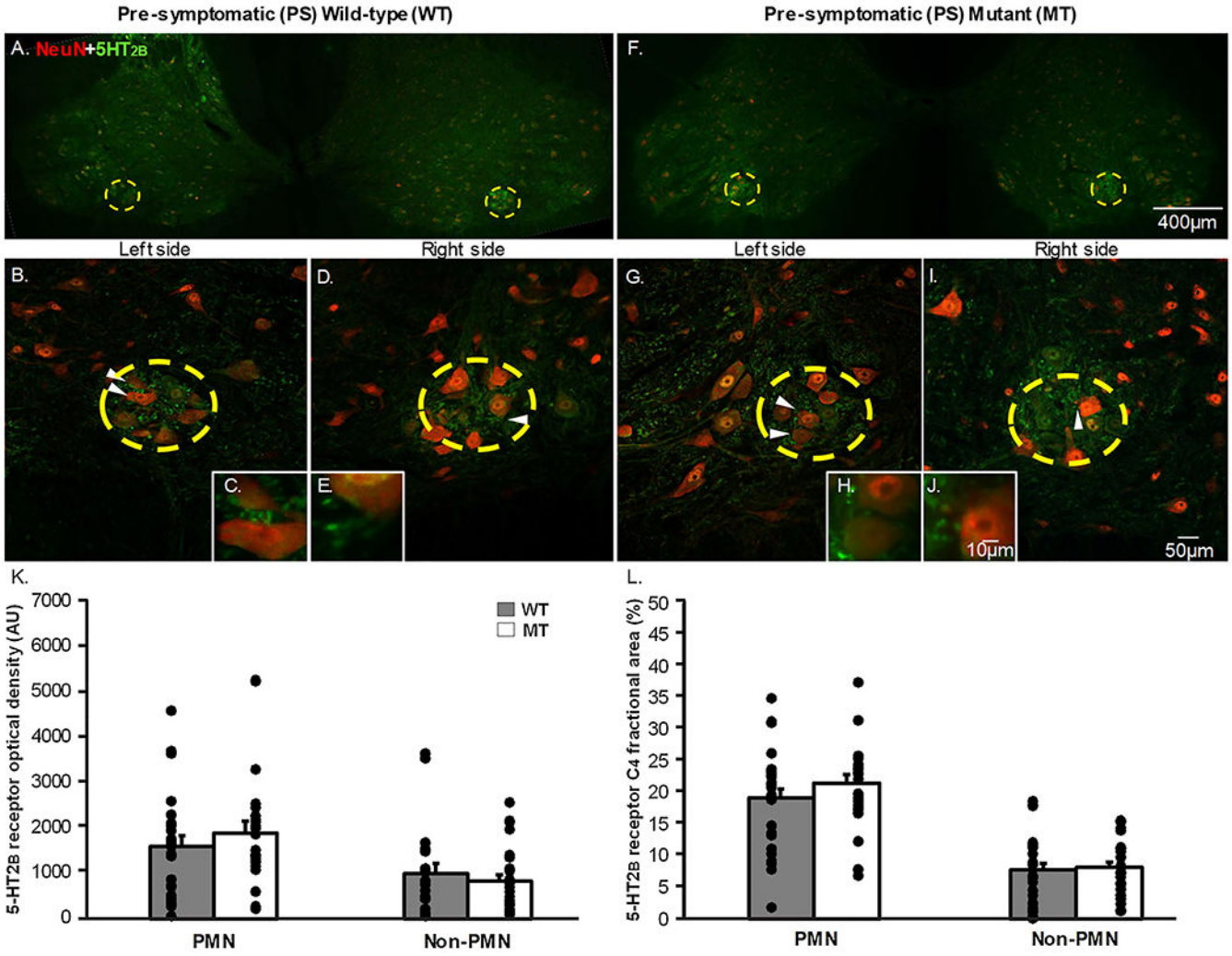

Figure 3: 5-HT$_{2B}$ receptor expression in the putative phrenic motor nucleus and non-phrenic ventral horn in pre-symptomatic wild-type and SOD1^{G93A} mutant in C$_4$ spinal cord sections.

The representative photomicrographs display motor neurons (NeuN; red) and 5-HT$_{2B}$ (green) receptor expression within the putative phrenic motor nucleus (yellow dashed circle) and the non-phrenic ventral horn (area outside of the yellow dashed circle) from rat C$_4$ spinal cord sections in pre-symptomatic (PS; *3A-J*) wild-type and SOD1^{G93A} (Mutant; MT; *3F-J*) rats at 4x (*3A&F*), 20x (*3B,D,G,I*), and 40x (*3C,E,H,J*) magnification. The white arrows in the 20x magnification images indicate what neurons are displayed in the 40x magnification images. 5-HT$_{2B}$ receptor expression is represented as optical density (AU; *3K*) and fractional area (*3L*) in the putative phrenic motor nucleus and non-phrenic ventral horn. 5-HT$_{2B}$ optical density and fractional area was not different in MT PS rats within the putative phrenic motor nucleus as well as within the non-phrenic ventral horn when comparing MT rats to age-matched WT littermates ($p>0.05$).

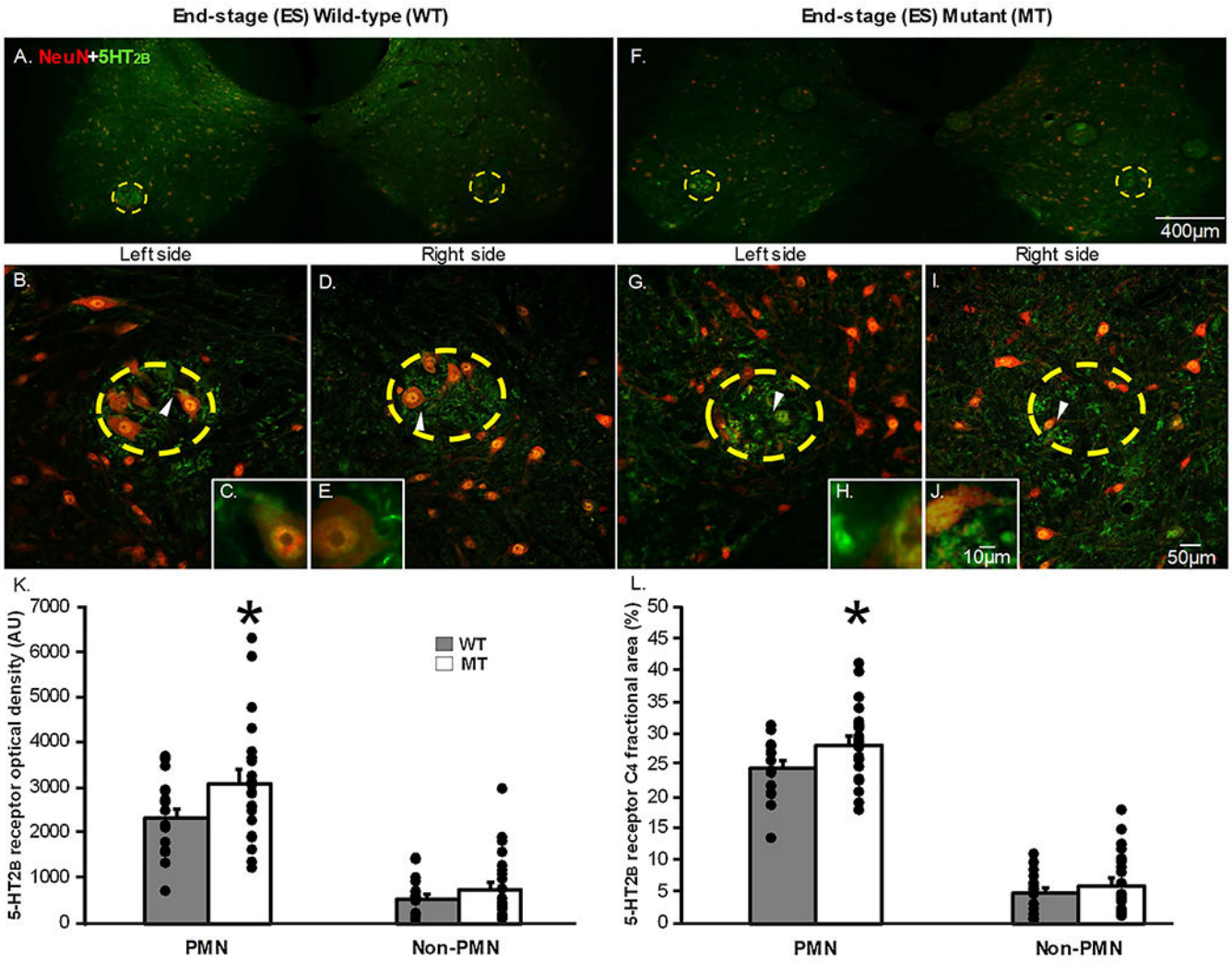

Figure 4: 5-HT$_{2B}$ receptor expression in the putative phrenic motor nucleus and non-phrenic ventral horn in end-stage wild-type and SOD1^{G93A} mutant in C$_4$ spinal cord slices.

The representative photomicrographs display motor neurons (NeuN; red) and 5-HT$_{2B}$ (green) receptor expression within the putative phrenic motor nucleus (yellow dashed circle) and the non-phrenic ventral horn from rat C$_4$ spinal cord sections (area outside of the yellow dashed circle) in end-stage (ES; *4A-J*) wild-type (WT; *4A-E*) and SOD1^{G93A} (Mutant; MT; *4F-J*) rats at 4x (*4A&F*), 20x (*4B,D,G,I*), and 40x (*4C,E,H,J*) magnification. The white arrows in the 20x magnification images indicate what neurons are displayed in the 40x magnification images. 5-HT$_{2B}$ receptor expression is represented as optical density (AU; *4K*) and fractional area (*4L*) in the putative phrenic motor nucleus (PMN) and non-phrenic ventral horn. 5-HT$_{2B}$ optical density ($p=0.032$) and fractional area ($p=0.04$) was significantly increased in MT ES rats within the putative phrenic motor nucleus when comparing MT rats to age-matched WT littermates at ES.