CHAPTER 6

Corticosteroid effects on airway neuroplasticity in a murine chronic asthma model

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Abstract

Airway neuroplasticity is a pathological feature of asthma that may contribute to airway hyperresponsiveness (AHR). Corticosteroids are known to reduce airway inflammation and improve lung function. However, their effect on airway neuroplasticity has not been investigated yet. This study aims to investigate the effects of budesonide, a corticosteroid that is commonly used in asthma therapy, on airway neuroplasticity. The role of budesonide on the neuronal marker class III β tubulin (βIII-tubulin) expression in the lung was evaluated in murine chronic asthma models using immunohistochemistry. Preventive budesonide administration, initiated from the first allergen exposure, dampened increased ovalbumin-induced neuronal growth. In contrast, when therapeutic budesonide was given only during the last 3 of 6 weeks OVA challenges, this effect was not observed. Both preventive and therapeutic intervention with budesonide equally inhibited eosinophil infiltration into the airways. Moreover, the anticholinergic drug tiotropium was able to reverse neuroplasticity in the therapeutic intervention model. Analysis of bronchial biopsy data from asthma patients with or without inhaled corticosteroids use showed no difference in a neuronal marker, vesicular acetylcholine transporter (VAChT), expression in asthma patients using ICS versus asthma patients not using ICS. The direct effect of budesonide on neuronal growth was investigated in vitro in neuronal-like cell line SH-SY5Y cells. Budesonide did not prevent BDNF-induced SH-SY5Y cell cholinergic differentiation. Collectively, these results indicate that corticosteroids dampen neuronal remodeling probably because of its effect on preventing eosinophil infiltration into the airways and not via its direct effect on neurons. Once neuronal remodeling is induced, corticosteroids are unable to reverse this process without associated muscarinic antagonist. Meanwhile, muscarinic antagonist alone is sufficient to reverse both airway inflammation and neuronal remodeling.

Keywords: Asthma, airway neuroplasticity, budesonide, corticosteroids

Introduction

Asthma is defined as a heterogenous disease affecting 1-18% of the population in different countries. It is characterized by chronic airway inflammation (1). Wheeze, shortness of breath, chest tightness and/or cough, and variable expiratory airflow obstruction are recognized clinical characteristics (1). Another important feature that is associated with asthma is airway hyperresponsiveness (AHR) to direct or indirect stimuli, e.g. allergen or irritant exposure, exercise, change in weather, or viral infections (1). Prolonged AHR is associated with airway remodeling, including increased airway smooth muscle mass, epithelial changes contributing to mucus hypersecretion, subepithelial changes in extracellular matrix deposition and bronchial microvascular remodeling (2–4). Recently, neuroplasticity or neuronal remodeling has been demonstrated in asthma as well (5).

Neuroplasticity is described as the ability of the nervous system to change its structure, function, and connection in response to stimuli (6). Eosinophils, the most common airway inflammatory cells in asthma, are increased around the airways, and their presence is associated with heightened parasympathetic and sensory nerve function in the airway, leading to bronchoconstriction and AHR (7,8). Recently it has been shown that the expression of vesicular acetylcholine transporter (VAChT), a cholinergic marker, in airway biopsies of asthma patients is higher compared to healthy controls (9). This is paralleled by an increased gene expression of tropomyosin receptor kinase B (TrkB), a receptor for brain-derived neurotrophic factor (BDNF) (9). Accordingly, inhibition of TrkB reduced cholinergic nerve density and the subsequent development of AHR in a murine chronic asthma model (9). The role of neurotrophins as mediators of airway neuroplasticity in asthma has been reviewed in detail by Kistemaker et al (9). Moreover, studies in animal models of asthma and human airway epithelial cells in vitro have suggested that cholinergic antagonism has protective effects on airway inflammation and remodeling, indicating a significant role of acetylcholine, either from neuronal or non-neuronal origin in asthma (10–13).

Inhaled corticosteroids (ICS) are the mainstay therapy in asthma, mainly used for their role in targeting chronic inflammation and, to a lesser extent, airway remodeling in asthma (1,14). In vitro and in vivo studies have demonstrated that dexamethasone, a potent synthetic corticosteroid, regulates BDNF expression and neuroplasticity in several areas of the central nervous system (CNS) relevant to CNS-related pathologies, such as depression, epilepsy, Alzheimer’s, Huntington’s, and Parkinson’s diseases (15–17). Despite the current knowledge on corticosteroids
interactions with neuroplasticity in the CNS, our knowledge on their regulatory effects in the airway nervous system is limited. In an *ex vivo* study of peripheral blood from asthma and allergic rhinitis patients, dexamethasone downregulated nerve growth factor (NGF) expression in eosinophils (18). Furthermore, the protein levels of NGF and BDNF in serum were higher in asthmatics without ICS treatment compared to the ICS treated and healthy control groups (19). However, whether corticosteroid treatment results in subsequent changes in airway neuroplasticity is unknown. An *in vitro* study showed that differentiated neuron PC12 is prone to axonal and developmental changes after exposure to corticosteroids treatment, suggesting corticosteroids potential in shifting neuronal growth (20). Therefore, we studied the role of inhaled budesonide in allergen-induced neuroplasticity and inflammation in a murine chronic asthma model. We found that inhaled budesonide can prevent, but not reverse ovalbumin-induced neuroplasticity.

**Materials and methods**

**Animals**

BALB/cByJ (Jax-strain) were purchased from Charles River. Female animals aged 8-12 weeks old were used for all experiments. Animals were group-housed with 4-8 animals per cage, under 12 hour light:dark cycles and received food and water ad libitum. Sample size for the study was calculated using G*Power 3.1.9.7 (downloaded from http://www.gpower.hhu.de/).

**Chronic asthma model**

Animals were sensitized to ovalbumin (Sigma-Aldrich) on days 1, 14, and 21, followed by intraperitoneal injection of 10 µg of ovalbumin emulsified in 1.5 mg aluminum hydroxide (Aluminject; Pierce Chemical) and diluted to 200 µl with phosphate buffered saline (PBS). For the budesonide prevention study, animals were randomly divided into 3 groups (N=8 each): Control (CTR), Ovalbumin (OVA), and Ovalbumin + Budesonide (OVA + BUD). Animals were exposed to aerosol of ovalbumin (1% in PBS) in OVA and OVA + BUD groups, and to saline in CTR group for 20 minutes twice weekly on consecutive days for 4 weeks as described previously (21). In the OVA + BUD group, budesonide (0.1 mM nebulizer concentration; Tocris Bioscience 2671, Bristol, UK) was administered by nebulization for 15 minutes twice weekly at 24 hours and 1-hour prior to each ovalbumin challenge for the entire 4-week period. A schematic detailing the challenge and treatment regimens is provided in Figure 1a. The aerosol was delivered to a Perspex exposure chamber (9 liter) by a Pari Box SX nebulizer driven by an airflow of 40 L/min, providing an aerosol with an output of 0.33 ml/min as described previously (22).

For the therapeutic budesonide study, animals were sensitized to ovalbumin (Sigma-Aldrich) as described in the prevention model above. Subsequently, animals were randomly divided into 5 groups (N=8 each): Control (CTR), Ovalbumin (OVA), Ovalbumin + Budesonide (OVA + BUD), Ovalbumin + Tiotropium (OVA + TIO), and Ovalbumin + Budesonide + Tiotropium (OVA + BUD + TIO). Animals were exposed to aerosol of ovalbumin (1% in PBS) in OVA, OVA + BUD, OVA + TIO, and OVA + BUD + TIO groups, and to saline in CTR group for 20 minutes twice weekly on consecutive days for 4 weeks. Budesonide (0.1 mM nebulizer concentration; Tocris Bioscience catalog number 2671, Bristol, UK) was administered by nebulization for 15 minutes twice weekly at 24 hours and 1-hour prior to each ovalbumin challenge at the last 3 weeks period. Tiotropium (0.1 mM; provided by Boehringer Ingelheim) was administered for 15 minutes twice weekly at 1-hour prior to each ovalbumin challenge during the last 3 weeks only. A schematic detailing the challenge and...
treatment regimens is provided in Figure 1.b. The aerosol delivery system is the same as described in the prevention model above. During the procedure, 1 animal of each of the CTR, OVA + BUD, and OVA + BUD + TIO groups were terminated because of prior established humane end-point criteria (more than 15% body weight reduction for more than a week).

**Lung harvesting**
Euthanasia was carried out via administration of 40 mg/kg of ketamine and 0.5 mg/kg of dexmedetomidine HCl (dexdormitor) interperitoneal. After euthanasia and exsanguination, lungs were harvested. The left lobe was removed and snap frozen for gene expression analysis. The right lung was inflated with 600 µl of 4% buffered PFA and fixed overnight immersed in the same solution at 4°C. The lungs were washed continuously with PBS for 48 hours and cryoprotected for 24 hours in 18% of sucrose solution and snap froze in liquid nitrogen for immunohistochemistry.

**Eosinophil staining in lung sections**
Cryosections of 5 µm thickness were dried for 30 minutes with a hair dryer and fixed in acetone for 10 minutes. Sections were then washed with 1X phosphate buffer saline (PBS) 3 times (3 minutes for each wash). Subsequently, sections were stained with diaminobenzidine (Sigma-Aldrich, Zwijndrecht, The Netherlands) diluted in 1X PBS with H₂O₂ and NaCN for 7 minutes. Sections were washed 2 times with 1X PBS and 2 times with demi water. Sections were further incubated for 2 minutes in hematoxylin and mounted with warm Kaiser’s glycerin on coverslips. The number of eosinophils within 50 µm around the airways was counted and adjusted for the length (mm) of the basement membrane. 5 airways were randomly selected and counted. Data analysis was done in a blinded manner using Microsoft Excel.

**Immunohistochemistry for βIII-tubulin in lung sections**
After fixation, cryosections of 12 µm thickness were blocked with 0.5% triton and 5% donkey serum in PBS for 1 hour. Next, they were incubated with primary antibody against βIII-tubulin (1:200 dilution; Cell Signaling Technology: D71G9 XP(R) Rabbit mAb) overnight at 4°C. Fluorophore conjugated secondary antibody (Donkey anti-rabbit 488) was added for 90 minutes at room temperature. Slides were mounted with Fluoroshield mounting medium with DAPI (Abcam). Images were acquired using a Leica DM4000 B microscope. βIII-tubulin positive area was quantified using ImageJ software (NIH) and normalized by the area (µm²) of airway basement membrane. 5 airways were randomly selected and counted. Data analyses were done in a blinded manner.

**Culture of SH-SY5Y cells**
SH-SY5Y cells were differentiated to express a cholinergic-like phenotype using protocols adapted from a previously published study (23). SH-SY5Y were expanded in growth medium (DMEM:F12 supplemented with 10%(v/v) FBS, 1% L-glutamine (L-glut), and 50 U/mL p/s). The maximum passage number used for experiments was p15. For cholinergic differentiation, the medium was changed to SH-SY5Y differentiation medium (DMEM:F12 supplemented with 1% FBS, 1% L-glut, 50 U/mL p/s, and 10 µM retinoic acid). On day-4, the differentiation medium was supplemented with BDNF (50 ng/ml, Peprotech 450-02) with or without budesonide (10 nM, Tocris Bioscience 2671). As controls, cell cultures were also kept with budesonide only or without any BDNF and/or budesonide. The cultures were maintained for five more days while changing the medium every other day before RNA extraction or immunofluorescence.
Gene expression analysis

Total RNA was isolated using the RNA purification kit NucleoSpin RNA (Macherey-Nagel 740955, Germany) according to the manufacturer’s guide. Cells were lysed by adding a mixture of buffer RA1 and dithiothreitol (Sigma-Aldrich, 3483-12-3). The yield of the isolated RNA was then measured using the NanoDrop 1000 spectrophotometer. Samples were analyzed by RT-PCR.

For RT-PCR analysis, equal amounts of total RNA were reverse transcribed into cDNA. cDNA was generated with cDNA synthesis kit (Promega) using M-MLV reverse transcriptase (Promega M1701) in Doppio thermal cycler (VWR, 732-2551). RT-PCR analysis were performed in PCRmax ECO48 Real-time qPCR system (PCRmax, UK) or Quantstudio 7 flex Real-Time PCR system (Thermo Fisher Scientific, 4485701). RT-PCR was performed with denaturation at 94°C for 30 seconds, annealing at 59°C for 30 seconds and extension at 72°C for 30 seconds for 45 cycles followed by 10 minutes at 72°C. RT-PCR data were analyzed using the comparative cycle threshold (Ct: amplification cycle number) method. Analysis of gene expression was performed with Eco Study software or Quantstudio Real-Time PCR software v1.2, respectively. The amount of target gene was normalized to the endogenous reference gene RPL13a and SDHA. Gene expression of target genes was normalized to house-keeping genes. The specific forward and reverse primers used are listed in table 1.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>VAChT</td>
<td>Forward – GTGGCCCATGTCCTCACTCA</td>
</tr>
<tr>
<td>TUBB3</td>
<td>Forward – GGAGATCGTGACACATCCAGG</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>Forward – TTCCCTGCGACAAACTCGGAC</td>
</tr>
<tr>
<td>RPL13a</td>
<td>Forward – TGTCTACGGCGAAGGACATC</td>
</tr>
<tr>
<td>SDHA</td>
<td>Forward – TGGAGCGGCGGATGGATGGAT</td>
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Staining of SH-SY5Y cells

SH-SY5Y cells were fixed in 4% paraformaldehyde (PFA) for 15 minutes at room temperature (RT) and washed 3x with 2X PBS. Samples were permeabilized using 0.3% Triton-X for 5 minutes at RT, washed 3x with Cyto-TBS, consisting of ddH2O supplemented with 20 mM Tris Base, 154.0 mM NaCl, 2 mM EGTA, and 2 mM MgCl2·6·H2O, and blocked for 1 hour with blocking buffer (Cyto-TBS supplemented with 2% bovine serum albumin (BSA)). The samples were incubated with β3-tubulin primary antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, USA) overnight in a humidified chamber at 4°C. The following day, samples were incubated with donkey anti-mouse Alexa Fluor 488 (1:1000, Invitrogen A21202) for 2 hours in the dark in room temperature and washed with 2X Cyto-TBST (0.1% Tween-20 in Cyto-TBS), 1X Cyto-TBS, and 1X ultrapure water. Samples were mounted using mounting medium with DAPI (Abcam, Cambridge, UK) overnight at 4°C. Images were acquired using a Leica DM4000 B microscope. βIII-tubulin positive of each group was represented.

Ethical statement

Data from human tissues were re-used and re-analyzed from Dragunas G et al. (2020)(9).

All animal experiments were performed in accordance with the national guidelines and approved by the University of Groningen Committee for Animal Experimentation (CCD license AVD105002016492; IVD protocol number 16492-01-004).

Statistical analysis

Data are represented as mean ± SEM. The statistical significance of differences between means of groups were determined by appropriate statistical tests as indicated in the text. Statistical analysis was made in Graphpad Prism version 9.3.1 (GraphPad Software). Differences were considered significant when P value <0.05.
Results

Preventive budesonide inhibits neuronal outgrowth and inflammation in murine chronic asthma model

To investigate the effect of preventive budesonide treatment on airway neuroplasticity in response to ovalbumin challenge in murine airways, sections were stained for βIII-tubulin as a pan-neuronal marker. Ovalbumin sensitization and challenge significantly increased the βIII-tubulin positive area in the airways (4-fold change) which was inhibited by preventive budesonide administration (66.5 %) (figure 2a-b).

In order to whether the observed effect on neuroplasticity could be related to the anti-inflammatory properties of budesonide, the number of eosinophils around the airways in these mice was also analyzed. Ovalbumin challenge induced a 17-fold increase in eosinophil numbers (figure 2c-d). This effect was prevented by budesonide by 64.9 %. Subsequently, we analyzed the correlation between the changes in βIII-tubulin positive areas and eosinophil numbers. The two markers were highly positively correlated (p<0.0001) (figure 2e).

Budesonide does not reverse ovalbumin-induced neuroplasticity

In view of the compelling effects of budesonide on the prevention of airway neuroplasticity in response to ovalbumin challenge, we next evaluated the effects of therapeutic budesonide on a background of already established disease. In this experiment, budesonide was given from week 4 to week 6 of the ovalbumin challenge. Although ovalbumin challenge increased the βIII-tubulin positive area in the airways (1.5-fold change), budesonide failed to significantly inhibit this effect with only 4.3 % reduction (figure 3a-b).

Although there was no effect of therapeutic budesonide on neuroplasticity in this model, its anti-inflammatory properties were preserved. This was evaluated by measuring eosinophil numbers around the murine lung airways. A significant induction in eosinophil numbers in response to ovalbumin challenge (7-fold change) was observed, which was reduced by therapeutic budesonide by 85.5 % (figure 3c-d). The contrast between these anti-neuroplasticity and anti-inflammatory effects was also shown by the lack of correlation between βIII-tubulin positive area and eosinophil numbers across these control, ovalbumin and ovalbumin + budesonide treatment groups (figure 3e).

Figure 2. Effect of preventive budesonide treatment on neuroplasticity and inflammation in the airways of chronically allergen-challenged mice. Mice were treated as described in figure 1a. Lungs were collected 24 hours after the last challenge. βIII-tubulin positive areas and eosinophil numbers were determined by immunohistochemistry. Quantification (a) and representative images (b) of βIII-tubulin positive areas as well as quantification (c) and representative images of eosinophil (d) are shown. Correlation of eosinophil numbers and βIII-tubulin positive areas were calculated by two-tailed Pearson correlation analysis (e). Data represent mean ± SEM. Five airways were analyzed for each animal, n=8 mice per group. Statistical analyses were performed with one-way ANOVA, "p<0.05, "p<0.01, ""p<0.001. Post-hoc analyses were done with Tukey’s multiple comparison test. Data points were normally distributed and checked with Saphiro-Wilk test and Kolmogorov-Smirnov test (α<0.05).
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To assess the reversibility of airway neuroplasticity to other drugs used for asthma treatment, we included tiotropium, a long-acting muscarinic antagonist, in these analyses as well. We found that tiotropium and its combination with therapeutic budesonide both decreased the βIII-tubulin expression, by 139.8% and 121.1% respectively (figure 3a-b). Similarly, significant anti-inflammatory effects were also observed in response to tiotropium alone and its combination with therapeutic budesonide, by 65.3% and 78.9% respectively (figure 3c-d).

ICS use and cholinergic nerve area in asthma
To evaluate the translational relevance of our findings, we used data previously obtained by Dragunas et al (9). Samples were derived from asthmatic patients and healthy controls. Asthmatics were further stratified based on the current use of inhaled corticosteroids (ICS). Stainings for the cholinergic marker VACHT+ area, normalized by alpha-smooth actin (α-SMA) area, are shown in Figure 4. Although there was a 1.9-fold increase of VACHT+ area in asthmatics (9), there was no difference between patients with or without current ICS treatment. This indicates that ICS have no direct effect on inhibiting cholinergic neuroplasticity in asthma patients.

Budesonide failed to prevent BDNF-induced SH-SY5Y cell cholinergic differentiation
To assess the direct effects of budesonide on cholinergic differentiation, we investigated its effects on neuronal cholinergic differentiation of SH-SY5Y cells. This is a neuronal-like cell line known to be responsive to retinoic acid and BDNF with effects on cholinergic marker gene expression. As depicted in figure 5 a-c, budesonide had no effect on BDNF-stimulated mRNA of the neuronal markers VACHT, TUBB3, and PGP9.5. In addition, no effect of budesonide was observed on βIII-tubulin positive area, whereas BDNF itself did have such effects (figure 5.d).

Figure 3. Effect of therapeutic budesonide on neuroplasticity and inflammation in the airways of chronically allergen challenged mice. Mice were treated as described in figure 1b. Lungs were collected 24 hours after the last challenge. βIII-tubulin positive areas and eosinophil numbers were determined by immunohistochemistry. Quantification (a) and representative images (b) of βIII-tubulin positive areas as well as quantification (c) and representative images of eosinophils (d) are shown. Correlation of eosinophil numbers and βIII-tubulin positive areas were calculated by two-tailed Pearson correlation analysis (e).

Data represent mean ± SEM. Five airways were analyzed for each animal, n=7-8 mice per group. Statistical analyses were performed with one-way ANOVA with *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, unpaired t-test with #p<0.05. Post-hoc analyses were done with Tukey’s multiple comparison test. Data points were normally distributed and checked with Shapiro-Wilk test and Kolmogorov-Smirnov test (α<0.05).
Discussion

In this study, we demonstrate that inhaled budesonide, as the main therapy in asthma maintenance, plays a role in allergen-induced neuroplasticity when it is administered preventively. Preventive budesonide significantly dampened increased ovalbumin-induced neuronal growth in murine chronic asthma model. In contrast, therapeutic budesonide had no such effect. Interestingly, both preventive and therapeutic administration of budesonide inhibited eosinophil infiltration into the airways, suggesting that budesonide consistently exhibits anti-inflammatory properties in both administration protocol. However, tiotropium, an anticholinergic, alone and in combination with budesonide reverse allergen-induced neuroplasticity and inflammation in the therapeutic treatment protocol, indicating that this is physiologically feasible. These results imply that corticosteroids prevent allergen-induced neuroplasticity but have no role in reducing neuroplasticity once it has been established. These findings are supported by the lack of effect of current ICS use on cholinergic nerve area in biopsies of asthma patients and by the lack of effect of budesonide on cholinergic differentiation of SH-SY5Y neuronal-like cells.

Growing evidence suggests that neuroplasticity in asthma includes alterations in afferent or sensory nerves, the integrating centers in the brain, notably in nucleus tractus solitarius (NTS), and efferent cholinergic nerves (5). Afferent sensory nerve endings in the subepithelial layer were exposed to airway lumen following epithelial damage in patients with asthma rendering higher sensitivity to outside stimulus (24). Similarly, in animal models of asthma, ovalbumin sensitization stimulated acute hypersensitivity of pulmonary C fibers and lowered action potentials of mechanosensitive A fibers, that might explain the mechanisms of this increased nerve sensitivity (25–27). Changes of NTS neurons were also reported after allergen exposure in animal models of asthma, indicating alterations in the central nervous system (28,29). Meanwhile, alterations in efferent neurons and parasympathetic ganglia by allergen exposure include decreased membrane potential and increased action potential generation, that subsequently leads to enhanced bronchoconstriction (30–32). Moreover, chronic allergen sensitization of airway nerves stimulates neuronal remodeling (8,9,33). Indeed, our study shows that ovalbumin challenge increased cholinergic nerve density as seen by elevated βIII-tubulin levels around the airways. This is in line with finding in patients with asthma that show elevated levels VAChT + area in lung biopsies compared to healthy controls (9).

Asthma is characterized by chronic inflammation with airway hyperresponsiveness (1,7). As airway hyperreactivity and bronchoconstriction are subject to neuronal (dys) regulation, it is of importance to understand the interaction between inflammation and...
neuroplasticity in asthma. Eosinophils, an airway inflammatory cell most commonly found in allergic asthma, increase both efferent parasympathetic and afferent sensory nerve function (7). Indeed, in guinea pigs, neuronal eotaxin-1 expression was stimulated following allergen challenge resulting in eosinophil migration to airway nerves, which was prevented by blocking the CCR3 receptor (34,35). The accumulated eosinophils around the airway nerve endings subsequently release major basic protein, which is an allosteric antagonist of parasympathetic inhibitory muscarinic 2 (M2) receptor, causing higher ACh release (36,37). In fact, dysfunction of the M2 receptor has been observed in patients with asthma (38–40). This eosinophil-derived major basic protein also stimulates pulmonary sensory neurons activity in response to capsaicin in rats, suggesting its wide range of effects in airway neuroplasticity (41). In line with these findings, we demonstrated that ovalbumin upregulated both eosinophil numbers and neuronal marker βIII-tubulin in the airway base membrane, indicating that chronic ovalbumin-challenge is an appropriate in vivo model to study the effect of budesonide in asthma inflammation and neuroplasticity.

However, although budesonide dampened the eosinophil infiltration both in the preventive and therapeutic treatment protocol, suggesting its intact anti-inflammatory effect in both models, its effect in inhibiting of βIII-tubulin was only observed in the preventive model and not in the therapeutic treatment model. The absence of a neuroplasticity inhibitory effect in the therapeutic treatment model was unexpected considering the mechanistic association of increased airway eosinophilia infiltration and neuroplasticity previously described.

Prior administration of dexamethasone inhibits the expression of neuronal-derived eosinophil protein adhesion (intercellular adhesion molecule 1; ICAM-1), airway reactivity, and M2 receptor dysregulation in allergen-sensitized guinea pig studies (42,43). Hence, corticosteroids effects on neuroplasticity may be exclusive to a preventive administration. A possible explanation for these observations is that the eosinophil-induced neuroplasticity took place in the initial allergen challenges which stimulated eosinophils migration into airways. Eosinophils are further activated and secrete mediators that promote neural growth and lower neurons’ activating threshold, giving rise to increased nerve growth and activity (7). Once the neuroplasticity is established, subsequent reduction of eosinophils by budesonide might not decrease airway neuroplasticity. This might explain the effectiveness of budesonide in preventing neuroplasticity in the preventive budesonide model and its absence of effect in the therapeutic budesonide model. This hypothesis is also supported by our finding in asthma patients that showed unchanged neuronal marker in patients treated with or without ICS.

Corticosteroids are suggested to have direct effects on neurons that further affects neuroplasticity (15–17,44,45). One of the feature of airway neuroplasticity in asthma is increased cholinergic density that is facilitated by BDNF/TrkB signaling (5,9). In line with the in vivo findings, we found no effect of budesonide on BDNF-induced cholinergic differentiation of SH-SYSY cells. SH-SYSY is a human neuroblastoma cell line that can be differentiated into neuronal-like cells with cholinergic characteristic and is widely used in nervous system disease model in vitro (23). Similarly, a recent study also reported that 5-day exposure to dexamethasone, a potent corticosteroid, did not alter cholinergic neuronal marker expressions in human pluripotent stem cell (hPSC)-derived airway cholinergic neurons (46). The absence of direct effects of corticosteroids on cholinergic neuronal differentiation may partly explain the lack of effect of budesonide in the therapeutic treatment model in vivo.

Increased activity in cholinergic signaling in asthma might be caused by airway neuroplasticity (47). Targeting cholinergic pathway by anticholinergics, such as tiotropium, results in bronchodilation, reduced mucus secretion, airway remodeling, and inflammation (48). However, little is known about the effects of anticholinergic drugs in reversing increased nerve density in response to allergen exposure. Our data demonstrate that tiotropium treatment significantly reduced expression of the neuronal marker βIII-tubulin in a murine chronic asthma model. This indicates that tiotropium is not only blocking cholinergic signaling by inhibiting muscarinic receptors but might have long lasting effect by reversing increased nerve density in asthma. Since no available therapy target in changes in asthma, further studies are needed to elucidate the mechanism and possible clinical relevance.

In conclusion, the results of the present study demonstrate that preventive treatment with budesonide has a protective effect against neuroplasticity that correlates with its anti-inflammatory properties. However, budesonide does not reverse established neuroplasticity. Therapeutic administration of tiotropium did both. These findings provide added insight into the role of corticosteroids in targeting specific asthma characteristics, as it is a mainstay controller therapy in asthma.

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Conflict of Interest
The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
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Author Contributions
SFR, RG, LK, and HK conceived and designed the study. SFR, ISTB, and GD performed all the experiments. SFR, ISTB, GD, and RG analyzed the data. SFR wrote the main manuscript text and prepared all figures. All authors reviewed the manuscript.

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GD declares no competing interest.

ISTB declares no competing interest.

RV declares no competing interest.

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