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Expression and function of NPSR1/GPRA in the lung before and after induction of asthma-like disease

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Allen, Irving C., Amy J. Pace, Leigh A. Jania, Julie G. Ledford, Anne M. Latour, John N. Snouwaert, Virginie Bernier, Rino Stocco, Alex G. Therien, and Beverly H. Koller. Expression and function of NPSR1/GPRA in the lung before and after induction of asthma-like disease. *Am J Physiol Lung Cell Mol Physiol* 291: L1005–L1017, 2006. First published July 7, 2006; doi:10.1152/ajplung.00174.2006.—A genetic contribution to asthma susceptibility is well recognized, and linkage studies have identified a large number of genes associated with asthma pathogenesis. Recently, a locus encoding a seven-transmembrane protein was shown to be associated with asthma in founder populations. The expression of the protein GPRA (G protein-coupled receptor for asthma susceptibility) in human airway epithelia and smooth muscle, and its increased expression in a mouse model of asthma, suggested that a gain-of-function mutation in this gene increased the disease risk. However, we report here that the development of allergic lung disease in GPRA-deficient mice is unaltered. A possible explanation for this finding became apparent upon reexamination of the expression of this gene. In contrast to initial studies, our analyses failed to detect expression of GPRA in human lung tissue or in mice with allergic lung disease. We identify a single parameter that distinguishes GPRA-deficient and wild-type mice. Whereas the change in airway resistance in response to methacholine was identical in control and GPRA-deficient mice, the mutant animals showed an attenuated response to thromboxane, a cholinergic receptor-dependent bronchoconstricting agent. Together, our studies fail to support a direct contribution of GPRA to asthma pathogenesis. However, our data suggest that GPRA may contribute to the asthmatic phenotype by altering the activity of other pathways, such as neurally mediated mechanisms, that contribute to disease. This interpretation is supported by high levels of GPRA expression in the brain and its recent identification as the neuropeptide S receptor.

neuropeptide S; G protein-coupled receptor; allergic lung disease; anaphylaxis

ASTHMA IS A COMPLEX genetic disorder with heterogeneous phenotypes and is strongly influenced by environmental factors. A strong genetic predisposition to asthma is well recognized. Over the past decade, linkage analyses for asthma susceptibility loci have identified several candidate regions. A recent addition to the pool of candidate genes associated with asthma susceptibility and atopy has been identified using genome-wide linkage scans and positional cloning on chromosome 7p15-p14 (19, 20). Founder populations from Finnish and French-Canadian cohorts were used for this study, which identified two candidate genes within this region. One, a putative gene with a large open reading frame, was designated

asthma-associated alternatively spliced gene 1. Expression analyses showed some weak hybridization to lung RNA; however, the open reading frame for this gene is not preserved in mouse. The second gene identified on the other strand of an overlapping region of DNA encodes a seven-transmembrane domain (7TM) protein. Termed G protein-coupled receptor for asthma susceptibility (GPRA), an asthma-associated single nucleotide polymorphism (SNP) identified in this study alters the primary structure of this gene: the polymorphism results in the substitution of an isoleucine for the asparagine located at position 107 of the protein in affected individuals. Excitement regarding this newly identified asthma candidate gene was fueled by the demonstration that the GPRA protein was expressed at high levels in the lung, particularly in tissues obtained from asthmatic patients.

Since this initial genetic study, GPRA polymorphisms have been found to be associated with asthma and atopy in other populations. Studies of multiple Western European populations revealed an association of GPRA haplotypes with asthma and atopy (18, 23). Likewise, assessments of a Chinese population revealed an association between a previously uncharacterized GPRA haplotype and methacholine-induced airway hyperresponsiveness, thus extending the association findings to a non-Caucasian population (7). In contrast to these original analyses and association studies, a high-resolution, fine-mapping study screening SNPs on chromosome 7p in German and Swedish populations as well as a phylogenetic analysis of GPRA-associated SNP haplotypes within a Korean population failed to show GPRA linkage or association with asthma (14, 28). Additional association studies in individuals of Northern European descent also failed to show association with the GPRA risk haplotypes and atopic dermatitis, a chronic recurring inflammatory skin disease characterized by high serum IgE levels and the recruitment of T helper 2 lymphocytes (31, 34).

The 7TM receptor encoded in the asthma-associated locus and termed GPRA was independently identified and characterized as the vasopressin receptor-related receptor 1 (VRR1) (9). VRR1 is expressed in the retina and has been mapped between markers associated with retinitis pigmentosa subtype 9 (9, 16). Likewise, GPRA has also been described as the receptor for neuropeptide S (NPSR1), a novel neuropeptide that potently modulates arousal and could also regulate anxiolytic-like effects. Both the ligand and the receptor were shown to be expressed at extremely high levels in the brain (37). Biochemical studies using cell lines have shown that GPRA/NPSR indeed couples to G proteins. Interestingly, these transfection studies in HEK-293 cells show that GPRA/NPSR couples to both G_q and G_s pathways, induces calcium mobilization, and

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increases adenylate cyclase activity (9). Which of these pathways dominates in nontransformed cells is not yet clear. However, similar studies have also demonstrated that the N107I polymorphism, although not altering ligand binding affinity, results in a gain-of-function mutation defined by an increase in agonist potency (4, 26).

The GPRA gene encodes a unique and recently deorphanized G protein-coupled receptor (GPCR) that was originally identified as GPR154. Alternative splicing of the gene results in variants that differ from one another in the primary amino acid structure of the cytoplasmic domain. Antibodies generated against COOH-terminal peptides of two of the GPRA isoforms demonstrated expression of the A isoform in the airways of both asthmatic and healthy individuals. In contrast, expression of the B isoform was detected in airway epithelial cells in healthy individuals and predominately in the airway smooth muscle cells in asthmatics (20, 35). Laitinen et al. also reported that GPRA expression could be detected in the mouse lung and that the expression increased in a mouse model of asthma (20). These data suggest that the mouse might provide a useful model for study of the role of GPRA in human disease. Toward this end, we report here the generation of a mouse line deficient in GPRA expression as well as the characterization of this mouse in the ovalbumin (OVA)-induced mouse model of allergic lung disease.

METHODS

Generation of GPRA-deficient mice. Segments of the NPSR1/GPRA encoding gene were amplified by PCR and used to create a plasmid capable of undergoing homologous recombination with the endogenous locus. Two 5-kb fragments of the *Npsr1/Gpra* gene were amplified using the following primer sets: 5'-GCGGC CGCAAGAT-GCCCAACCAAGTAAGAAATC-3' and 5'-GTGACCTAGGTAG-AGGCATAC AGCAGGACAA-3' and 5'-GGTACCCGGGCCAT-GGGGAACAGAACGGAGAT-3' and 5'-GCAATTGAGCCCCAC CAAGCAAACCTGT-3'. The fragments were then cloned 5' and 3' of the neomycin gene in the pXena vector. This targeting plasmid is designed to replace a 744-bp region containing the majority of exon 4 with the neomycin cassette. Exon 4 includes regions of the gene encoding the third transmembrane-spanning domain and regions of the i2 intracellular loop. The plasmid was linearized and introduced into embryonic stem cells derived from 129/SvEv mice, and transformants were isolated using standard methodologies (24). A DNA probe corresponding to the region immediately 5' of the targeted region generated by PCR (5'-GCTCATGTGTTTCTTTCCTTATCT-3' and 5'-ACCTCCCATGCC CACTCGT-3') was used to identify targeted ES cells by Southern blot. A second probe, corresponding to DNA encoding exon 4, was generated by PCR (5'-CCATCGTTTAC-CCCATGAAG-3' and 5'-CCTGGTACCCCAACAGTAGC-3') and was used to verify the loss of the region of the gene during the homologous recombination event. ES cells carrying the correctly modified locus were used to generate chimeric animals, which in turn were bred to 129/SvEv, C57BL/6, or BALB/c mice. Those carrying the mutant allele were identified by either Southern blot analysis using the probes described above or by PCR analysis (common: 5'-GT-GGGTACATGAGAAGGTTAGGAG-3'; endogenous: CCTTATC-CTCAAACCAAG TAT-3'; targeted: AAATGCCTGCTCTT-TACTGAAGG) of DNA prepared from tail biopsies. We designate this mutation GPRA^{Δ94-159}; however, in the interest of brevity, we will refer to mice homozygous for the mutation as GPRA^{-/-}. All studies were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by an independent Institutional Animal Care and Use Committee according to guidelines of the University of North Carolina at Chapel Hill.

Northern and RT-PCR analysis of Gpra RNA present in GPRA^{Δ94-159} homozygous animals. Total RNA was isolated from the brains of GPRA^{-/-} and GPRA^{+/+} mice by phenol/chloroform extraction using RNABee (Tel-test) as instructed by the manufacturer. For Northern blot analysis, 20 µg of total RNA were electrophoresed on a 1.1% formaldehyde, 1.2% agarose gel and transferred to an Immobilon-NC nitrocellulose membrane (Millipore). After transfer, the filters were hybridized with a full-length [α -³²P]dCTP random-labeled *Npsr1/Gpra* cDNA probe in Quick-Hyb (Stratagene) for 1 h at 68°C. The mRNA from the GPRA^{-/-} animals was also used to generate cDNA by reverse transcriptase PCR, using the primers TGGGAACTCT-GTTGTGCTG (forward) and GAGATGAGCCCTCGGTTGTA (reverse). The resultant cDNA product was cloned into the TA Cloning Vector pCR 2.1 (Invitrogen, Carlsbad, CA). Subsequent sequencing verified the formation of a splice variant lacking exons 3 and 4.

Measurement of airway reactivity in conscious mice. Airway reactivity was assessed by evaluating enhanced pause (Penh) at baseline and after increasing doses of methacholine (MCh). The Penh calculation is based on inspiratory pressure, expiratory pressure, and expiratory time, and its use as a measure of airway reactivity has been previously validated (11). Spontaneously breathing, unrestrained, conscious mice were placed in a Biosystem XA whole body plethysmograph (Buxco Electronics, Troy, NY). Animals were placed in individual 80-ml chambers, and each chamber was ventilated by bias airflow at 0.2 l/min. Baseline Penh was assessed for 5 min, followed by a 2-min aerosolization of vehicle (PBS) and increasing doses of MCh (12, 25, and 50 mg/ml; Sigma-Aldrich, St. Louis, MO). The response for each aerosol challenge was assessed for 2 min.

Measurement of airway reactivity in intubated mice. Mice were anesthetized with 70–90 mg/kg pentobarbital sodium (American Pharmaceutical Partners, Los Angeles, CA), tracheostomized, and mechanically ventilated at a rate of 300 breaths/min, tidal volume of 6 ml/kg, and positive end-expiratory pressure of 3–4 cmH₂O with a computer-controlled small-animal ventilator (Scireq, Montreal, Canada). Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide. Following baseline assessments, mice were exposed to aerosol challenges by directing the inspiratory line through the aerosolization chamber of an ultrasonic nebulizer connected through a sideport in the ventilator circuit. Animals were ventilated at a rate of 200 breaths/min for 30 s with a tidal volume of 0.15 ml. Immediately following the aerosol challenge, the nebulizer was isolated from the inspiratory circuit, and the original mechanical ventilation was resumed. Forced oscillatory mechanics (FOM) were determined every 10 s for the following 3 min. Briefly, following passive expiration, a broadband (1–19.625 Hz) volume perturbation was applied to the lungs while the pressure required to generate the perturbations was assessed. The resultant pressure and flow data were fit into a constant phase model as previously described (12). Similar to other studies assessing FOM, we confined our analysis to airway resistance or R_{aw} (Rn; Newtonian resistance), which assesses the flow resistance of the conducting airways; G (tissue damping), which reflects tissue resistance; and H (tissue elastance), which reflects the tissue rigidity (33).

Induction and assessment of allergic airway inflammation. To assess OVA-induced airway inflammation, groups of mice were sensitized by intraperitoneal injection of 20 µg of OVA (Grade V, Sigma) emulsified in 2.25 mg of aluminum hydroxide (Sigma) in a total volume of 200 µl, on days 1 and 14. Mice were challenged (45 min) via the airways with OVA (1% in saline) for 5 days (days 21–25) using an ultrasonic nebulizer (DeVillbiss Health Care, Somerset, PA). Control mouse groups received the two OVA immunizations but were challenged with aerosolized saline. Airway reactivity was assessed 24 h after the final aerosol OVA or saline challenge (day 26).

Following airway assessments, mice were euthanized, and ~1 ml of blood was collected by cardiac puncture. Blood was allowed to coagulate, and the serum was collected. Total IgE levels were determined by ELISA (ICN Biomedicals). Bronchoalveolar lavage (BAL) was performed five times with 1.0 ml of sterile Hank's buffered saline

solution each time. The number of cells present in the BAL fluid was determined using a hemacytometer. A differential cell count was conducted on a cytospin prepared from 150 μ l of BAL fluid and stained with either fast green and neutral red or Diff-Quik solution (Sigma). The remaining BAL fluid was centrifuged to remove cells, and the IL-13 level in the supernatant was determined using ELISA (R&D Systems).

For histopathological examination, lungs were fixed by inflation (20-cm pressure) and immersion in 10% formalin. To evaluate airway eosinophilia, fixed lung slices were subjected to hematoxylin and eosin (H&E) staining. To assess goblet cell hyperplasia, serial sections of the left lobes of the lungs that yield maximum longitudinal visualization of the intrapulmonary main axial airway were analyzed following Alcian blue/periodic acid-Schiff reaction (PAS) staining. To avoid bias for a certain region, and to consistently view the identical region in all slides, a 2-mm length of airway, located midway along the length of the main axial airway, was digitally imaged. Using ImageJ software (National Institutes of Health, National Technical Information Service, Springfield, VA), the area and length of the AB/PAS-stained region in the sections were measured and the data were expressed as the mean volume density ($V_v = \text{nl/mm}^2$ basal lamina $\pm$ SE of AB/PAS-stained material within the epithelium) as previously described (18).

Induction and assessment of LPS-induced acute airway inflammation. To assess LPS-induced airway inflammation, groups of mice were anesthetized by isoflurane inhalation, and LPS (Sigma) isolated from *Escherichia coli* (sterile serotype 0111:B4) was instilled intratracheally as previously described (32). Control mouse groups received an intratracheal dose of saline. Rectal temperatures and baseline Penh were assessed every 4 h following LPS administration and were utilized as surrogate markers of inflammation. Cohorts of mice were euthanized 18–24 h post-LPS exposure, and BAL fluid was collected as described above. Total cell counts were determined, and differential staining was conducted as described above. The leukocyte composition of the BAL fluid was determined based on morphological criteria.

Passive airway anaphylaxis. Mice were sensitized by intravenous injection with 20 μ g of human monoclonal anti-dinitrophenyl (DNP) IgE (Sigma) in 200 μ l of sterile PBS. Twenty-four hours after anti-DNP IgE injection, mice were anesthetized, tracheostomized, and mechanically ventilated as previously described. Baseline airway mechanics were assessed for 2 min, and passive systemic anaphylaxis was induced via intravenous injection of human DNP-albumin (Sigma) in 250 μ l of sterile PBS. Immediately following anaphylaxis induction, R_{aw} was assessed for 3 min. Control mice received either an IgE injection and no DNP-albumin or DNP-albumin and no IgE. Age- and sex-matched mast cell-deficient mice (*Kit^{W-sh}/Kit^{W-sh}*, The Jackson Laboratory) were used to confirm mast cell participation in this assay.

NPSR1/GPRA expression analysis. Total RNA was isolated from various tissues and cells, including the lungs from naïve and OVA-challenged mice (see OVA protocol above), various human cell lines, and primary cells derived from the airways. Samples of epithelial/fibroblast cells from asthmatic and control airways were generated from lung biopsies as previously described (25) and generously provided by F. Goulet, Laval University, Quebec, Canada. *Npsr1/Gpra* expression was analyzed by Northern blot, as described above, or by quantitative RT-PCR (TaqMan) using commercially available primer sets. Total RNA was purified via a Qiaprep RNeasy Kit (Qiagen), and the quality of the RNA was evaluated with an Agilent 2100 Bioanalyzer. RNA (5–10 μ g) was reverse transcribed using the High-Capacity cDNA Archive Kit (Applied Biosystems) according to the manufacturer's instructions. cDNA was amplified with Taqman PCR Universal Master Mix (Applied Biosystems) using the Applied Biosystems 7900 HT Fast Real-Time PCR System. All samples were run in quadruplicate, and relative expression was determined by normalizing samples to β -actin, 18S rRNA, GAPDH, β 2M, and HPRT

housekeeping genes. *Muc5AC* expression was also assessed as described above in selected tissues. Data were analyzed using the comparative C_t method ($\Delta\Delta C_t$). All primer/probe sets were commercially available and obtained from Applied Biosystems. For human cells, RNA was isolated and *NPSR1/GPRA* expression was quantified as described above, with the following exceptions. Cells or biopsy-derived samples were first lysed by addition of TRIzol reagent (GIBCO), and RNA was extracted according to the manufacturer's instructions prior to purification using the Qiaprep Rneasy mini kit (Qiagen). Samples were run in duplicate and normalized to *GAPDH* expression.

Statistical analysis. Data are presented as means $\pm$ SE. A random effects model followed by the Tukey-Kramer honestly significant difference (HSD) test was utilized to assess dose response data. ANOVA followed by Tukey-Kramer HSD for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by the Student's two-tailed *t*-test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Targeted disruption of the *Gpra* gene. NPSR1/GPRA is highly conserved; both the structure of the gene and the primary structure of the protein are highly similar between mice and humans. Previous data have demonstrated that an Asn107Ile polymorphism is found at higher frequency in the affected human population (20). However, in all the mouse genomes examined, including inbred and wild-derived strains, the amino acid encoded at position 107 corresponds to Ile. Interestingly, this Ile is also present in the rat, dog, cow, and chimp *Npsr1/Gpra* genes. Thus, the gain-of-function mutation associated with asthma in humans is the common, if not the only, allele present in other species. Therefore, mice express the form of NPSR1/GPRA associated with disease in humans. However, we reasoned that if a modest increase in activity of this receptor leads to enhanced disease in humans, complete loss of expression might attenuate disease in a mouse model of asthma.

A targeting vector was designed that would disrupt the normal expression of this gene (Fig. 1A). Homologous recombination of the targeting plasmid with the endogenous gene removes the majority of exon 4 while leaving the splice acceptor site intact. Exon 4 encodes the majority of the third transmembrane-spanning domain and a portion of the i2 intracellular loop. Therefore, this deletion removes regions of the GPRA protein that are critical for ligand binding. A variety of studies have demonstrated that loss of the third transmembrane domain of GPCRs prevents ligand coupling and induces major conformational changes. Examination of the litters resulting from the intercross of heterozygous animals revealed the presence of animals homozygous for the mutant allele at expected frequencies (Fig. 1B). Southern analysis of DNA prepared from tail biopsies from these animals verified that the recombination event had resulted in the deletion of the majority of exon 4, as no hybridization was detected with a probe specific for this region of the *Npsr1/Gpra* gene. No gross anatomical or morphological differences were observed in GPRA-deficient mice. GPRA-deficient mice demonstrated normal blood cell composition and blood chemistry. (Supplemental data for this article is available online at the *American Journal of Physiology-Lung Cellular and Molecular Physiology* web site.) Analysis of leukocyte populations from the thymus, spleen, and lymph nodes with T cell, B cell, and macrophage

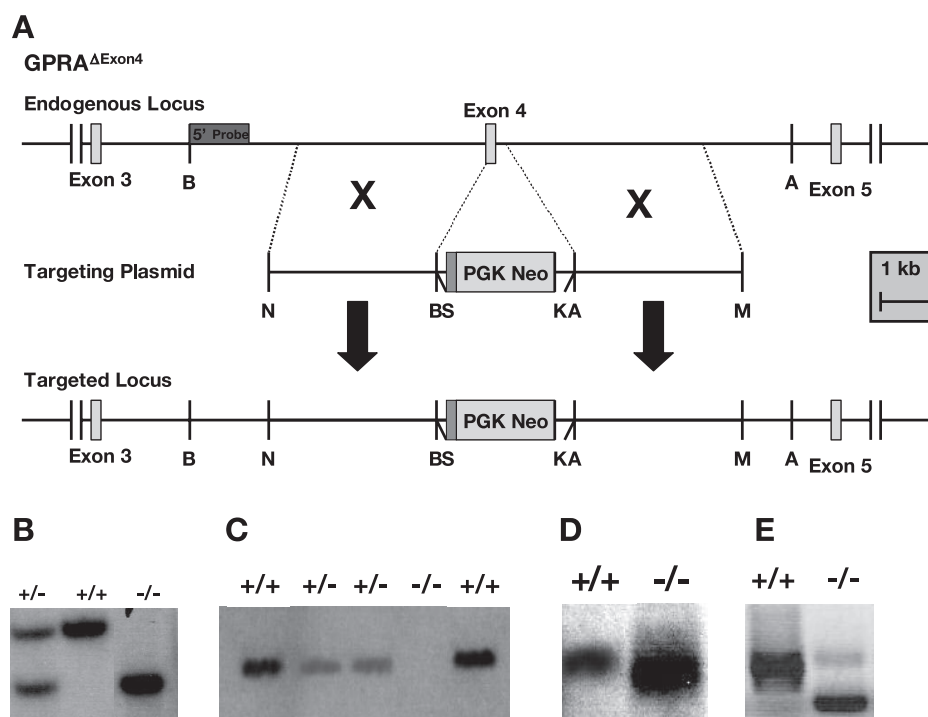


Fig. 1. Generation of a mouse line with a deletion in the *Gpra* gene. **A:** restriction maps of the endogenous *Gpra* locus, the *Gpra* targeting construct, and the targeted locus after homologous recombination with the targeting plasmid. Upon homologous recombination, the *Gpra*^{Δ94-159} construct replaces a 744-bp region of the *Gpra* gene, which includes the majority of exon 4, with the selectable marker gene neomycin (neo). This deletion is predicted to remove the 3rd transmembrane domain and second exoloop of the G protein-coupled receptor. Relevant restriction sites are abbreviated as follows: B, *Bam*HI; N, *Not*I; S, *Sal*I; K, *Kpn*I; A, *Apa*I; M, *Mfe*I. **B:** Southern blot analysis of offspring generated by the intercross of mice heterozygous for the *Gpra* mutant allele. A 521-bp genomic fragment that hybridizes upstream of the targeted region was used as a probe to detect the change in *Bam*HI restriction fragment length that occurs with proper integration of the targeting construct. **C:** Southern blot analysis of the *Gpra*^{Δ94-159} mice to confirm the deletion of the desired region. A 631-bp genomic fragment that includes exon 4 was used as a probe to confirm the loss of this region in the *Gpra*^{Δ94-159} homozygous animals. **D:** Northern blot analysis of *Gpra* expression in the brains of wild-type and *Gpra*^{Δ94-159} homozygous animals. Abundant *Gpra* RNA is observed in wild-type mice, whereas a smaller *Gpra* transcript resulting from the deletion of exon 4 is seen in RNA prepared from *Gpra*^{Δ94-159} mice. **E:** Analysis with a cyclooxygenase-2-specific probe indicates equal RNA sample loading (not shown). **E:** analysis of the truncated *Gpra* mRNA expressed by the *Gpra*^{Δ94-159} animals by RT-PCR. RT-PCR of RNA from wild-type animals yields a fragment of 604 bp, whereas a fragment of ~406 bp was amplified from the homozygous mutant cDNAs. Subsequent cloning and sequencing of the resultant cDNA from the *Gpra*^{Δ94-159} animals confirmed that an alternative splicing event joining exon 2 to exon 5 has occurred. If transcribed, this would encode a protein lacking exons 3 and 4.

markers revealed no aberrations in development of the immune system in the GPRA-deficient animals (Supplemental data).

To verify that the recombination event had indeed resulted in the alteration of the *Npsr1/Gpra* transcript, RNA was prepared from the brains of GPRA^{-/-} and wild-type animals and analyzed by Northern blot (Fig. 1C). The *Npsr1/Gpra* mRNA present in preparations from GPRA^{-/-} animals is of lower-molecular-weight, but similar in abundance, to the native mRNA present in wild-type mice (Fig. 1C). To verify that the RNA transcript from the GPRA-deficient mice lacks the coding information included in the region of the gene lost during the homologous recombination event, cDNA was prepared from the control animals and mice homozygous for the mutation. The region of the transcript extending from exon 2 to exon 5 was then amplified, cloned, and sequenced. This analysis indicated that the transcript present in the mutant animals consisted of a splice variant lacking exons 3 and 4. Because the mouse codon orthologous to Ile107 is located in exon 3 of the mouse gene, an additional consequence of this splice variant formation is the loss of this codon in the mutant mice. This splice variant has recently been identified in a human lung epithelial carcinoma cell line (NCI-H358) and was termed GPRA-F (AY310332) (33). Transfection of this transcript into

COS-1 cells yielded a protein; however, analysis of these transfected cells showed that GPRA-F failed to properly integrate into the cell membrane and is not a functional GPCR (35). We therefore refer to the mice homozygous for the mutant allele as GPRA^{-/-} mice.

Airway mechanics in naïve GPRA^{-/-} mice after exposure to bronchoconstricting agents. Previous studies reported that GPRA was expressed by airway smooth muscle cells (20). These studies also suggested that the expression of the Asn107Ile variant by these cells contributes to the pathogenesis of asthma (20). To begin to test this hypothesis, we determined the impact of complete loss of functional GPRA on basal airflow and basal airway mechanics. In addition, we determined whether loss of GPRA modulates the changes in breathing patterns or airway mechanics observed upon exposure to bronchoconstricting agents such as MCh. We also determined whether bronchoconstriction observed after passive anaphylaxis is altered in mice lacking functional NPSR1/GPRA.

Breathing patterns of wild-type and NPSR1/GPRA-deficient mice were analyzed by whole body plethysmography. Studies were carried out using wild-type and GPRA^{-/-} mice on three different genetic backgrounds. The first group of animals analyzed consisted of 129/SvEv mice and coisogenic

GPRA^{-/-} animals. In addition, GPRA^{-/-} mice and wild-type littermates were generated after the backcross of the mutation onto the C57BL/6 and BALB/c genetic backgrounds for three generations. No difference in baseline Penh was observed in any of the GPRA-deficient populations, and MCh elicited a similar increase in Penh in the GPRA-deficient mice compared with their genetically matched controls (Supplemental data).

We next determined whether changes in airway mechanics could be observed in naïve GPRA^{-/-} mice. For this analysis, we utilized a computer-controlled small animal ventilator, highly sensitive pressure transducers, and software (Flexivent) to record airway opening pressures, volume, and airflow. Changes in lung mechanics were determined using the constant phase model. Three different parameters were compared: R_{aw} , G, and H. No difference in these parameters was observed between 129/SvEv and coisogenic GPRA^{-/-} naïve unchallenged mice (Fig. 2). Exposure of the mice to MCh resulted in an increase in all three parameters, in particular, airway and tissue resistance. The magnitude of these changes did not differ significantly between the wild-type and mutant mouse lines. Similar studies comparing GPRA^{-/-} BALB/c and C57BL/6 mice to their respective genetically matched controls also failed to identify a role for NPSR1/GPRA in changes in lung mechanics in response to MCh (data not shown). Exposure of the mice to serotonin (5-HT) also resulted in an increase in R_{aw} , G, and H. However, similar to the MCh responses, the magnitude of these changes did not differ significantly between the wild-type and mutant mouse lines (data not shown).

Activation of mast cells by IgE and antigen results in release of potent bronchoconstricting agents including leukotrienes, prostaglandins, serotonin, and histamine. As these mediators contribute to the reversible airway obstruction characteristic of asthma, it was of interest to determine whether loss of GPRA altered the changes in lung mechanics measured after mast cell degranulation (Fig. 3). Passive anaphylaxis was induced in GPRA^{-/-} 129/SvEv mice and in coisogenic controls by injection of animals with monoclonal antibody to DNP. Twenty-four hours later, mice were anesthetized, tracheostomized, and mechanically ventilated. After establishment of baseline airway mechanics, mice received antigen (DNP) intravenously, and the change in airway mechanics was measured. Passive anaphylaxis resulted in a large increase in R_{aw} (Fig. 3A). A robust increase in R_{aw} was also observed in the GPRA-deficient mice, but the magnitude of this change did not differ from the coisogenic control animals. A smaller change in G and H was observed after induction of passive anaphylaxis (data not shown). Again, no difference was observed in these parameters between GPRA^{-/-} and control animals. As expected, the airway mechanics of mice that received either antibody or antigen alone did not change significantly from baseline (Fig. 3A), nor was there a significant change in lung mechanics observed upon treatment of mast cell-deficient *Kit^{W-sh}/Kit^{W-sh}* mice with antigen and antibody (Fig. 3B).

GPRA-deficient mice demonstrate normal allergic airway inflammation responses. Sensitization of mice with OVA followed by exposure of the animals to aerosols of antigen results in development of lung inflammation, which models some aspects of asthma. GPRA expression in the lung was reported to increase sevenfold after induction of a similar model of allergic lung disease (20). It was therefore reasonable to assume that functions of GPRA in the lung might be high-

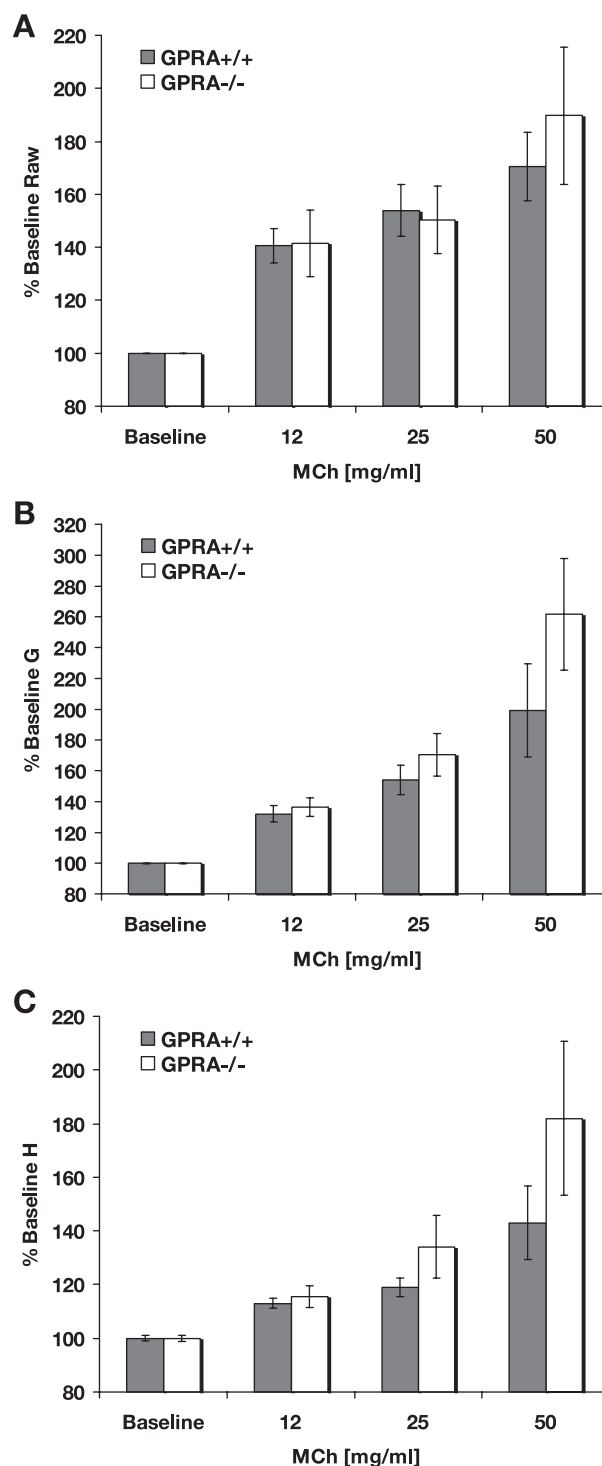


Fig. 2. Changes in airway physiology of wild-type and GPRA^{-/-} mice in response to methacholine (MCh). Airway resistance (R_{aw} ; A), tissue damping (G; B), and tissue elastance (H; C) were assessed in 129/SvEv coisogenic wild-type and GPRA^{-/-} mice in response to MCh. The percent change in R_{aw} , G, and H from baseline increased significantly in all animals regardless of genotype. GPRA^{+/+} mice, $n = 15$; GPRA^{-/-} mice, $n = 9$.

lighted in animals with this disease. Allergic lung disease was induced in 129/SvEv and coisogenic GPRA^{-/-} mice, and the development of inflammation and changes in airway mechanics were assessed in the two groups 24 h after the final exposure to antigen (Fig. 4).

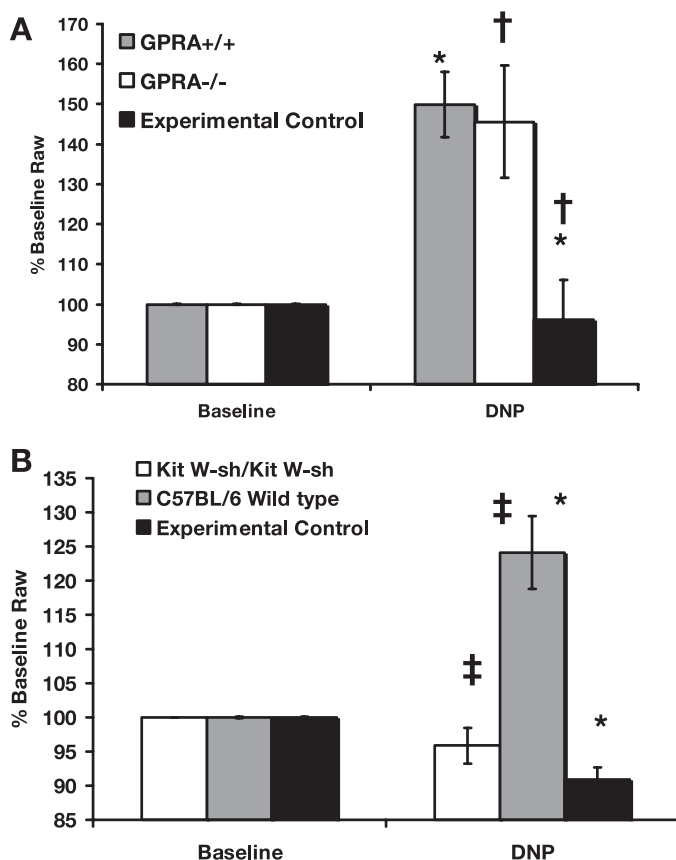


Fig. 3. Mast cell-mediated airway anaphylaxis in wild-type and GPRA^{-/-} mice. Mast cell function and changes in R_{aw} in response to mast cell degranulation were assessed in a model of passive systemic anaphylaxis. A: dinitrophenyl (DNP) administration caused a significant increase in R_{aw} in all mice regardless of genotype. No significant increases in either G or H were observed (data not shown). GPRA^{+/+}, $n = 4$; GPRA^{-/-}, $n = 4$; experimental controls, $n = 4$ (* $P < 0.05$; † $P < 0.05$). B: to confirm that this airway anaphylaxis is indeed an assay of mast cell function, mast cell-deficient mice (Kit^{W-sh}/Kit^{W-sh}) were also assessed. The mast cell-deficient mice failed to demonstrate an increase in R_{aw} in response to DNP administration. Mast cell deficient, $n = 10$; C57BL/6 wild type, $n = 7$; experimental controls, $n = 2$ (§ $P < 0.001$; * $P < 0.05$).

The total number of cells present in the BAL fluid was significantly increased in both the wild-type and GPRA^{-/-} mice following OVA challenge. However, no significant difference was noted between the wild-type and GPRA^{-/-} mice (Fig. 4A). Furthermore, no difference could be identified in the total leukocyte composition of the BAL fluid assessed by morphological criteria (data not shown). IL-13 levels are increased in the BAL fluid after induction of allergic airway disease and are critical to the development of airway hyperresponsiveness (AHR) and goblet cell metaplasia/hyperplasia. IL-13 levels were assessed via ELISA in BAL fluid collected from OVA-immunized and either OVA- or saline-challenged animals (Fig. 4B). A significant increase in IL-13 was observed in OVA-immunized/OVA-challenged mice regardless of the genotype of the mice (Fig. 4B).

Histological and morphometric assessment of the lungs of mice that were sensitized and exposed to antigen revealed similar increases in the number of goblet cells in GPRA-deficient and wild-type mice (Fig. 4D). As expected, few PAS+ cells were observed in the OVA-immunized/saline-

challenged mice (data not shown). In mouse models of asthma, the induction of pulmonary *Muc5ac* gene expression correlates with goblet cell hyperplasia/metaplasia and the increased production of airway mucus. To further evaluate mucus production in GPRA-deficient mice, *Muc5ac* expression was assessed by quantitative RT-PCR (TaqMan) on RNA prepared from the lungs of OVA- and saline-challenged animals (Fig. 4E). A significant increase in *Muc5ac* expression was observed in OVA-immunized/OVA-challenged mice regardless of genotype (Fig. 4E). However, again no difference was observed between the wild-type and GPRA-deficient mice. As expected, no increase in *Muc5ac* expression was observed in OVA-immunized/saline-challenged mice (Fig. 4E).

In addition to finding an association between GPRA and the asthmatic phenotype, recent studies have also suggested an association between polymorphisms in GPRA and elevated serum IgE levels (18, 20, 23). Increased serum IgE is observed in the OVA model of allergic lung disease, and, not surprisingly, the IgE is specific for OVA. Total IgE levels were assessed via ELISA in serum collected from OVA-immunized and either OVA- or saline-challenged animals (Fig. 4C). As expected, a significant increase in total IgE was observed in OVA-immunized/OVA-challenged mice; however, the magnitude of this increase was not significantly different in the GPRA^{-/-} animals (Fig. 4C).

NPSR1/GPRA deficiency has no effect on the induction of AHR in OVA model of allergic airway disease. As discussed above, no difference was observed in the airway mechanics of the naïve NPSR1/GPRA mice in response to constricting agents such as MCh (Fig. 2) and serotonin (data not shown). In addition, the increase in airway resistance during passive anaphylaxis was not affected by the absence of functional NPSR1/GPRA (Fig. 3). However, it is possible that contribution of this protein to airway physiology becomes apparent only in the inflamed lung. We therefore examined baseline airway mechanics and the change in airway mechanics in response to MCh in 129/SvEv mice and coisogenic GPRA^{-/-} animals after induction of allergic airway disease (Fig. 4, F–H). None of the three described parameters, R_{aw} , G, or H, differed between the GPRA^{-/-} animals and similarly treated controls.

GPRA-deficient mice demonstrate normal inflammatory responses in an LPS-mediated acute model of airway inflammation. Endotoxin, a constituent of gram-negative bacteria, and its functional derivative LPS, are ubiquitous in the environment and particularly concentrated in several occupational, industrial, and domestic settings. Several lines of evidence suggest that inhalation of LPS causes an inflammatory response and increases airway reactivity in asthmatics. This raises the possibility that GPRA could contribute indirectly to the development of asthma by altering the sensitivity of individuals to other immunological stimuli in the lung such as LPS. GPRA^{-/-} mice and coisogenic 129 controls were treated with 50 μ g of LPS intratracheally, and the resultant changes in body temperature, breathing patterns (Penh), and inflammation were monitored. Control animals of both genotypes were treated with vehicle. Previous studies indicated that maximum changes in these three parameters are observed in 129 mice 16–20 h after exposure to LPS. As seen in Fig. 5, A and B, administration of LPS induced a dramatic drop in body temperature and a significant increase in baseline Penh, regardless of genotype.

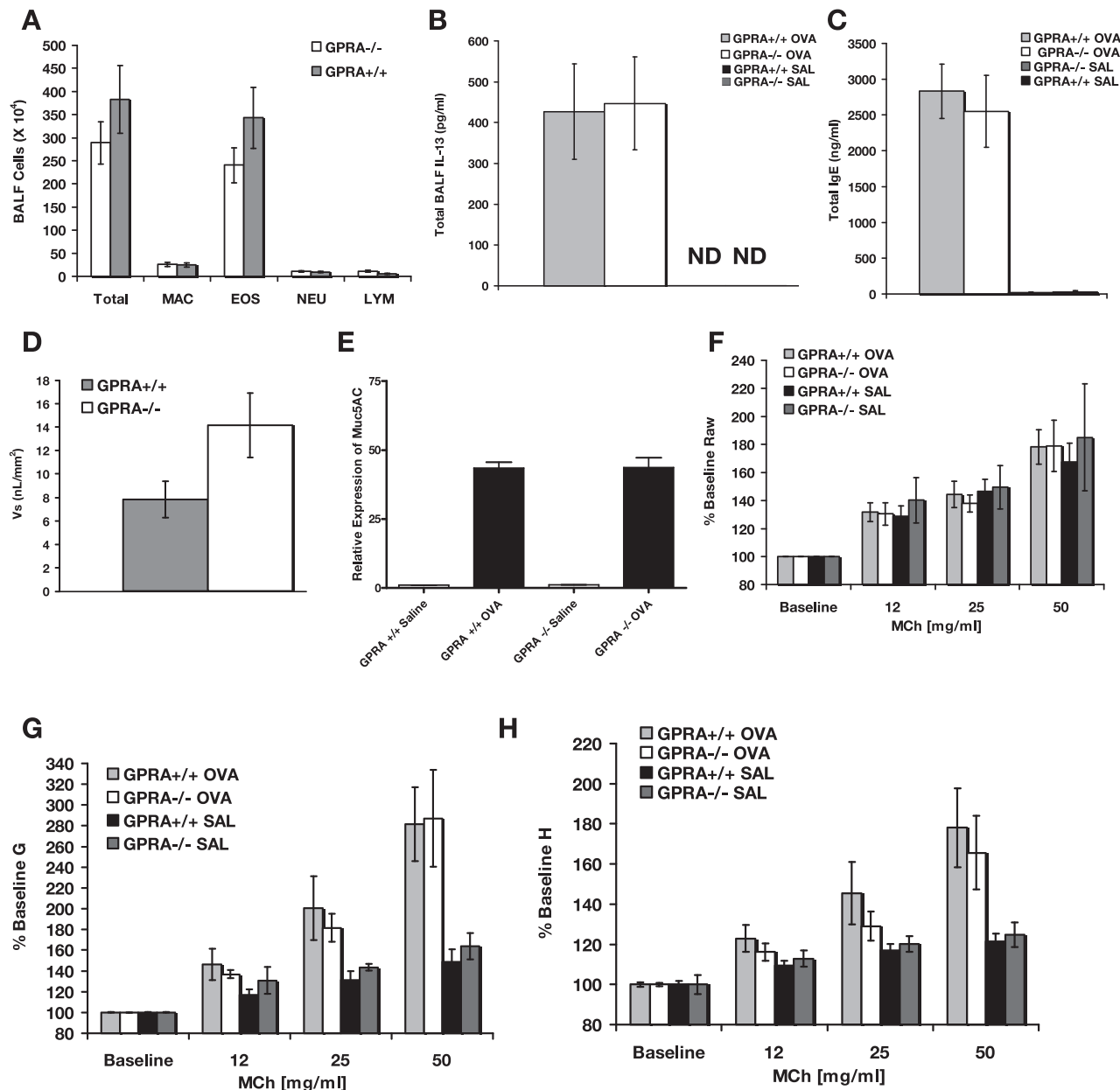


Fig. 4. Allergic airway inflammation in wild-type and GPRA^{-/-} mice. Allergic lung disease was induced in 129/SvEv coisogenic wild-type and GPRA^{-/-} mice. A: increases of similar magnitude were observed in the cellularity and composition of bronchoalveolar lavage fluid (BALF) between the ovalbumin (OVA)-challenged GPRA^{-/-} and wild-type mice. OVA GPRA^{+/+}, *n* = 16; OVA GPRA^{-/-}, *n* = 14; saline (SAL) GPRA^{+/+}, *n* = 9; SAL GPRA^{-/-}, *n* = 9. Histological scoring of hematoxylin- and eosin-stained lung slices demonstrated a significant increase in inflammatory cells present in the lungs of OVA-sensitized and challenged mice regardless of genotype (data not shown). B: a significant increase in total BALF IL-13 levels was detected following OVA sensitization and challenge regardless of genotype. IL-13 levels from the OVA-immunized/saline challenged mice were below the detection limits of this assay. OVA GPRA^{+/+}, *n* = 16; OVA GPRA^{-/-}, *n* = 14; SAL GPRA^{+/+}, *n* = 9; SAL GPRA^{-/-}, *n* = 9. C: a significant increase in total serum IgE levels was detected following OVA sensitization and challenge regardless of genotype. Total serum IgE levels failed to increase above baseline levels in the OVA-sensitized/saline challenged mice, and no significant differences were detected between genotypes. No significant differences were observed between GPRA^{+/+} and GPRA^{-/-} mice; however, a slightly larger increase was observed in the GPRA^{-/-} mice (*P* = 0.11). OVA GPRA^{+/+}, *n* = 16; OVA GPRA^{-/-}, *n* = 14; saline data not shown. D: sections through the main bronchiole of the left lobe of the lung were stained with Alcian blue/periodic acid-Schiff reaction (AB/PAS), and the volume of AB/PAS-stained mucosubstance per square millimeter of basal lamina (V_s) was determined. A significant increase in airway mucus production was observed in mice of both genotypes. No significant differences in mucus production were observed between GPRA^{+/+} and GPRA^{-/-} mice; however, a slightly larger increase was observed in the GPRA^{-/-} mice (*P* = 0.11). OVA GPRA^{+/+}, *n* = 16; OVA GPRA^{-/-}, *n* = 14; saline data not shown. E: a significant increase in *Muc5AC* expression was detected with no significant differences observed between GPRA^{+/+} and GPRA^{-/-} mice. Relative expression was determined by standardizing samples to the 18S housekeeping gene. OVA-sensitized/saline challenged mice, *n* = 3/genotype; OVA-sensitized/OVA-challenged mice, *n* = 3/genotype. The change in respiratory mechanics, assessed as R_{aw} (F), G (G), and H (H), in intubated mice was determined in response to MCh for OVA-immunized/OVA-challenged and OVA-immunized/saline-challenged wild-type and GPRA^{-/-} mice. Dose-dependent increases in R_{aw}, G, and H were observed in all mice regardless of genotype. Wild-type and GPRA^{-/-} OVA-immunized and challenged mice demonstrated an increase in airway hyperresponsiveness as determined by increased G and H. OVA GPRA^{+/+}, *n* = 16; OVA GPRA^{-/-}, *n* = 14; SAL GPRA^{+/+}, *n* = 5; SAL GPRA^{-/-}, *n* = 3. MAC, macrophage; EOS, eosinophil; NEU, neutrophil; LYM, lymphocyte; ND, not detected.

LPS exposure results in recruitment of neutrophils to the lung. No significant difference was observed in either the number or composition of cells present in the BAL fluid as assessed by morphological criteria (Fig. 5C).

GPRA is highly expressed in the central nervous system with only minimal airway expression. The inability to discern changes in airway mechanics and in the development of allergic lung disease in GPRA-deficient mice was surprising given the high levels of GPRA expression reported in the lung and the elevation in GPRA expression in this model of asthma. It was therefore of interest to reexamine this finding and verify that this was indeed the case in coisogenic mice on the 129 genetic background, which were utilized in the majority of our experiments. The $2^{-\Delta\Delta C_t}$ method was used for analysis of expression of NPSR1 after induction of allergic lung disease. ΔC_t is defined as the difference between the threshold cycle (Ct) of the gene of interest and the internal reference (housekeeping) gene of choice. Internal reference genes are genes whose expression is not expected to change as a result of the experimental manipulation, such as the induction of allergic lung disease. Because their expression remains constant, internal references can be used to normalize expression of the gene of interest, NPSR1. This normalization corrects for differences in NPSR1 expression in the allergic lung that might be due to slight variations in quality or quantity of RNA between preparations. Before initiating these studies, we first tested a number of commonly used internal reference (housekeeping) genes to determine their suitability for examination of changes of gene expression in the lung after induction of allergic lung disease. To do this, we examined the number of cycles required to detect expression (fluorescence above background) of the gene in the healthy and inflamed lung (Ct). We found that after ~20 cycles, β -actin expression was detected in the healthy lung. However, when RNA was prepared from the inflamed tissue, β -actin mRNA was less abundant, and over 30 cycles were required to detect expression (Ct = 30; Fig. 6A). The lower abundance of the β -actin mRNA could reflect changes in cells of the lung or the fact that much of the RNA present in the inflamed lung is derived from infiltrating leukocytes. Regardless, this change in Ct made β -actin an inappropriate internal reference gene when comparing healthy and inflamed lungs. Likewise, increased Ct for *GAPDH*, *HPRT*, and β 2M were also observed in the inflamed lung, and use of these housekeeping genes would, therefore, overestimate increases of the test gene expression. We found that 18S ribosomal RNA was the only RNA whose expression itself did not significantly change after induction of allergic airway disease (Fig. 6A). To our surprise, the levels of *Gpra* expressed in the naïve lungs were extremely low, and these levels did not increase in the inflamed lung when 18S was used to normalize expression (Fig. 6B). To reconcile this with previous reports, we next determined whether use of other internal reference genes could lead to the supposition that expression of GPRA is increased in the inflamed mouse lung. When other housekeeping genes were utilized in the analysis, such as β -actin, there was no difficulty in reporting an increase in *Npsr1/Gpra* expression in the lungs of mice with allergic airway disease (Fig. 6C). To verify that our primer sets were appropriate for the analysis, we examined other tissues, including various regions of the brain and the retina. The relative levels of expression of *GPRA* in these tissues mimicked those reported by Xu et al. (37) during an

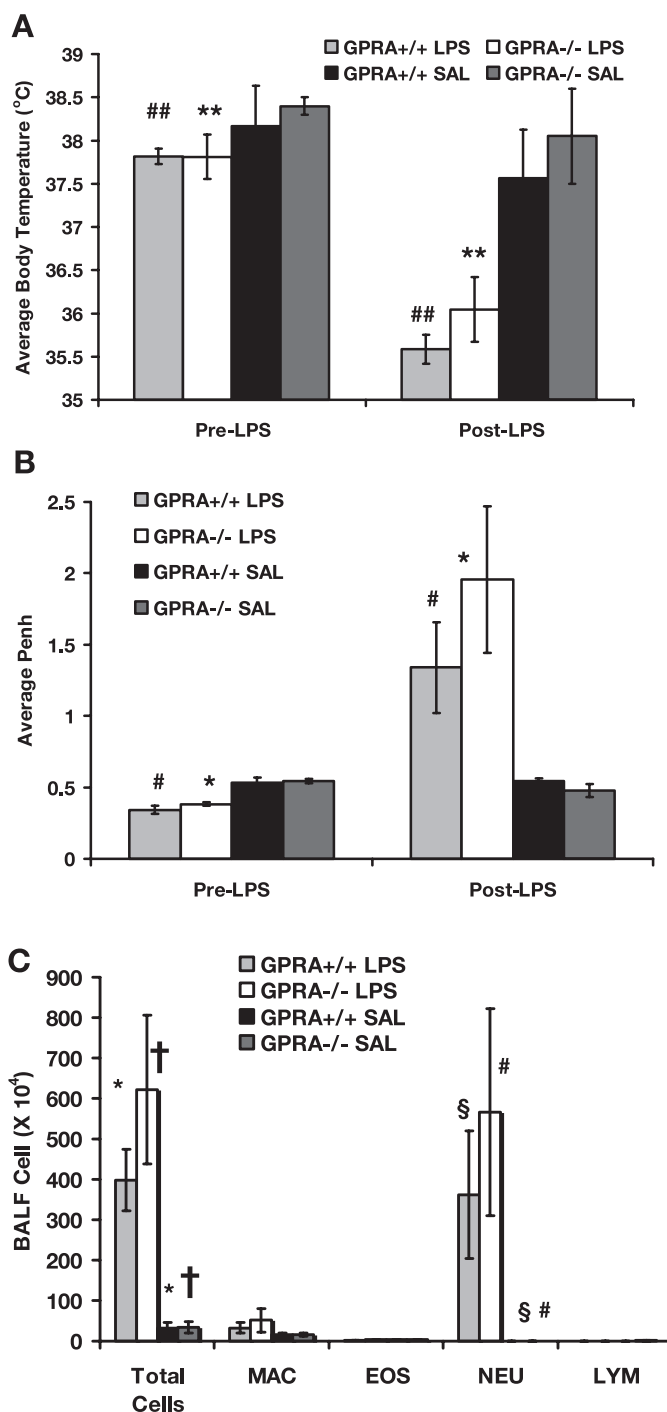


Fig. 5. LPS mediated acute lung inflammation in wild-type and GPRA^{-/-} mice. Acute lung inflammation was induced in C57BL/6 wild-type and GPRA^{-/-} mice. Mice were challenged via intratracheal instillation with either LPS (1 mg/ml) or sterile saline. A: LPS induced a significant drop in body temperature in both wild-type and GPRA^{-/-} mice. LPS GPRA^{+/+}, $n = 14$; LPS GPRA^{-/-}, $n = 13$; SAL GPRA^{+/+}, $n = 3$; SAL GPRA^{-/-}, $n = 3$ ($*P < 0.005$; $##P < 0.005$). B: LPS induced a significant increase in baseline enhanced pause (Penh) in both wild-type and GPRA^{-/-} mice. LPS GPRA^{+/+}, $n = 14$; LPS GPRA^{-/-}, $n = 13$; SAL GPRA^{+/+}, $n = 3$; SAL GPRA^{-/-}, $n = 3$ ($*P < 0.01$; $#P < 0.01$). C: increases of similar magnitude were observed in the cellularity and composition of BALF in LPS-challenged wild-type and GPRA^{-/-} mice. LPS GPRA^{+/+}, $n = 11$; LPS GPRA^{-/-}, $n = 10$; SAL GPRA^{+/+}, $n = 3$; SAL GPRA^{-/-}, $n = 3$ ($*P < 0.05$; $\$P < 0.05$; $\dagger P < 0.05$; $\#P < 0.05$).

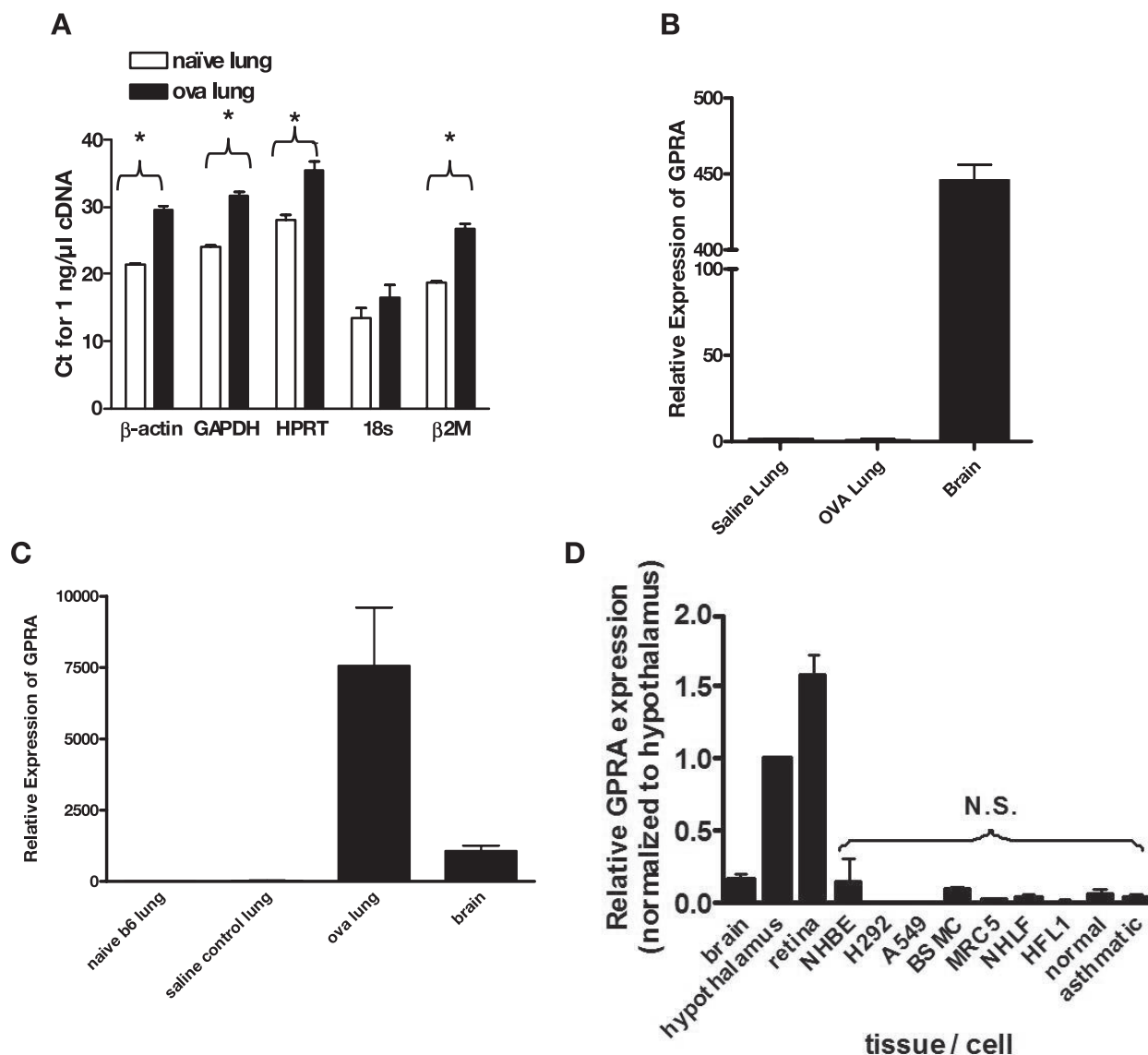


Fig. 6. GPRA expression in human and mouse tissues. **A**: a comparison of threshold cycles (Ct) for common housekeeping genes used to normalize RT-PCR data. OVA inflammation significantly alters housekeeping gene expression. The 18S gene appeared to be the only appropriate housekeeping gene assessed for use in normalizing RT-PCR data from the inflamed lung (* $P < 0.05$ compared with naïve lung). **B**: no increase in *Gpra* transcript levels was detected following challenges with 1% OVA. Mice were sensitized to OVA and subjected to aerosol challenges with either OVA or saline. The lung and trachea were removed immediately following the final aerosol challenge, and total RNA was extracted. *Gpra* expression was assessed by quantitative RT-PCR (TaqMan) using commercially available primer sets. Whole brains were removed from naïve animals to serve as positive controls for real-time assessments. Relative expression was determined by standardizing samples to the 18S housekeeping gene. OVA-sensitized/saline-challenged mice, $n = 3$; OVA-sensitized/OVA-challenged mice, $n = 3$; naïve mice, $n = 3$. **C**: analysis of *Gpra* expression assessed by quantitative RT-PCR and normalized with β -actin. **D**: GPRA expression in human tissues and cells. Levels of GPRA cDNA were measured in cDNA libraries of brain, hypothalamus, or retina, or in various airway-derived cells and cell lines following conversion of mRNA to cDNA using reverse transcriptase. GPRA levels were assessed by quantitative PCR (TaqMan) using commercially available primer sets. Relative expression was determined by standardizing samples to GAPDH levels and normalizing to a hypothalamus cDNA library. "Normal" and "asthmatic" refer to cells obtained from biopsies of non-asthmatic ($n = 4$) and asthmatic ($n = 8$) individuals, respectively. For the indicated cells and cell lines (N.S.), the measured cycle thresholds were indistinguishable from those obtained in identical samples that had not been treated with reverse transcriptase, with the exception of 1 of 3 NHBE samples and 2 out of 4 normal samples, for which very low but significant levels were detected. Each bar represents the mean $\pm$ SD of 2–8 independent samples. A representative experiment is shown.

extensive evaluation of the expression of NPSR1 in the mouse. Thus the primers and conditions used in the studies detect differences in *GPRA* expression between various tissues. Although the results shown in Fig. 6 were obtained using mice on the 129 genetic background subjected to the OVA-challenged model described herein, we also failed to observe an upregulation of the *GPRA* gene and ligand (NPS) in BALB/c mice subjected to OVA- and IL-13-challenged models of asthma in

which robust inflammation, mucus, and airway hyperreactivity endpoints were observed (data not shown).

GPRA is expressed in retina and hypothalamus, but not in human lung cells. Our expression analysis fails to support the expression of substantial levels of GPRA in the mouse lung or the previously reported increase in this expression after induction of allergic lung disease. It was therefore of interest to reexamine the expression of NPSR1/GPRA in humans, since

expression in humans but not in the mouse would simply suggest that the mouse is not the appropriate model for studying the contribution of this gene to asthma. Initial reports indicated that this GPCR is expressed in both epithelial cells and airway smooth muscle cells in the lung (20, 35). Analysis of human airway-derived cell lines and primary cells by quantitative PCR failed to detect significant levels of GPRA mRNA in various epithelial (A549, H292, NHBE), smooth muscle (BSMC), and fibroblast (MRC5, HFL1, NHLF) cell lines and primary cells, with the exception of one out of three samples of NHBE cells that showed low but significant expression. In contrast, significant GPRA expression was observed in commercial brain, retina, and hypothalamus cDNA libraries (Fig. 6D). As additional controls, we observed high levels of GPRA mRNA in GPRA-A and GPRA-B-transfected CHO cells (not shown), ruling out the possibility that the probe and primers used for quantitative PCR in this study are specific to only one of the two GPRA isoforms. Since the possibility exists that expression of this GPCR is upregulated only in lungs of asthmatics, we also examined mRNA levels in cells derived from lung biopsies (graciously provided by F. Goulet, Laval University, Quebec, Canada) from both normal controls ($n = 4$) and asthmatics ($n = 8$). These samples, containing epithelial cells and fibroblasts, were prepared as previously described (25). Again, there was no significant expression of GPRA in either normal or asthmatic lung, with the exception of two of the normal samples that contained significant but very low mRNA levels (Fig. 6D). These data are in contradiction with previous findings (35) showing not only that GPRA expression is increased in asthmatics but also that the GPRA-B isoform is ubiquitously expressed in a variety of tissues and cell lines.

GPRA may participate in an indirect, neurally mediated mechanism of smooth muscle constriction. High levels of NPSR1/GPRA are expressed in the brain and by neuronal cell lines. This raises the interesting possibility that the linkage of the polymorphism in the NPSR1/GPRA receptor to asthma reflects a contribution of the nervous system, perhaps through cholinergic pathways, to the development of asthma. To begin to address this possibility, we examined the change in airway responsiveness of the 129/SvEv GPRA^{-/-} mice and their genetic controls to thromboxane. Previous studies have demonstrated that the increase in airway and tissue resistance in the mouse in response to this agent is dependent on an intact cholinergic pathway (2). As seen in Fig. 7, we assessed changes in airway reactivity utilizing FOM in the coisogenic 129Sv/Ev mice. As described previously, mice were subjected to aerosolized vehicle (data not shown), followed by dose response challenges of U-46619 (10^{-5} to 10^{-3} M). U-46619 challenge induced a robust increase in airway reactivity, regardless of genotype (Fig. 7). Increasing doses of U-46619 generated equivalent increases in G and H (Fig. 7, B and C); however, GPRA-deficient mice demonstrated a modest but significant attenuation in R_{aw} at the highest concentration of U-46619 tested (10^{-3} M; Fig. 7A). This decrease in the R_{aw} response and lack of differences in either G or H following U-46619 challenge is interesting because it suggests a role for GPRA in mediating vagal nerve responses in the conducting airways. In the mouse, airway innervation is higher in the conducting airways and significantly decreases toward the peripheral airways. Thus these combined data suggest a poten-

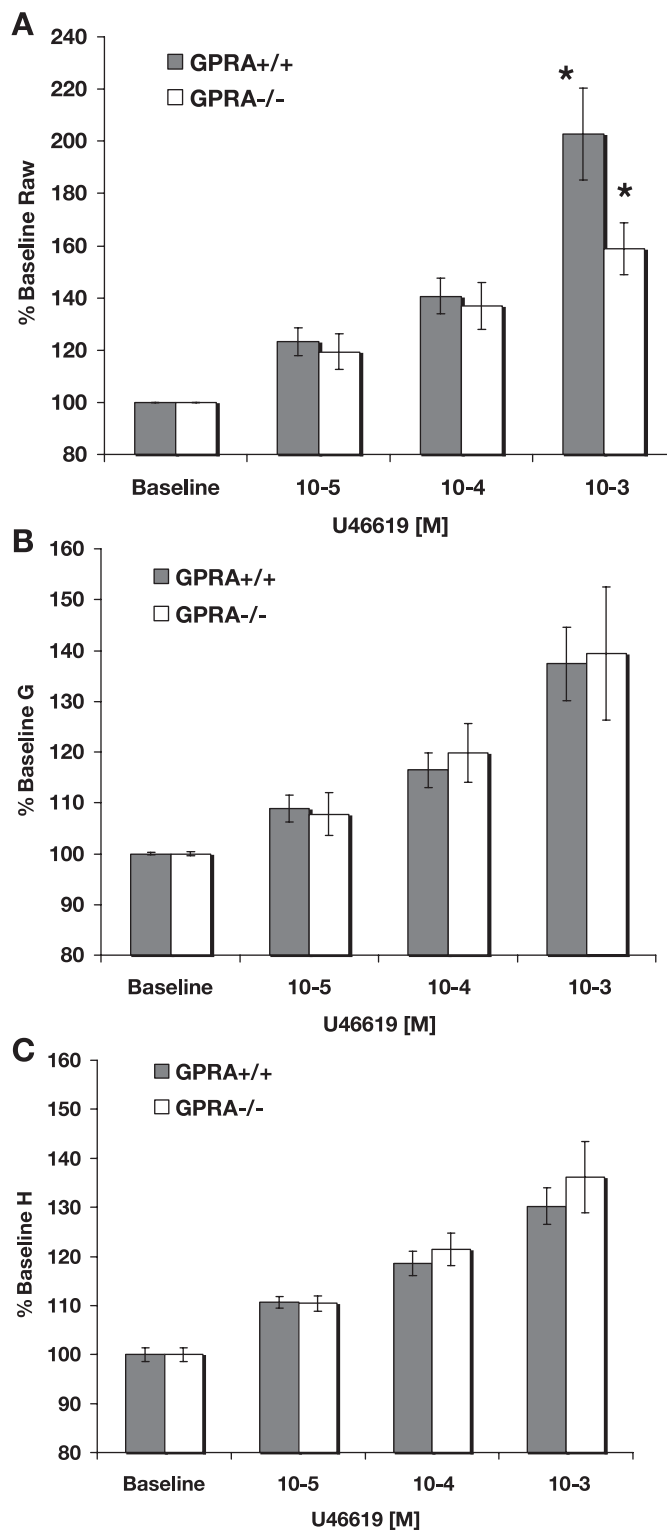


Fig. 7. Changes in airway physiology of wild-type and GPRA^{-/-} mice in response to a thromboxane A₂ analog (U-46619). R_{aw} (A), G (B), and H (C) were assessed in wild-type and GPRA^{-/-} mice in response to U-46619, which induces airway smooth muscle constriction through an indirect, neurally mediated mechanism. The percent change in R_{aw} , G, and H from baseline increased significantly in all animals regardless of genotype. However, GPRA-deficient mice demonstrated a significantly attenuated R_{aw} response following U-46619 challenge. GPRA^{+/+} mice, $n = 17$; GPRA^{-/-} mice, $n = 16$ (* $P = 0.038$).

tial role for GPRA in indirect mechanisms affecting airway smooth muscle constriction.

DISCUSSION

Previous studies reported a dramatic increase in the expression of NPSR1/GPRA in the mouse lung after induction of allergic lung disease, suggesting that the mouse was likely to provide a model for the study of the role of this gene in the pathogenesis of asthma. As a first step to examine the functional significance of GPRA in the development of disease in the mouse lung, we generated a mouse lacking the functional receptor. A deletion mutation introduced into the exon encoding a portion of the third transmembrane domain not only results in the loss of codons encoding amino acid residues 94–159 from the mouse genome but also results in an alteration in the splicing pattern of the gene, such that the major transcript remaining in the mutant mouse line is predicted to produce an isoform incapable of integration into the cell membrane (previously characterized in Ref. 35). Despite the loss of functional NPSR1/GPRA, we detected no difference in the development of allergic lung disease in these mice. Neither qualitative nor quantitative differences in the cellular infiltrate, nor the development of AHR in response to MCh, distinguished the mutant mice from littermate controls.

A number of explanations are possible for the failure to observe differences in the development of disease between the GPRA^{-/-} and littermate control mice. First, it is possible that, whereas loss of the receptor has little impact on the development of disease because of compensatory pathways, the expression of an allele encoding a gain-of-function mutation in GPRA would result in increased signaling and might result in more severe disease. Unfortunately, this hypothesis is not easily tested in the mouse. As discussed above, all mouse strains examined to date express the disease-associated *Gpra* allele. Given our difficulty in observing a measurable consequence as a result of the complete loss of GPRA, it is unlikely that introducing the codon for asparagine at position 107 will alter the development of disease in the mouse asthma model.

We cannot rule out the possibility that the inflammation models we have chosen for testing the role of GPRA lack the sensitivity for discerning the contribution of GPRA to disease development. There are ample examples in the literature that have demonstrated the reliance on a particular immunization protocol or on a particular mouse strain for the ability to distinguish the role of a particular cell type or molecule in allergic lung inflammation. For example, the contribution of the mast cell to allergic lung disease is only observed in a chronic OVA inflammation model when mice are sensitized with antigen free of alum (36). Likewise, the dependence of allergic airway disease on the eosinophil (21, 13) was more apparent when C57BL/6 mice were used rather than BALB/c. Further studies with the GPRA^{-/-} mice should allow us to rule out these possibilities.

A third possibility is suggested by our analysis of expression of GPRA in the lung of BALB/c, C57BL/6, and 129/SvEv mice before and after induction of airway disease, as well as our analysis of expression of the GPRA ligand NPS in select tissues. In all cases, quantitative PCR failed to detect expression of GPRA: the signal obtained from the tissue was observed only after 35–36 cycles and is thus outside of the range

that we believe provides reliable detection of expression. Consistent with these data, Northern blot analysis of RNA prepared from either naïve lung or lung tissue obtained from mice with allergic airway disease failed to detect expression of GPRA (data not shown), although expression in RNA prepared from the brain was easily observed. Our findings are similar to those of Xu et al. (37), who observed high levels of GPRA/NPSR1 expression in the brain but very low expression in the naïve lungs. A possible explanation for the differences in our results and those published by Laitinen et al. (20) is the method used in the analysis of the quantitative PCR, specifically the choice of the housekeeping genes used to normalize the results. The importance of the choice of housekeeping gene with which to normalize expression in tissue with an active and ongoing inflammatory response has been discussed previously (3, 6, 8). Consistent with this, we show that the Ct at which the expression of a variety of common housekeeping genes, including *β-actin* and *GAPDH*, are detected varies significantly between OVA- and saline-challenged lung samples. Changes in levels of housekeeping genes in this model are consistent with previous reports demonstrating that *β-actin*, *GAPDH*, and elongation factor-1 α are inappropriate for normalizing mRNA levels in diseased airways and can lead to erroneous results regarding gene expression in the lung (6, 8). Of the housekeeping genes included in our study, 18S rRNA was the only normalizer appropriate for assessing mRNA expression levels in OVA-treated lungs. In fact, upon normalizing our data sets to *β-actin*, we found enhanced *GPRA* expression in the OVA-treated lungs similar to that previously reported by Laitinen et al. (20). Although it is possible that GPRA is expressed at low levels in the lung epithelia and/or airway smooth muscle, our studies do not support the expression at the levels previously reported nor do they indicate that these levels change dramatically in the inflamed lung. This raises the possibility that the failure to observe a change in the development of allergic lung disease in the GPRA^{-/-} mice reflects a differential expression pattern of this gene in mouse and human. However, our further studies of expression of GPRA in human tissues do not support this interpretation.

The excitement over this latest asthma candidate gene was based, not only on the strong genetic evidence derived from two independent founder populations, but also on the expression of the gene in airway epithelial and smooth muscle cells, and, importantly, the changes in expression patterns of two of the isoforms in the airways of asthmatics. However, contrary to the previously published report, we could find no evidence for substantial expression of *GPRA* in normal lung samples or in tissue biopsies from the asthmatic lung by quantitative PCR. The earlier observations were based largely on the expression of GPRA detected by antibodies specific for the two isoforms of GPRA. We cannot rule out the possibility that the differences between our findings reflect an extreme disconnect between mRNA and protein levels. However, inconsistent with this interpretation, Laitinen et al. (20) present Northern analysis of lung tissue demonstrating detection of two, albeit faint, bands corresponding to the GPRA isoforms with a GPRA-specific probe. We cannot explain these discrepancies. However, consistent with our studies of the allergic mouse airways, extensive analysis of tissues and cell lines fails to support a model in which substantial levels of GPRA are expressed by either human epithelium or airway smooth muscle cells.

Whereas genetic studies identified GPRA as a potential risk factor for asthma, studies by Xu and colleagues (37) simultaneously identified GPRA as the NPSR. These studies demonstrated that NPSR1/GPRA is highly expressed in the brain, with the highest levels detected in the hypothalamus. They further demonstrated that central administration of NPS results in increased locomotor activity, altered sleep states, and increased anxiolytic-like effects in mice, thus suggesting that NPSR1/GPRA can play a role in arousal and anxiety (37). This raises the interesting possibility that NPSR1/GPRA may affect airway function as a result of its role in the nervous system. A number of reports suggest that the pathogenesis of many chronic inflammatory diseases including atopic dermatitis, rheumatoid arthritis, and asthma can be modulated by stress and emotion. For example, studies have reported that some asthmatics demonstrate a reduction in pulmonary function in response to increased anxiety levels evoked by exposure to emotionally charged films (27), listening to stressful interactions (17), and participating in a sustained stressful life event (final academic examinations) (22). Increasing evidence suggests that the biological basis for these observations involves alterations of the stress response, which can contribute to dysfunctional interactions between the neuroendocrine and immune systems. These interactions are likely mediated by the hypothalamo-pituitary-adrenal (HPA) axis via regulation of circulating concentrations of corticosteroid hormones, such as corticosterone (reviewed in Ref. 10). These hormones are potent modulators of both immune and neuronal mechanisms. Interestingly, studies in rats have demonstrated that intracerebroventricular and paraventricular nucleus administration of NPS significantly increases plasma levels of adrenocorticotrophic hormone and corticosterone (30). A recent study of mice lacking corticotropin-releasing hormone also demonstrated the ability of this pathway to modulate inflammation in a mouse model of allergic lung disease (29). This raises the possibility that polymorphisms in the *NPSR1/GPRA* gene could alter the activity of the HPA axis and in this manner impact the risk for the development of asthma, although increased activity would be predicted to inhibit, rather than enhance, inflammatory disorders.

The high expression of NPSR1/GPRA in the nervous system suggests a second possible mechanism by which altered function of this receptor might influence the pathogenesis of asthma. The human airway is highly innervated, and expression of GPRA, either on sensory or cholinergic neurons, could influence the tone of the airway smooth muscle or the response to stimuli. Although not specifically addressed, it is possible that various neuronal populations in the lung express NPSR1/GPRA. This expression would be difficult to detect by analysis of total RNA but could dramatically impact the pathogenesis of asthma and particularly the development of AHR. In this regard, it was of interest that the only difference discerned between the wild-type and GPRA^{-/-} mice was an attenuation in airway resistance in response to the thromboxane A₂ analog U-46619. Analysis of a large cohort of 129/SvEv mice and coisogenic NPSR1/GPRA^{-/-} animals demonstrated a small but significant decrease in the change in airway resistance. The difference appears to be confined to the central airways, as no significant difference was observed in the G and H parameters, which are sensitive to changes in the distal lung. Previous studies have demonstrated that U-46619 facilitates airway

smooth muscle constriction through an M₃ muscarinic acetylcholine receptor-dependent mechanism (2). It has been suggested that this mechanism likely involves afferent and efferent neural signaling (1, 2, 15). This leaves open the possibility that polymorphisms in NPSR1/GPRA could alter neurally mediated mechanisms affecting smooth muscle constriction and airway function and in this way increase the risk for asthma.

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DISCLOSURES

V. Bernier, R. Stocco, and A. G. Therien are currently employed by Merck Frost Canada Ltd. in the Department of Biochemistry and Molecular Biology.

REFERENCES

1. Aizawa H, Takata S, Shigyo M, Matsumoto K, Koto H, Inoue H, and Hara N. Effect of BAY u3405, a thromboxane A₂ receptor antagonist, on neuro-effector transmission in canine tracheal tissue. *Prostaglandins Leukot Essent Fatty Acids* 53: 213–217, 1995.
2. Allen IC, Hartney JM, Coffman TM, Penn RB, Wess J, and Koller BH. Thromboxane A₂ induces airway constriction through an M₃ muscarinic acetylcholine receptor-dependent mechanism. *Am J Physiol Lung Cell Mol Physiol* 290: L526–L533, 2006.
3. Barber RD, Harmer DW, Coleman RA, and Clark BJ. GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiol Genomics* 21: 389–395, 2005.
4. Bernier V, Stocco R, Bogusky MJ, Joyce JG, Parachoniak C, Grenier K, Arget M, Mathieu MC, O'Neill GP, Slipetz D, Crackower MA, Tan CM, and Therien AG. Structure/function relationships in the neuropeptide S receptor: molecular consequences of the asthma-associated mutation N107I. *J Biol Chem* (June 20, 2006). 10.1074/jbc.M603691200.
5. Cressman VL, Hicks EM, Funkhouser WK, Backlund DC, and Koller BH. The relationship of chronic mucin secretion to airway disease in normal and CFTR-deficient mice. *Am J Respir Cell Mol Biol* 19: 853–866, 1998.
6. Dheda K, Huggett JF, Bustin SA, Johnson MA, Rook G, and Zumla A. Validation of housekeeping genes for normalizing RNA expression in real-time PCR. *Biotechniques* 37: 112–114, 116, 118–119, 2004.
7. Feng Y, Hong X, Wang L, Jiang S, Chen C, Wang B, Yang J, Fang Z, Zang T, and Xu X. G protein-coupled receptor 154 gene polymorphism is associated with airway hyperresponsiveness to methacholine in a Chinese population. *J Allergy Clin Immunol* 117: 612–617, 2006.
8. Glare EM, Divjak M, Bailey MJ, and Walters EH. Beta-actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels. *Thorax* 57: 765–770, 2002.
9. Gupte J, Cutler J, Chen JL, and Tian H. Elucidation of signaling properties of vasopressin receptor-related receptor 1 by using the chimeric receptor approach. *Proc Natl Acad Sci USA* 101: 1508–1513, 2004.
10. Haddad JJ, Saade NE, and Safieh-Garabedian B. Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis. *J Neuroimmunol* 133: 1–19, 2002.
11. Hamelmann E, Schwarze J, Takeda K, Oshiba A, Larsen GL, Irvin CG, and Gelfand EW. Noninvasive measurement of airway responsiveness in allergic mice using barometric plethysmography. *Am J Respir Crit Care Med* 156: 766–775, 1997.
12. Hantos Z, Adamczak A, Govaerts E, and Daroczy B. Mechanical impedances of lungs and chest wall in the cat. *J Appl Physiol* 73: 427–433, 1992.

13. Humbles AA, Lloyd CM, McMillan SJ, Friend DS, Xanthou G, McKenna EE, Ghiran S, Gerard NP, Yu C, Orkin SH, and Gerard C. A critical role for eosinophils in allergic airways remodeling. *Science* 305: 1776–1779, 2004.
14. Immervoll T, Loesgen S, Dutsch G, Gohlke H, Herbon N, Klugbauer S, Dempfle A, Bickeboller H, Becker-Follmann J, Ruschendorf F, Saar K, Reis A, Wichmann HE, and Wjst M. Fine mapping and single nucleotide polymorphism association results of candidate genes for asthma and related phenotypes. *Hum Mutat* 18: 327–336, 2001.
15. Karla W, Shams H, Orr JA, and Scheid P. Effects of the thromboxane-A2 mimetic, U46619, on pulmonary vagal afferents in the cat. *Respir Physiol* 87: 383–396, 1992.
16. Keen TJ, Inglehearn CF, Green ED, Cunningham AF, Patel RJ, Peacock RE, Gerken S, White R, Weissenbach J, and Bhattacharya SS. A YAC contig spanning the dominant retinitis pigmentosa locus (RP9) on chromosome 7p. *Genomics* 28: 383–388, 1995.
17. Kolbe J, Garrett J, Vamos M, and Rea HH. Influences on trends in asthma morbidity and mortality: the New Zealand experience. *Chest* 106: 211S–215S, 1994.
18. Kormann MS, Carr D, Klopp N, Illig T, Leupold W, Fritzsche C, Weiland SK, von Mutius E, and Kabesch M. G-protein-coupled receptor polymorphisms are associated with asthma in a large German population. *Am J Respir Crit Care Med* 171: 1358–1362, 2005.
19. Laitinen T, Daly MJ, Rioux JD, Kauppi P, Laprise C, Petays T, Green T, Cargill M, Haahtela T, Lander ES, Laitinen LA, Hudson TJ, and Kere J. A susceptibility locus for asthma-related traits on chromosome 7 revealed by genome-wide scan in a founder population. *Nat Genet* 28: 87–91, 2001.
20. Laitinen T, Polvi A, Rydman P, Vendelin J, Pulkkinen V, Salmikangas P, Makela S, Rehn M, Pirskanen A, Rautanen A, Zucchelli M, Gullsten H, Leino M, Alenius H, Petays T, Haahtela T, Laitinen A, Laprise C, Hudson TJ, Laitinen LA, and Kere J. Characterization of a common susceptibility locus for asthma-related traits. *Science* 304: 300–304, 2004.
21. Lee JJ, Dimina D, Macias MP, Ochkur SI, McGarry MP, O'Neill KR, Protheroe C, Pero R, Nguyen T, Cormier SA, Lenkiewicz E, Colbert D, Rinaldi L, Ackerman SJ, Irvin CG, and Lee NA. Defining a link with asthma in mice congenitally deficient in eosinophils. *Science* 305: 1773–1776, 2004.
22. Liu LY, Coe CL, Swenson CA, Kelly EA, Kita H, and Busse WW. School examinations enhance airway inflammation to antigen challenge. *Am J Respir Crit Care Med* 165: 1062–1067, 2002.
23. Melen E, Bruce S, Doekes G, Kabesch M, Laitinen T, Lauener R, Lindgren CM, Riedler J, Scheynius A, van Hage-Hamsten M, Kere J, Pershagen G, Wickman M, and Nyberg F. Haplotypes of G protein-coupled receptor 154 are associated with childhood allergy and asthma. *Am J Respir Crit Care Med* 171: 1089–1095, 2005.
24. Mohn A and Koller BH. *DNA Cloning 4*, edited by Glover DM and Hames BD. New York: Oxford, 1995, p. 143–184.
25. Paquette JS, Tremblay P, Bernier V, Auger FA, Laviolette M, Germain L, Boutet M, Boulet LP, and Goulet F. Production of tissue-engineered three-dimensional human bronchial models. *In Vitro Cell Dev Biol Anim* 39: 213–220, 2003.
26. Reinscheid RK, Xu YL, Okamura N, Zeng J, Chung S, Pai R, Wang Z, and Civelli O. Pharmacological characterization of human and murine neuropeptide S receptor variants. *J Pharmacol Exp Ther* 315: 1338–1345, 2005.
27. Ritz T, Steptoe A, DeWilde S, and Costa M. Emotions and stress increase respiratory resistance in asthma. *Psychosom Med* 62: 401–412, 2000.
28. Shin HD, Park KS, and Park CS. Lack of association of GPRA (G protein-coupled receptor for asthma susceptibility) haplotypes with high serum IgE or asthma in a Korean population. *J Allergy Clin Immunol* 114: 1226–1227, 2004.
29. Silverman ES, Breault DT, Vallone J, Subramanian S, Yilmaz AD, Mathew S, Subramaniam V, Tantisira K, Pacak K, Weiss ST, and Majzoub JA. Corticotropin-releasing hormone deficiency increases allergen-induced airway inflammation in a mouse model of asthma. *J Allergy Clin Immunol* 114: 747–754, 2004.
30. Smith KL, Patterson M, Dhillon WS, Patel SR, Semjonous NM, Gardiner JV, Ghatei MA, and Bloom SR. Neuropeptide S stimulates the hypothalamo-pituitary-adrenal axis and inhibits food intake. *Endocrinology* 147: 3510–3518, 2006.
31. Soderhall C, Marenholz I, Nickel R, Gruber C, Kehrt R, Rohde K, Griffioen RW, Meglio P, Tarani L, Gustafsson D, Hoffmann U, Gerstner B, Muller S, Wahn U, and Lee YA. Lack of association of the G protein-coupled receptor for asthma susceptibility gene with atopic dermatitis. *J Allergy Clin Immunol* 116: 220–221, 2005.
32. Speyer CL, Rancilio NJ, McClintock SD, Crawford JD, Gao H, Sarma JV, and Ward PA. Regulatory effects of estrogen on acute lung inflammation in mice. *Am J Physiol Cell Physiol* 288: C881–C890, 2005.
33. Tomioka S, Bates JH, and Irvin CG. Airway and tissue mechanics in a murine model of asthma: alveolar capsule vs. forced oscillations. *J Appl Physiol* 93: 263–270, 2002.
34. Veal CD, Reynolds NJ, Meggitt SJ, Allen MH, Lindgren CM, Kere J, Trembath RC, and Barker JN. Absence of association between asthma and high serum immunoglobulin E associated GPRA haplotypes and adult atopic dermatitis. *J Invest Dermatol* 125: 399–401, 2005.
35. Vendelin J, Pulkkinen V, Rehn M, Pirskanen A, Raisanen-Sokolowski A, Laitinen A, Laitinen LA, Kere J, and Laitinen T. Characterization of GPRA, a novel G protein-coupled receptor related to asthma. *Am J Respir Cell Mol Biol* 33: 262–270, 2005.
36. Williams CM and Galli SJ. Mast cells can amplify airway reactivity and features of chronic inflammation in an asthma model in mice. *J Exp Med* 192: 455–462, 2000.
37. Xu YL, Reinscheid RK, Huitron-Resendiz S, Clark SD, Wang Z, Lin SH, Brucher FA, Zeng J, Ly NK, Henriksen SJ, de Lecea L, and Civelli O. Neuropeptide S: a neuropeptide promoting arousal and anxiolytic-like effects. *Neuron* 43: 487–497, 2004.

Expression and function of NPSR1/GPRA in the lung before and after induction of asthma-like disease

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Online Data Supplement

Figure Legends

Table E1. Complete Blood Counts. The red blood cell, white blood cell and platelet composition of both GPRA^{+/+} and GPRA^{-/-} mice fall within normal reference ranges. RBC, Red blood cells; HGB, Hemoglobin; HCT, Hematocrit; MCV, Mean Corpuscular Volume; MCH, Mean Corpuscular Hemoglobin; MCHC, Mean Corpuscular Hemoglobin Concentration; RDW, Red Cell Distribution Width; PLT, Platelets Count; MPV, Mean Platelet Volume; WBC, White Blood Cells; LYM, Lymphocytes; MON, Monocytes; EOS, Eosinophils; GRA, Granulocytes. All mice are on the 129SvEv coisogenic background. GPRA^{+/+} (n = 4); GPRA^{-/-} (n = 4)

Figure E1. FACS Scan Analysis. No significant differences were detected in the immune cell composition between GPRA^{-/-} and GPRA^{+/+} mice on the 129/SvEv coisogenic background. Thymus: GPRA^{+/+} (n = 1), GPRA^{-/-} (n = 1); Spleen: GPRA^{+/+} (n = 4), GPRA^{-/-} (n = 4); Lymph Nodes: GPRA^{+/+} (n = 3), GPRA^{-/-} (n = 3).

Figure E2. Methacholine response in conscious, unrestrained wild type and GPRA^{-/-} mice. Changes in enhanced pause (Penh) of GPRA^{+/+} and GPRA^{-/-} mice in response to MCh was assessed in mice backcrossed onto BALB/c (**A**), 129/SvEv (**B**) and C57BL/6 (**C**) backgrounds. Following baseline measurements, the airways were exposed to aerosolized vehicle (not shown) followed by increasing doses of aerosolized MCh (12 mg/ml to 50 mg/ml). The average Penh increased significantly in all groups of mice regardless of genotype or strain. GPRA^{+/+} mice: BALB/c, n = 3; 129/SvEv, n = 15; C57BL/6, n = 9; GPRA^{-/-} mice: BALB/c, n = 4; 129/SvEv, n = 7; C57BL/6, n = 7.

Table E1

	WBC (L 10 ³ /min ³)	RBC (10 ⁶ /min ³)	PLT (H 10 ³ /min ³)	HGB (g/dl)	HCT (%)
GPRA ^{+/+}	2.13 ± 0.33	7.86 ± 2.10	979.67 ± 130.56	12.33 ± 2.92	41.07 ± 11.39
GPRA ^{-/-}	1.67 ± 0.12	8.53 ± 0.14	990.67 ± 40.92	13.13 ± 0.26	43.37 ± 0.29

	MCV (fl)	MCH (pg)	MCHC (g/dl)	RDW (L %)	MPV (fl)
GPRA ^{+/+}	51.67 ± 0.88	16.17 ± 0.87	31.27 ± 2.22	14.1 ± 0.25	5.53 ± 0.12
GPRA ^{-/-}	51 ± 0.58	15.4 ± 0.21	30.33 ± 0.43	12.9 ± 0.15	5.3 ± 0.06

	LYM (L 10 ³ /min ³)	MON (L 10 ³ /min ³)	GRA (L 10 ³ /min ³)
GPRA ^{+/+}	1.37 ± 0.15	0.13 ± 0.07	0.63 ± 0.12
GPRA ^{-/-}	1.27 ± 0.12	0.1 ± 0	0.3 ± 3.14 X 10 ⁻⁹

	MON (%)	GRA (%)	EOS (%)	LYM (%)
GPRA ^{+/+}	8.23 ± 1.32	22.2 ± 2.80	5.03 ± 0.77	69.57 ± 4.12
GPRA ^{-/-}	7.1 ± 0.49	13.87 ± 2.80	3.57 ± 0.35	79.03 ± 1.41

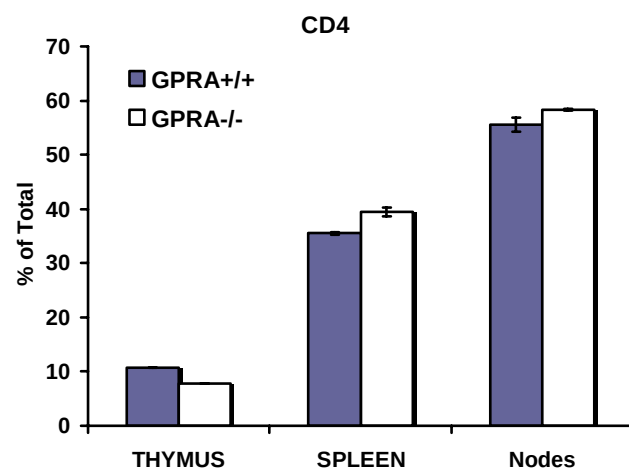
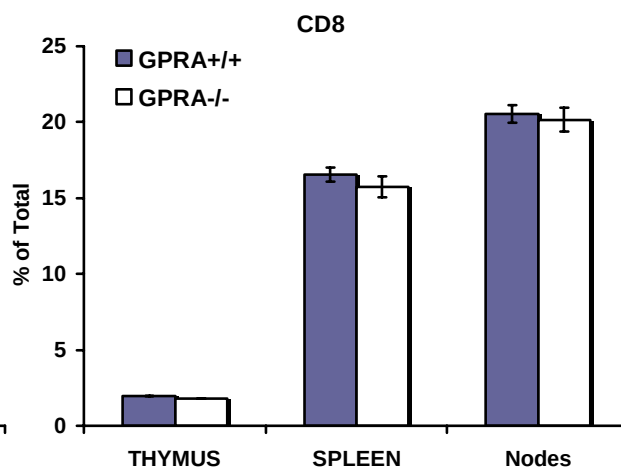
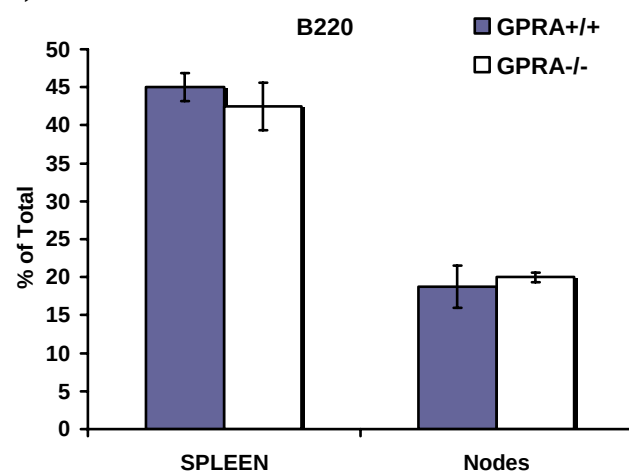
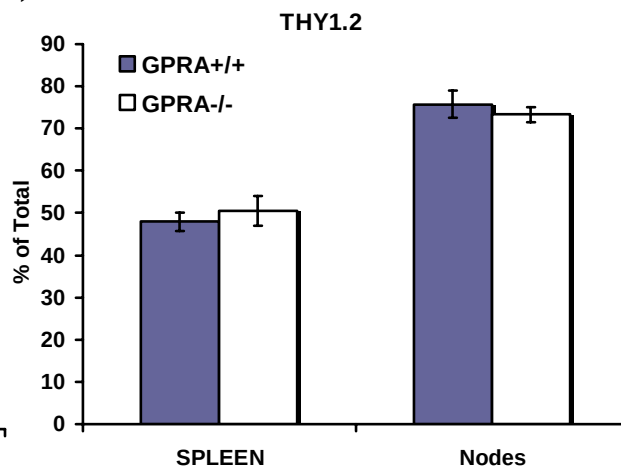
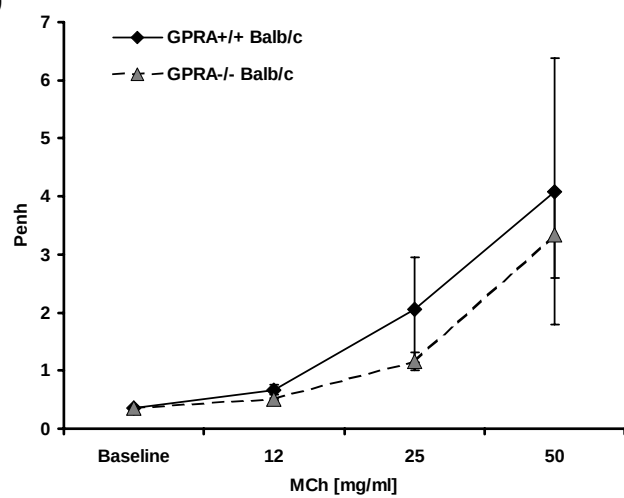
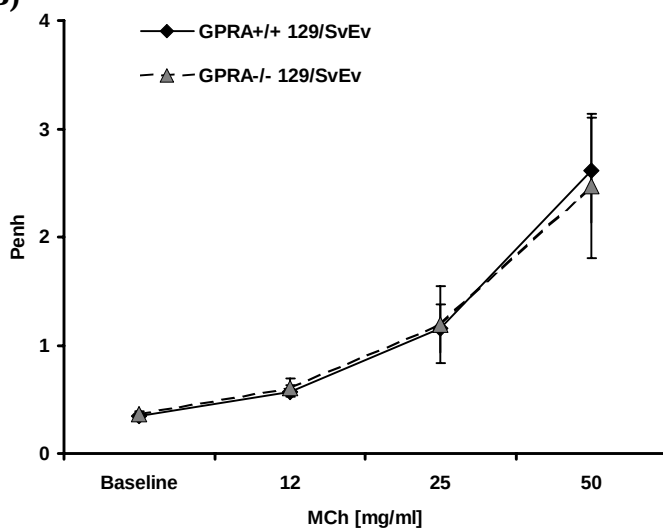
Figure E1**A)****B)****C)****D)**

Figure E2

A)



B)



C)

