Synergistic antipruritic effects of gamma aminobutyric acid A and B agonists in a mouse model of atopic dermatitis

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Background: Despite recent insights into the pathophysiology of acute and chronic itch, chronic itch remains an often intractable condition. Among major contributors to chronic itch is dysfunction of spinal cord gamma aminobutyric acidergic (GABAergic) inhibitory controls.
Objectives: We sought to test the hypothesis that selective GABA agonists as well as cell transplant-derived GABA are antipruritic against acute itch and in a transgenic mouse model of atopic dermatitis produced by overexpression of the T_H2 cell-associated cytokine, IL-31 (IL-31Tg mice).
Methods: We injected wild-type and IL-31Tg mice with combinations of GABA-A (muscimol) or GABA-B (baclofen) receptor agonists 15 to 20 minutes prior to injection of various pruritogens (histamine, chloroquine, or endothelin-1) and recorded spontaneous scratching before and after drug administration. We also tested the antipruritic properties of intraspinal transplantation of precursors of GABAergic interneurons in the IL-31Tg mice.
Results: Systemic muscimol or baclofen are antipruritic against both histamine-dependent and -independent pruritogens, but the therapeutic window using either ligand alone was very small. In contrast, combined subthreshold doses of baclofen and muscimol produced a significant synergistic antipruritic effect, with no sedation. Finally, transplant-mediated long-term enhancement of GABAergic signaling not only reduced spontaneous scratching in the IL-31Tg mice but also dramatically resolved the associated skin lesions.
Conclusions: Although additional research is clearly needed, existing approved GABA agonists should be considered in the management of chronic itch, notably atopic dermatitis. (J Allergy Clin Immunol 2017;140:454-64.)

Key words: *Atopic dermatitis, baclofen, chronic itch, GABA, GABAergic progenitor cell transplants, muscimol, pruritogens*

Atopic dermatitis (AD), an inflammatory, relapsing chronic pruritic skin disease, is an often intractable form of chronic itch that negatively impacts the quality of life of millions of patients.[1] Unfortunately, because chronic itch conditions have very different etiologies, most treatments have poor outcomes and are accompanied by unacceptable adverse side effects, notably sedation.[2] Clearly, a better understanding of the pathophysiology of these chronic itch conditions is critical to designing successful therapeutic strategies.

Studies of the etiology of chronic itch[3] generally focus on changes in skin and immune dysfunction. However, there is now considerable evidence for a contribution of primary afferent pruritoceptors that transmit itch messages to spinal cord and brainstem circuits engaged by and that regulate these messages.[4] Of particular interest are studies demonstrating commonalities in the mechanisms underlying nerve injury-induced neuropathic pain and itch and the possibility that comparable approaches may be appropriate for their management.[5]

Although there is evidence for specificity in the transmission of itch and pain messages at the level of the primary afferent nociceptor and pruritoceptor,[6,7] both pain and itch are under spinal cord inhibitory interneuron-mediated control. For example, loss of spinal cord gamma aminobutyric acid (GABA) or glycinergic function is a major contributor to the spontaneous pain and hypersensitivity that develops following nerve injury.[8-10] Moreover, persistent scratching, a manifestation of chronic itch, occurs in the *Bhlhb5* mutant mouse, in which there is dramatic loss of dorsal horn GABAergic inhibitory interneurons.[11] Ablation of glycinergic interneurons also induces excessive scratching and pain.[12] And in a model of dry skin–induced scratching in the mouse, GABA and glycine receptor antagonists can block scratching-induced inhibition of firing in superficial dorsal horn neurons.[13] Finally, in patients, acute withdrawal of intrathecal baclofen, a GABA-B receptor agonist, can induce pruritus.[14]

Given the evidence for a potential contribution of GABA agonists in the management of pruritus, it is surprising that there are no studies that assessed their utility in preclinical or clinical conditions. Here, we demonstrate that both GABA-A and

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Abbreviations used
AD: Atopic dermatitis
CQ: Chloroquine
ED_{50}: Median effective dose
GABA: Gamma aminobutyric acid
GAD: Glutamic acid decarboxylase
GFP: Green fluorescent protein
GRP: Gastrin-releasing peptide
GRPR: Gastrin-releasing peptide receptor
IL-31Tg: IL-31 overexpressing transgenic mouse
ip: Intraperitoneal
MGE: Medial ganglionic eminence
NGST: Normal goat serum in PBS with 0.3% Triton
PPTA: Preprotachykinin-A
TRPV1: Transient receptor potential cation channel subfamily V member 1

GABA-B agonists are not only effective in models of acute itch, but we also show that systemic administration of very low doses of these agonists has synergistic antipruritic effects in IL-31 overexpressing transgenic mouse, a model of AD[15] that is refractory to antihistamines[1,16] and thus particularly difficult to manage. Most importantly, the antipruritic synergy could be produced without concomitant sedation. Finally, we show that sustaining high levels of GABA inhibition can be achieved using intraspinal transplantation of cortical GABAergic interneuron precursor cells. The transplants not only attenuated spontaneous scratching but also dramatically reduced skin lesions in the IL-31 overexpressing transgenic mouse (IL-31Tg) mice.

METHODS

Animals

Male C57BL6/J mice purchased from Jackson Laboratories (Bar Harbor, Maine) were used for all experiments unless otherwise stated. IL-31 transgenic mice were a generous gift from ZymoGenetics/Bristol-Myers Squibb (Seattle, Wash). The IL-31Tg mice were generated as previously described.[15] All experiments were approved by the University of California San Francisco Institutional Animal Care and Use Committee and conducted in accordance with the *NIH Guide for the Care and Use of Laboratory Animals.*

Pharmacology

Based on our previous studies,[7] we used a minimum of 4 to 5 wild-type C57BL6 male mice per group in acute itch studies. For chronic itch studies, because scratching levels vary among IL-31Tg mice, to decrease variability, we only used mice with skin lesions and who exhibited >100 scratching bouts (over 30 minutes) at the nape of the neck. Mice received intraperitoneal (ip) injections of baclofen (1-4.0 mg/kg in saline) or muscimol (0.3-2.0 mg/kg), and motor coordination/sedation was subsequently evaluated using the rotarod test. Only nonsedating doses were subsequently used for behavioral analyses (≤2.0 mg/kg for baclofen and ≤1.25 mg/kg for muscimol). For acute itch studies, we administered the following pruritogens subcutaneously into the nape of the neck: histamine (500 μg/100 μL), endothelin-1 (25 ng/100 μL) and chloroquine (100 or 200 μg/100 μL). To quantify scratching behavior, mice were habituated, individually, in plexiglass cylinders for 1 hour. Mice were then injected with baclofen or muscimol (ip) and 15 to 20 minutes later with the pruritogen. Behavior (scratching) was monitored by video recording over the next 30 minutes.

To assess the antipruritic effects of baclofen and muscimol in IL-31Tg mice, we injected these mice with baclofen (2.0 mg/kg, ip) or muscimol (1.25 mg/kg, ip) and recorded the scratching behavior for up to 1 hour (muscimol) or 6 hours (baclofen). To assess the synergistic effects of the baclofen-muscimol combinations, we injected IL-31Tg mice with a subthreshold dose of baclofen (1.0 mg/kg; ip) 20 minutes prior to a subthreshold dose of muscimol (1.0 mg/kg; ip) and recorded scratching bouts for the next 60 minutes. Motor performance of the IL-31Tg mice injected with baclofen, muscimol, or the combination was also evaluated with the rotarod test. In all behavioral analyses, the investigator scoring the behavior was blind to treatment and codes were only broken after all scoring was completed.

Statistical analyses

Behavioral and anatomical data are expressed as means ± SEMs, where n represents the number of mice. Raw data obtained in the course of the study were analyzed with a 2-way ANOVA followed by a Bonferroni *post hoc* test. Asterisks (*) indicate statistically significant differences between groups: $*P < .05$; $**P < .01$; $***P < .001$.

Cell transplantation

Medial ganglionic eminence (MGE) cells were dissected and transplanted into the spinal cord of IL-31Tg mice, as previously described.[17] Briefly, green fluorescent protein (GFP)-expressing cells from the MGE were harvested from E13.5 embryos, manually dissociated and resuspended in culture medium. One group of animals received MGE cells (MGE group) and one group received medium only (control group). After hemilaminectomy of the C4 to C8 vertebra, we transplanted cells (50,000) unilaterally over 2 segments of the cervical spinal cord. Photographs of the lesions were taken before and once a week for 4 weeks after transplantation.

Immunohistochemistry

Mice were perfused with 10 ml of PBS followed by 30 ml of ice-cold 10% formalin. Spinal cord and lumbar dorsal root ganglia were dissected, postfixed 3 to 4 hours at 4°C, and cryoprotected overnight in phosphate-buffered 30% sucrose. Frozen cryostat sections of spinal cord and dorsal root ganglia were cut at 25 or 14 μm, respectively. After 1 hour of incubation in 10% normal goat serum in PBS with 0.3% Triton (NGST), the sections were incubated overnight in primary antibody diluted in 1% NGST. The following day, the sections were washed 3 times with PBS, and then incubated 1 hour in secondary antibody (Alexa-488 or Alexa-594, diluted 1:1000 in 1% NGST). After washing 3 times in PBS, sections were mounted and coverslipped with Fluoromount G. Sections were viewed with a Nikon Eclipse fluorescence microscope (Tokyo, Japan) and images were collected with a Zeiss confocal microscope (Oberkochen, Germany). Brightness and contrast were adjusted using Adobe Photoshop, version 6.0 (San Jose, Calif).

Antibodies

We used the following antibodies: rabbit anti-GFP (1:2000; Molecular Probe, Eugene, Ore), rabbit anti-Fos (1:4000; Oncogene Research Products, Cambridge, Mass), chicken anti-GFP (1:2000; Abcam, Cambridge, United Kingdom), rabbit anti-GABA (1:2000; Sigma, St Louis, Mo), mouse antiparvalbumin (1:2000; Sigma), and rabbit antineuropeptide Y (1:2000; gift from J. Allen).

Counts of Fos^+ neurons

Labeled cell bodies were counted from digitized images by an experimenter who was blind to treatment. The percentage of Fos^+ cells was determined by counting all Fos^+ cell bodies in the dorsal horn of 10 spinal cord sections. Both ipsilateral and contralateral sides were counted in 3 mice per experimental group. To calculate the percentage of Fos^+ neurons, we divided the number of Fos^+ neurons ipsilateral to the transplant by the number of Fos^+ neurons on the contralateral side and multiplied by 100. Values are presented as means ± SEMs. Statistical significance was assessed by Student *t* test. $P < .05$ was considered significant and is indicated with an asterisk (*).

Skin histology and scoring

Skin biopsies were collected from transplanted and control IL-31Tg mice 4 weeks posttransplant. Skin samples were processed as previously

described.[18] Briefly, tissue was fixed in 4% paraformaldehyde for 1 hour at room temperature and then embedded in paraffin. Six-micron sections were stained with hematoxylin and eosin or with toluidine blue for mast cells. A dermatologist, who was blind to treatment, scored the alterations in skin structure. Epidermal thickening was scored semiquantitatively on a 0 to 10 scale ranging from 0 (normal; orthokeratosis) to 10 (maximal pathology). A similar 0 to 10–point scale was used to quantify inflammatory cell infiltrate in the dermis. A score of 0 represented the absence of leukocytes and a score of 10 represented a very dense leukocyte infiltration. For mast cell numbers, images of toluidine blue–stained skin were taken with a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) and counted using Image J (National Institutes of Health, Bethesda, Md), by an experimenter who was blind to treatment.

Quantitative real-time PCR

We used Trizol (Invitrogen, Thermo Fisher Scientific, Waltham, Mass) to extract mRNA from cervical, thoracic, and lumbar spinal cord. For transplanted animals, tissues were only collected from the cervical spinal cord (ipsilateral) 4 weeks after transplantation. We reverse-transcribed 200 ng of purified mRNA into cDNA using oligo deoxythymines and Superscript III (Invitrogen). The mRNA levels for gastrin releasing peptide (GRP), gastrin releasing peptide receptor (GRPR), glutamic acid decarboxylase (GAD) 65/67, GABA-A and GABA-B receptor subunits, preprotachykinin-A (PPTA), and β-actin were quantified with a Realplex[2] real-time PCR system (Eppendorf, Hamburg, Germany) using SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK). Cycle threshold data were analyzed with a comparative cycle threshold method using β-actin as an internal standard. Ratios of gene to β-actin mRNA were compared and analyzed by Student *t* test. The following primers were designed using NCBI Primer-BLAST (5′->3′) (National Center for Biotechnology Information, Bethesda, Md):

GRP-F: CCGGTGTCGACAGGCGCAG
GRP-R: TCAGCCGCATACAGGGACGG
gR-F: AGTGGGGGTGTCTGTCTTCACACT
GRPR-R: TCAGGGCATGGGATGCCTGGAT
$GABA_AR\alpha1$-F: TGGCCCACAACATGACCATGCC
$GABA_AR\alpha1$-R: ACGGCGTGGCTCTCTGGTCC
$GABA_AR\alpha2$-F: TGGCCCACACACATGACCATGCC
$GABA_AR\alpha2$-R: TCGGTTCTGGCGTCGTTGCAC
$GABA_AR\beta1$-F: GCCCTCAGAAAAGGGAGCGAGC
$GABA_AR\beta1$-R: CTCGATGCTGGCGCTGTCGT
$GABA_AR\beta2$-F: TGGCTCAAACGGTCTCGGGGT
$GABA_AR\beta2$-R: ACATCAAAGGGGCAGCGGCGS
$GABA_BR1$-F: ACCCTGCCAACACCCGAAGC
$GABA_BR1$-R: CGCACTCCTGAACGGCCACC
$GABA_BR2$-F: CCGTGGGCTACACAACCGCC
$GABA_BR2$-R: TGGGTCCGGCTCCATGCTGT
GAD65-F: CAGCAGTGCCCAGGCTCATCG
GAD65-R: GGTGGTTCCAGCTGTGGCACTC
GAD67-F: CCGCCACAAACTCAGCGGCA
GAD67-R: TGGCGGCCACACTGAATCGC

RESULTS

Previous studies emphasized the limitations of GABA agonists, in a variety of preclinical and clinical conditions, because of their side effects, namely sedation.[19-21] For this reason, we first used the rotarod test to establish nonsedative doses of muscimol and baclofen, GABA agonists that selectively target the GABA-A and B receptors, respectively. Fig 1, *A* illustrates that baclofen is nonsedating at doses < 3.0 mg/kg and muscimol at doses < 1.5 mg/kg. Next, we analyzed the antipruritic effect of different, nonsedating doses of baclofen or muscimol administered 15 to 20 minutes prior to an injection of histamine (500 μg) at the nape of the neck (Fig 1, *B*), or of the histamine receptor-independent pruritogens, chloroquine (CQ) (200 μg) (Fig 1, *C*), and endothelin-1 (25 ng) (Fig 1, *D*). We found that the therapeutic window for the antipruritic effects of baclofen is small. Thus, 2.0 mg/kg baclofen significantly reduced the scratching induced by all pruritogens, but doses ≤ 1.5 mg/kg of baclofen had no antipruritic effects. The highest dose tested (3.0 mg/kg) completely blocked histamine-induced scratching. However, as this dose was sedating, a direct antipruritogen effect could not be concluded.

Muscimol also reduced (at 0.75 mg/kg) or largely eliminated (at 1.0 mg/kg) scratching provoked by 200 μg CQ (Fig 1, *C*). Even lower doses of muscimol (0.3 mg/kg) were effective against histamine-induced scratching (Fig 1, *B*). In contrast, only doses ≥ 1.25 mg/kg reduced endothelin-1-provoked scratching (Fig 1, *D*). Importantly, only doses of muscimol ≥ 1.5 mg/kg were sedating (Fig 1, *A*). Taken together, these results demonstrate that systemic administration of either GABA-A or GABA-B agonists has profound antipruritic effects against both histaminergic and nonhistaminergic pruritogens, but their therapeutic window is relatively small.

Our findings indicate that muscimol and baclofen are effective at reducing acute itch that results from pruritogen-induced increased activity of primary afferent pruritoceptors. To determine whether the GABA agonists retain their therapeutic value in the setting of a chronic itch condition that is driven from the periphery, we used the IL-31Tg mice, in which there is a persistent increase in the activity of primary afferent pruritoceptors. In the IL-31Tg mice, a lymphocyte-specific promoter drives overexpression of IL-31, a T_H2-cell-derived cytokine that induces scratching by engaging sensory pruritoceptors that express the IL-31 receptor A.[22] The chronic itch phenotype in these mice develops after 8 weeks of age and mimics AD, which in humans is also associated with increased atopic skin levels of IL-31.[23,24] In mice, the phenotype is manifest by significant unremitting scratching, eventual excoriation, and skin lesions.

In the IL-31Tg mice, we first tested the efficacy of a single, nonsedating, systemic dose of baclofen (2.0 mg/kg) or muscimol (1.25 mg/kg) against spontaneous scratching. Compared with saline, baclofen reduced scratching after 60 minutes and this lasted up to 6 hours (Fig 2, *A*). In contrast, muscimol was antipruritic within 30 minutes of injection, but the effect lasted <1 hour (Fig 2, *B*). Importantly, in these mice, neither the muscimol nor the baclofen dose had sedating effects in the rotarod test (Fig 2, *C*). We conclude that pharmacological activation of either GABA-A or GABA-B receptors can significantly reduce pruritogen-evoked scratching and perhaps most importantly ameliorate spontaneous scratching in an atopic dermatitis model of chronic itch.

Synergistic interactions potentiate the antipruritic effects of GABA agonists

Although our results demonstrate that GABA agonists have potent antipruritic properties, their relatively small therapeutic window will likely limit their utility in patients. Indeed, clinical studies have shown that sedation is often the cause for discontinuing baclofen treatment in the management of spasticity. Therefore, with a view to overcoming this limitation, we asked whether we could identify a nonsedating synergistic antipruritic

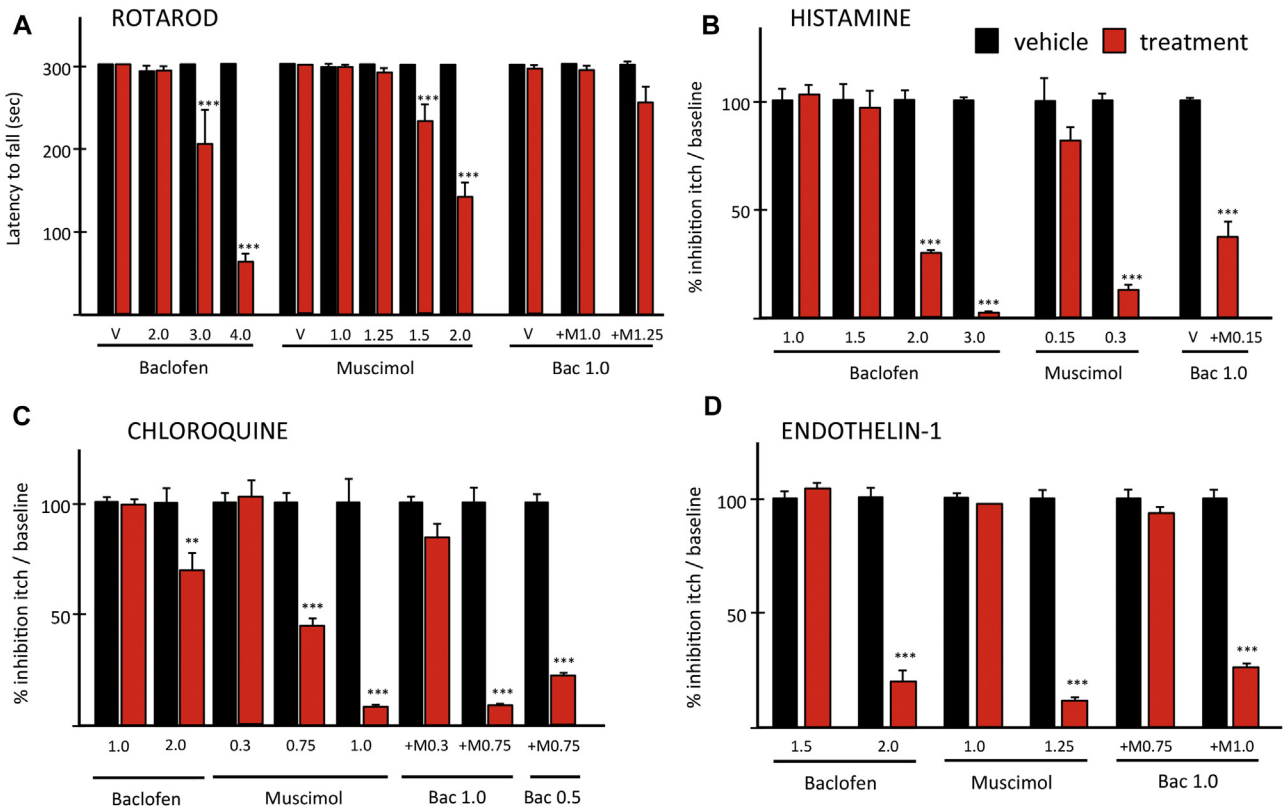

FIG 1. Synergistic antipruritic interaction of GABA-A and GABA-B receptor agonists. **A,** Baclofen (*Bac*) ≥ 3.0 mg/kg or muscimol ≥ 1.5 mg/kg (*red*) are sedating. Nonsedative baclofen or muscimol against histamine- **(B)**, chloroquine- **(C)**, and endothelin-1 **(D)**. Coadministering nonsedative baclofen and muscimol against histamine **(B)** and endothelin-1 **(D)**. Combined subthreshold baclofen and ED_{50} muscimol against chloroquine **(C)**. Data are means ± SEMs; $^{**}P < .005$, $^{***}P < .001$; 2-way ANOVA. *Bac 1.0*, Baclofen 1.0 mg/kg; *V*, vehicle.

interaction using a combination of subthreshold doses of baclofen and muscimol. We first examined dose combinations against various pruritogens, in wild-type mice, and found that neither 1.0 mg/kg baclofen nor 0.15 mg/kg muscimol, when administered alone, has antipruritic effects against any of the pruritogens (Fig 1). However, their co-administration significantly reduced both histamine- and endothelin-1-triggered scratching (Fig 1, *B* and *D*). Importantly, although we could not find a combination of subthreshold doses of baclofen and muscimol that was effective against the 200 μg CQ (Fig 1, *C*), coadministration of a subthreshold dose of baclofen (1.0 mg/kg) with a median effective dose (ED_{50}) of muscimol (0.75 mg/kg) was antipruritic, and in fact, more efficacious than the same dose of muscimol administered alone. Of note, the combined dose of baclofen and muscimol was as effective as a higher dose of muscimol (1.0 mg/kg) administered alone. Interestingly, even lower doses of baclofen (0.5 mg/kg) in combination with the ED_{50} muscimol dose significantly retained an antipruritic effect against 200 μg CQ (Fig 1, *C*). Taken together, these results demonstrate that ineffective doses of baclofen, when combined with a subthreshold or near ED_{50} dose of muscimol have significant antipruritic effects in models of acute itch. Most importantly, these same combinations were not sedating (Fig 1, *A*).

Finally, we asked whether a combination of subthreshold doses of baclofen and muscimol is also effective against chronic itch. Here we coinjected baclofen (1.0 mg/kg) and muscimol (1.0 mg/kg) into the nape of the neck of the IL-31Tg mice and measured spontaneous scratching. Although these doses administered separately were completely ineffective against chronic pruritus in the IL-31Tg mice, again the combination revealed a nonsedating, synergistic interaction against spontaneous scratching (Fig 2, *B* and *C*).

Although these systemic, pharmacologic approaches significantly reduced spontaneous scratching in the IL-31Tg mice, the antipruritic effects are temporary. As a result there is no resolution of the associated skin pathology characteristic of the condition. With a view to translating these pharmacological approaches to a long-term management regime, we next asked whether permanently sustaining high levels of GABA inhibition could not only reduce scratching but also ameliorate the associated skin lesions in the IL-31Tg mice. To this end, we transplanted precursors of embryonic cortical GABAergic interneurons derived from the MGE into the spinal cord of the IL-31Tg mice.

Spinal cord molecular changes in the IL-31Tg mice

In the *Bhlhb5* mutant mouse model of neuropathic itch,[11] there is a profound loss of GABAergic interneurons in the dorsal horn of the spinal cord. Not surprisingly, there is a corresponding significant decrease of GAD65 and GAD67, the major biosynthetic enzymes for GABA.[25] To determine whether there is any alteration in GABAergic biochemistry in the IL-31Tg mice, we used quantitative PCR to analyze the expression levels of various receptors and enzymes involved in GABAergic

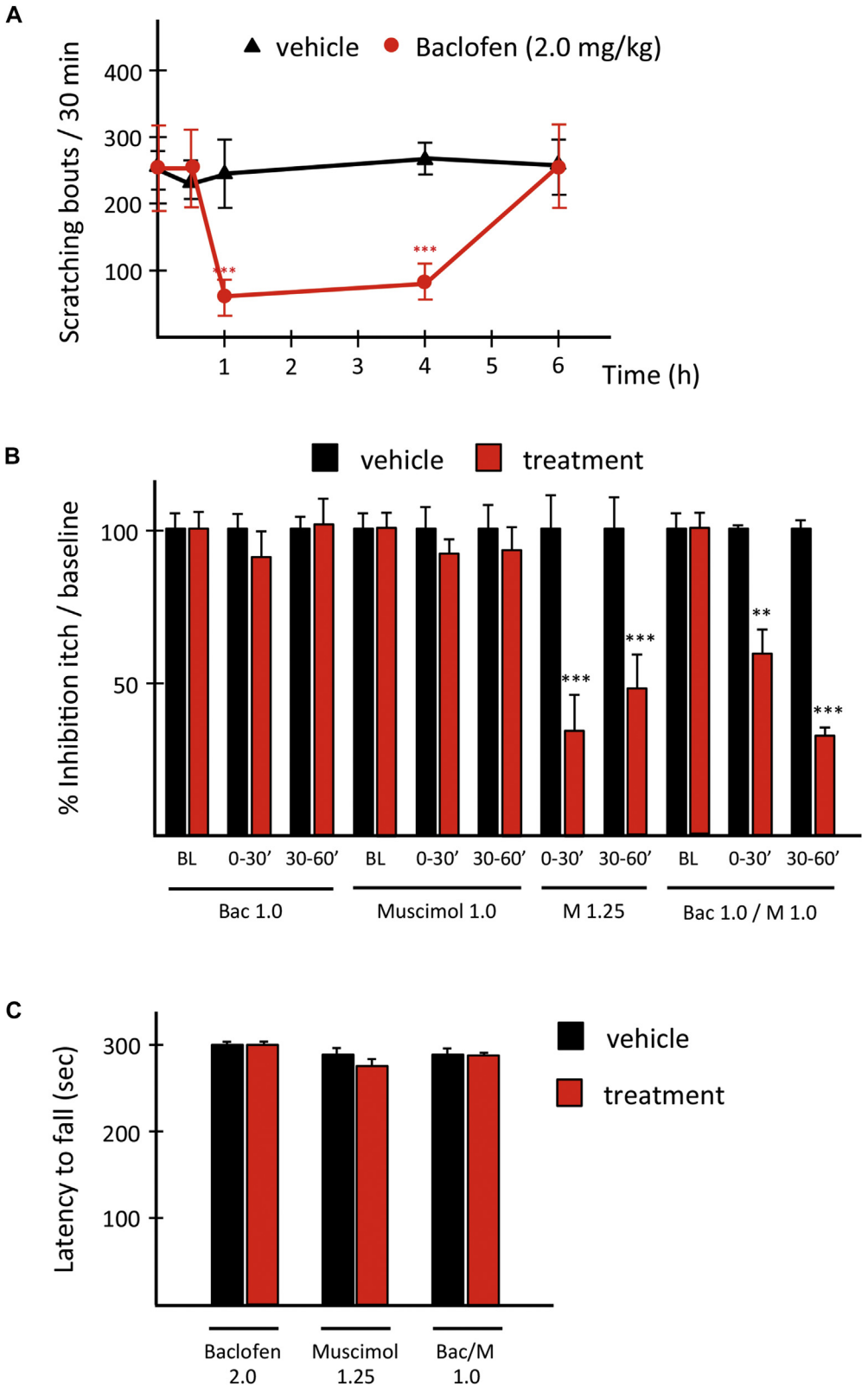

FIG 2. A synergistic interaction of GABA-A and GABA-B receptors is antipruritic against chronic itch. Compared with saline (*black*), systemic baclofen (*red*; **A**) or muscimol **(B)** in IL-31Tg mice is antipruritic. Systemic coadministration of subthreshold baclofen and muscimol (*M*) synergize against spontaneous scratching and these doses are not sedating **(C)**. Data are means ± SEMs; $^{**}P < .005$, $^{***}P < .001$; 2-way ANOVA. *BL*, Baseline.

signaling. In distinct contrast to the *Bhlhb5* mutant mice, we found no significant difference between wild-type and IL-31Tg mice in spinal cord mRNA levels of GAD65 or GAD67 (Fig 3, *A*). The major GABA-A receptor subunits (α1, α2, β1, and β2) were also unaffected (Fig 3, *B*). On the other hand, in spinal cord segments that receive inputs from regions with skin lesions, we found a significant decrease (~2-fold) in mRNA levels of the B1 subunit of the GABA-B receptor. B1 mRNA levels in the IL-31Tg mice at other spinal cord levels did not differ when compared with those levels in wild-type mice. The modest decrease of the B2 subunit of the GABA-B receptor was not significant. Thus, although GABA synthesis is unchanged in the IL-31Tg mice, GABAergic signaling may, nevertheless, be reduced secondary to decreased GABA-B receptor expression.

We also analyzed spinal cord mRNA levels of GRP and GRPR, both of which have been implicated in the transmission of itch signals carried by sensory pruritoceptors.[26-30] Compared with wild-type mice, in the IL-31Tg mice, GRP mRNA levels are significantly increased (~4-fold, $P = .047$) in segments of cervical spinal cord corresponding to dermatomes with lesions (Fig 3, *C*). GRPR levels did not change. We also observed a significant increase in the spinal cord expression levels of PPTA mRNA, the precursor of substance P, a peptide that provokes significant scratching after intrathecal administration.[31] Levels of the substance P receptor, NK1R, did not change. Taken together, our analysis indicates that peripheral overexpression of IL-31 results in spinal cord upregulation of the pro-pruritoceptive peptides, GRP and substance P, and concurrent downregulation of a putative antipruritoceptive receptor, the B1 subunit of the GABA-B receptor. We hypothesize that these biochemical changes together contribute to chronic itch in the IL-31Tg mice.

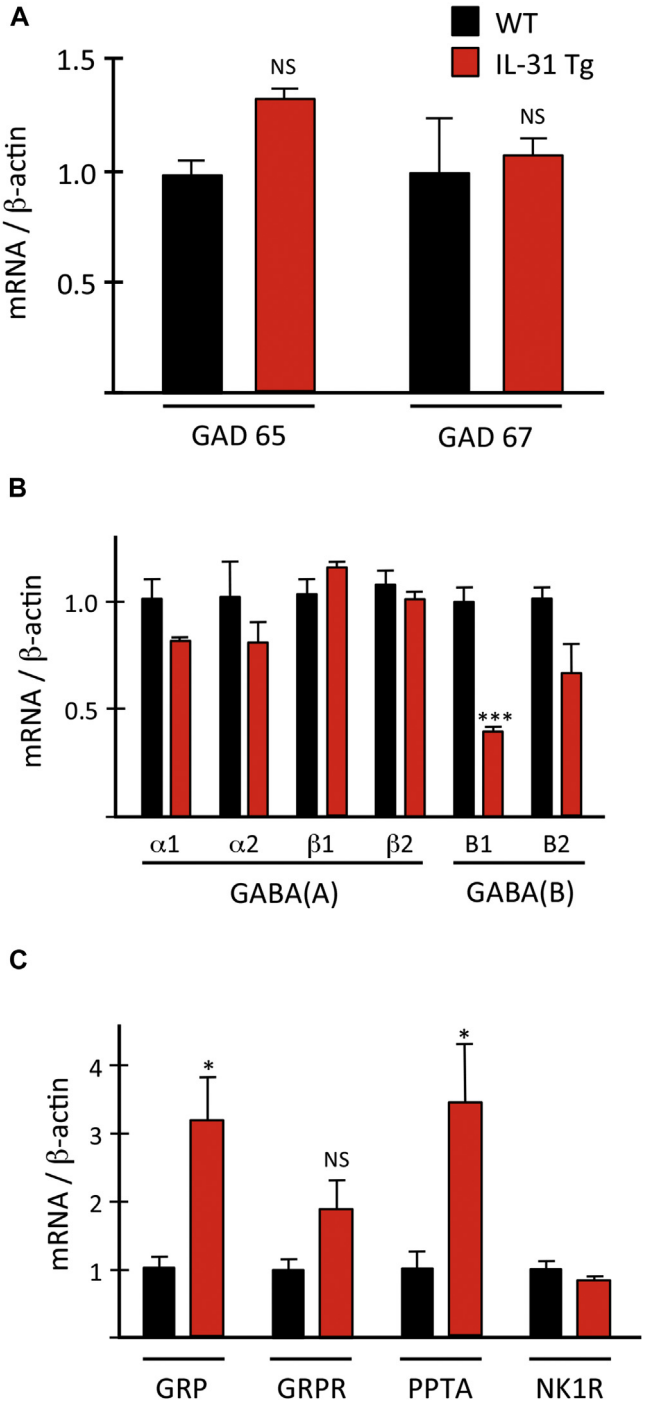

FIG 3. Upregulation of propruritic and downregulation of antipruritic genes in the spinal cord of IL-31Tg mice. **A-C,** In IL-31Tg mice (*red*), spinal cord mRNA levels of the GABA-B1 receptor **(B)** decreased; GRP and PPTA mRNA **(C)** increased. Expression of GAD 65/67 **(A)**, GABA-A receptor subunits **(B)**, or GRPR and NK1 receptor (*NK1R*) **(C)** did not change. Data are means ± SEMs; $*P < .05$; $***P < .001$; Student *t* test. *NS*, Not significant; *WT*, wild type.

Long-term GABAergic inhibition ameliorates skin lesions in IL-31Tg mice

Given the association between chronic itch and decreased GABA-B receptor expression in the IL-31Tg mice, we next tested whether the IL-31Tg mice respond to MGE cell transplantation. Here, we studied IL-31Tg mice with bilateral lesions in the nape of the neck. Importantly, lesions never resolve spontaneously. We transplanted MGE cells unilaterally into the cervical spinal cord (C4-C8), so that scratching of the skin ipsilateral and contralateral to the transplants could be compared. A control group received transplant medium only. We monitored spontaneous scratching and severity of the skin lesions for 4 weeks posttransplantation.

Within 2 weeks of MGE transplantation, we recorded substantially reduced skin lesions ipsilateral to the transplant (Fig 4, *A-F*). In several IL-31Tg mice there was complete resolution of the skin lesions and regrowth of hair, of note only ipsilateral to the transplant. Neither the severity of lesions contralateral to the transplant, nor lesions in control animals ever decreased (Fig 4, *G-I*). Interestingly, skin lesions often persisted in dermatomes immediately adjacent to recovered areas, demonstrating that the MGE transplants exert a topographic, rather than systemic effect. The improvement of the skin ipsilateral to the transplant was associated with a significant (~50%) reduction of scratching in the MGE-transplanted mice (Fig 4, *J*), which began 2 weeks posttransplant and remained low for the following 2 weeks. In contrast, spontaneous scratching did not change in control mice. We conclude that intraspinal MGE transplantation in IL-31Tg mice markedly reduces spontaneous scratching, a likely consequence of reduced itch, which in turn leads to gradual resolution of the skin lesions.

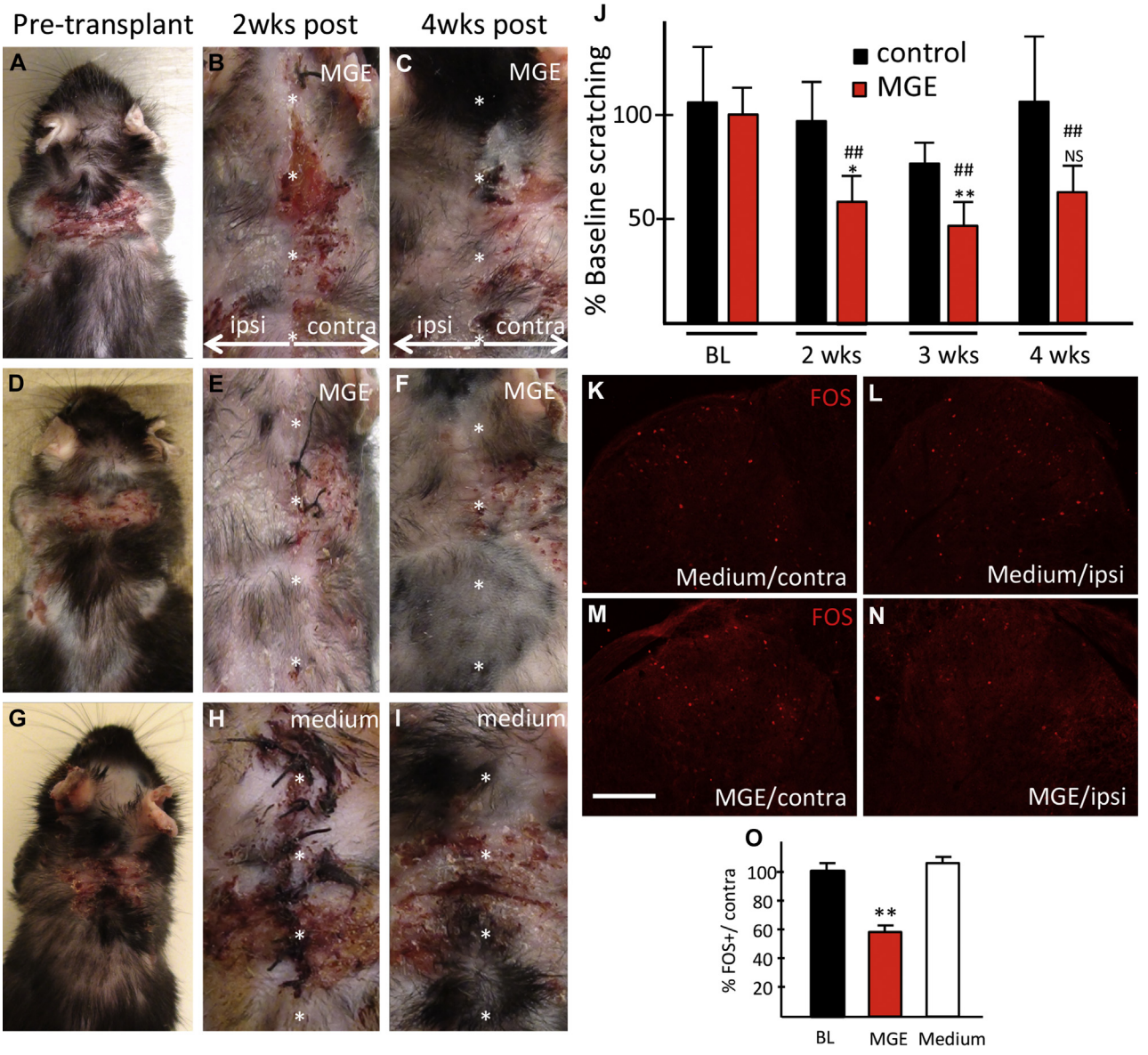

FIG 4. Transplant-mediated reduction of scratching, skin lesions, and spinal cord activity in IL-31Tg mice. Skin lesions **(A-I)** and scratching **(J)** (n = 10 vs 9 controls) reduced only after MGE transplant. Data are means ± SEMs; *relative to baseline; #relative to control; $*P < .05$; $**P < .005$; $^{\#\#}P < .05$; 2-way ANOVA and Bonferroni *post hoc.* **K-O,** MGE transplants reduced scratching-induced dorsal horn Fos+ neurons (**N**; *red bar* in **O**). Data are means ± SEM; $*P < .05$; Student *t* test. Bar = 100 μm.

Skin histology

Compared with the contralateral side (arrows in Fig 5, *A*), skin from the transplanted side had a thinner stratum corneum, with reduced epidermal thickening (arrows in Fig 5, *B*), which was confirmed histologically (scores: contralateral: 6 ± 0.56 vs ipsilateral: 3 ± 0.58) (Fig 5, *E*, left). Scratching-induced wounds were also reduced, as was erythrocyte extravasation. However, regardless of treatment, mast cell number did not change (Fig 5, *C*, *D*, and *F*) and there was no difference in inflammatory cell infiltrate (Fig 5, *E*, right). Importantly, in medium-injected, control mice, we found no difference in the histology between ipsilateral and contralateral skin (data not shown). We conclude that intraspinal transplant of MGE cells results in an overall improvement but not complete normalization of previously lesional skin structure, a likely consequence of MGE-induced reduction of scratching.

MGE cells differentiate into GABAergic interneurons

In mice that received an MGE transplant in the cervical spinal cord, we detected a large number of GFP^+ MGE cells throughout the cervical enlargement (segments C4-C8) (Fig 6), as well as GFP^+ processes that extended rostrally and caudally, well beyond the transplant site (see Fig E1 in this article's Online Repository at www.jacionline.org). Many MGE cells also expressed GABA (Fig 6, *A-C*) and markers of subpopulations of GABAergic interneurons, namely parvalbumin (Fig 6, *D-F*) and neuropeptide

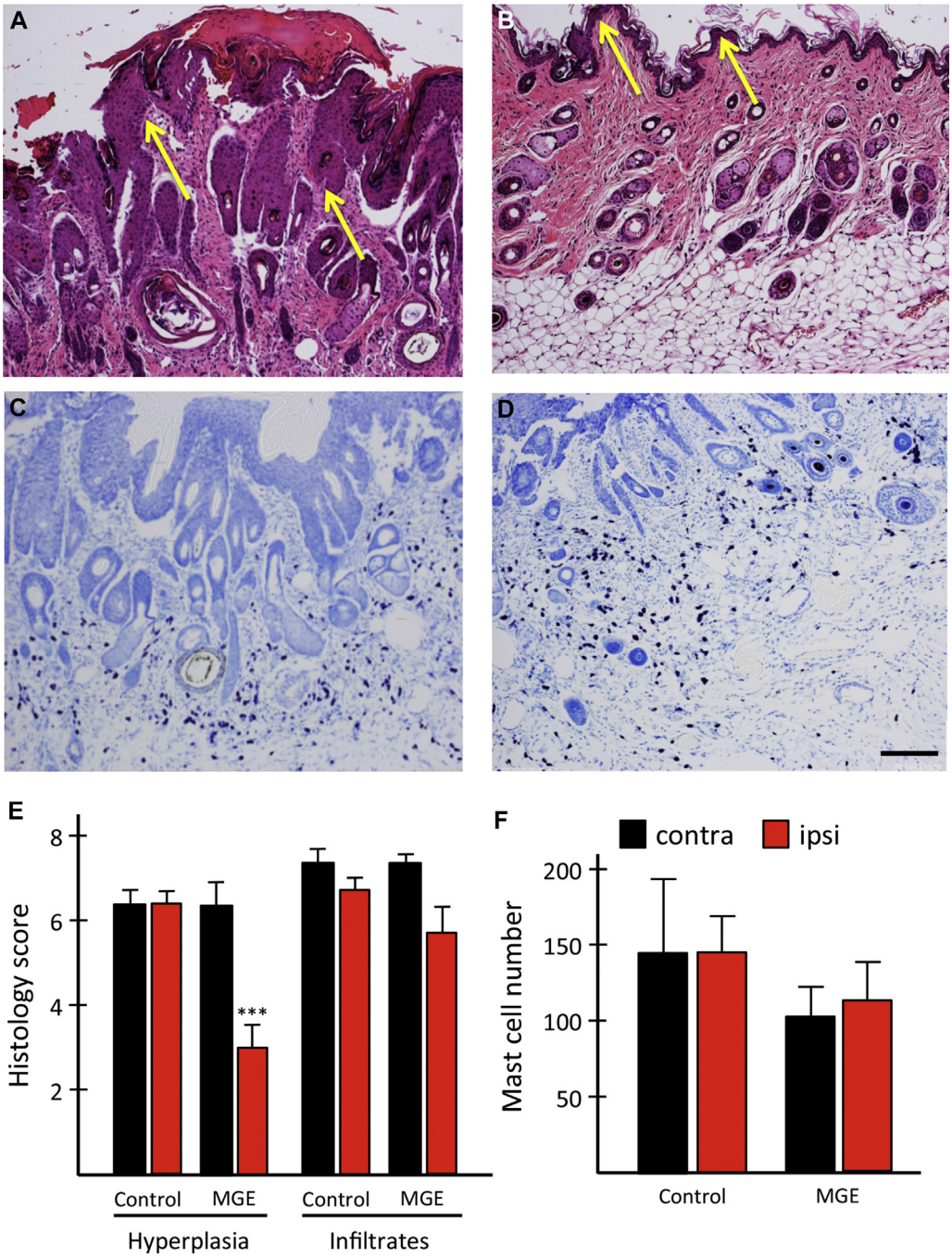

FIG 5. MGE transplants ameliorate skin pathology. **A-F,** Scratching associated hyperparakeratosis, epidermal thickening (hyperplasia), and looser upper dermis (**A,** *yellow arrows*; **E,** n = 3) are reduced after MGE transplantation **(B)**. Neither inflammatory infiltrate (**A, B, E,** n = 3) nor mast cell number differed in control (**C,** n = 3) and transplanted mice (**D, F,** n = 4). Data are means ± SEMs; ***P < .001; Student t test. Bar = 145 μm.

Y (Fig 6, *G-I*). Together, our results indicate that MGE transplants survived, differentiated into GABAergic interneurons and integrated in the spinal cord of the IL-31Tg mice.

GABA signaling and neuronal activity in the IL-31Tg mice

To better understand the mechanisms that underlie the MGE-mediated antipruritic effects, we assessed the effects of the MGE cell transplants in the spinal cord biochemistry of the IL-31Tg mice. We hypothesize that scratching-induced activation of MGE cells in the IL-31Tg mice results in spinal cord release of the MGE-derived GABA, which in turn should reduce the levels of neuronal activity in the spinal cord. To test this hypothesis, we immunostained spinal cord for the immediate early gene *Fos*, a marker of activated neurons, in both transplanted and control IL-31Tg mice. Fig 4, *K-N* illustrate that scratching indeed dramatically induced Fos in the dorsal horn of the IL-31Tg

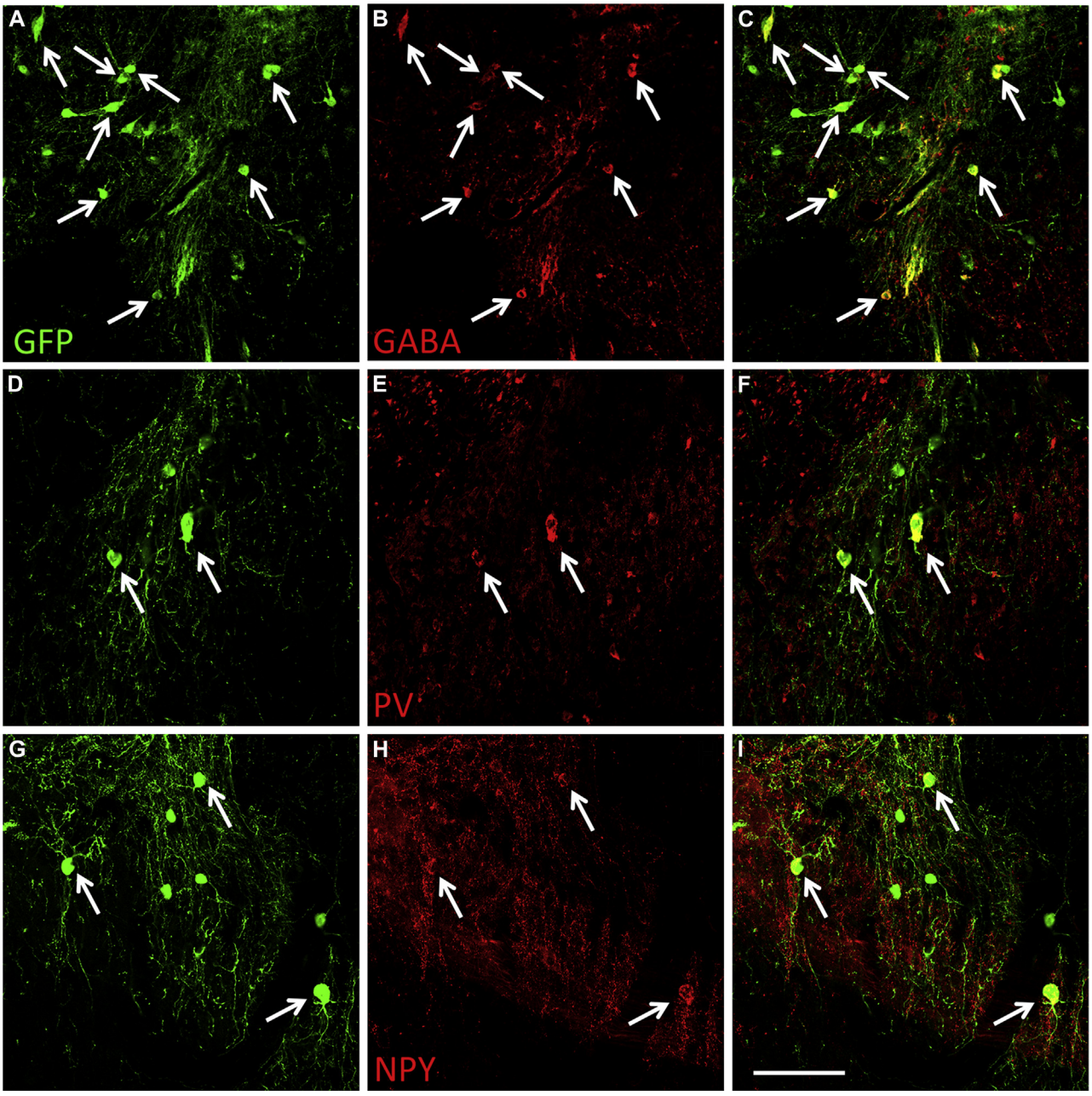

FIG 6. Spinal cord transplanted MGE cells differentiate into GABAergic interneurons. **A-I,** GFP^+ (*green*) MGE cells survived in the spinal cord of IL-31Tg mice **(A, D, G)** and expressed markers of subpopulations of GABAergic interneurons, including GABA **(B)**, parvalbumin **(E)**, and neuropeptide Y **(H)**. Merged imaged are shown in **C, F,** and **I**. Bar = 100 μm.

mice, particularly in cord segments that receive inputs from skin with lesions. MGE cell transplants significantly reduced Fos expression (by ~50%, $P < .003$) (Fig 4, *N* and *O*) ipsilateral to the transplant, indicating reduced neuronal activity and thus less drive in itch circuitry. In contrast, there was no reduction in the number of Fos^+ neurons in the control IL-31Tg mice (Fig 4, *L* and *O*).

Next, we measured dorsal horn GAD65 and GAD67 mRNA before and after MGE transplantation and found comparable levels in transplanted and nontransplanted mice, indicating that the MGE transplants do not increase GAD mRNA levels above normal (see Fig E2 in this article's Online Repository at www.jacionline.org). Thus, although release of GABA from MGE cells is critical to the presumptive GABA-B receptor signaling deficit in the IL-31Tg mice, our results suggest that the transplants do not act as therapeutic pumps that continuously release GABA, which could be concluded if GAD levels were increased. Rather we suggest that it is the integration of the cells into the spinal cord circuits that underlies their inhibitory effects.

DISCUSSION

As there is considerable preclinical evidence that GABAergic circuits regulate the transmission of pruritoceptive messages at

the level of the spinal cord and that dysfunction of GABA signaling contributes to chronic itch,[4,11,32] it is surprising, to our knowledge, that neither preclinical nor clinical studies examined the therapeutic effects of GABA agonists against either acute or chronic itch. Baclofen, a prototypical GABA-B agonist, has proven effective in several preclinical pain studies,[33,34] and when administered by the intrathecal route, it is routinely used in patients with multiple sclerosis, for its antispasticity properties.[35] Baclofen is also occasionally prescribed, in France[36] to treat addictive disorders. Muscimol, on the other hand, although not approved by the US Food and Drug Administration, has been evaluated in a recent clinical trial to treat epilepsy (clinicaltrials.gov NCT00005925). Our present preclinical findings are, therefore, the first to show that baclofen and muscimol, in fact, have profound antipruritic properties in models of both acute and chronic itch and that their antipruritic effects can be produced without concomitant sedation. We also show that sustaining high levels of GABA-mediated inhibition by MGE cell transplantation has significant utility in the management of a chronic inflammatory itch condition (namely atopic dermatitis) as the transplants not only reduced spontaneous scratching, but also dramatically reduced the incidence and severity of the associated skin lesions.

Although baclofen and muscimol have different onset latency, duration of action, and potency, we found that both agonists effectively reduce the scratching evoked by pruritogens that engage both histamine-dependent and -independent pathways. It appears, therefore, that GABA receptor-mediated inhibitory controls can regulate the transmission of itch messages generated by most subset of afferents, a conclusion that is consistent with the fact that loss of dorsal horn GABAergic interneurons increases the scratching evoked by a wide variety of pruritogens.[11] Given that baclofen and muscimol were administered systemically, it remains to be determined whether their site of action is spinal and/or supraspinal. Interestingly, a recent study reported that direct microinjection of muscimol into the central nucleus of the amygdala has antipruritic effects against both acute (serotonin) and chronic (dry skin model) itch.[37] As supraspinal antinociceptive actions of GABA receptor agonists have been reported,[38] it is likely that systemic administration of GABA agonists has concurrent spinal and supraspinal actions. Somewhat disappointingly, but consistent with studies reporting baclofen-related side effects, we found a very small therapeutic window using systemic GABA agonists, due to sedation. Importantly, however, we showed that this limitation was mitigated with synergistic combinations of low doses of baclofen and muscimol. Indeed, doses near the ED_{50} or even subthreshold doses of muscimol potentiated the antipruritic effects of nonsedating, subthreshold doses of baclofen in acute pruritus.

Perhaps more importantly, we showed that the combination of low doses of GABA-A and GABA-B agonists is also significantly antipruritic in a model of chronic inflammatory itch. Chronic itch in the IL-31Tg mice differs considerably from the neuropathic itch that develops in *Bhlhb5* mutant mice, in which there is a selective loss of spinal cord GABAergic inhibitory interneurons. In contrast, the AD-like condition produced in the IL-31Tg mice involves both peripheral and central mechanisms. First, there is enhanced release of IL-31 in the skin,[15] which we previously reported exerts its effect by activating a small subset of transient receptor potential cation channel subfamily V member 1 (TRPV1)-positive primary afferent pruritoceptors that express the IL-31 receptor. As capsaicin-mediated deletion of these pruritoceptors significantly reduced scratching provoked by injection of IL-31,[22] we conclude that a peripheral mechanism contributes to the IL-31-mediated itch phenotype. Here, we also uncovered significant changes in spinal cord circuits in the IL-31Tg mice. Not only is there a significant increase in message levels of GRP, a neuropeptide expressed by excitatory interneurons that engage propruritic dorsal horn circuitry,[27] but we also recorded a significant decrease of postsynaptic GABA-B1 receptor subunit message. We suggest that these pathophysiological changes in the IL-31Tg mice result in an enhanced excitatory and decreased inhibitory "drive" that underlies a central hyperexcitability state that is critical to the chronic itch phenotype, including that observed in patients with AD.[39]

Consistent with this observation, spinal cord Fos expression in the absence of an applied stimulus was significantly increased in segments that receive inputs from skin with lesions. As the MGE cell transplants significantly reduced the Fos expression as well as the excessive scratching, we suggest that the inhibitory control exerted by the transplanted cells reduced the activity of hyperactive spinal cord circuits. Interestingly, despite the reduced scratching in transplanted animals, neither inflammatory infiltrate nor mast cell numbers changed. We conclude, therefore, that the MGE transplants can overcome both the increased peripheral primary afferent and central excitatory drive that occurs in this model of chronic itch. This approach differs considerably from the more commonly prescribed treatments for AD, namely anti-inflammatory medications that have a predominant peripheral site of action. Taken together with our previous studies,[5] the present findings suggest that MGE transplantation is a disease-modifying approach that can repair a GABAergic neuronal dysfunction. Our study also demonstrates that sustaining high levels of spinal cord GABAergic inhibition, whether pharmacologically or using cell-based therapies, can be very effective against chronic itch conditions with a variety of etiologies. Importantly, as there are reports that long-term use of baclofen can increase sedation liability, future preclinical studies should determine whether repeated administration of GABA agonists, either alone or in combination, can mimic the prolonged effects of the transplants without concomitant increase in sedation. Although baclofen is approved for clinical use, the choice of GABA-A agonist will require additional study to determine which is most appropriate for a clinical trial. Of interest are possible combinations with benzodiazepines, which bind to and regulate GABA-A receptors.

Conclusions

Although there are reports of synergistic actions of GABA agonists and morphine in rodents[38,40] and humans,[41] this is the first demonstration that GABA agonists acting at different GABA receptors potentiate. This is also the first report of their preclinical use in the treatment of acute and chronic itch. We believe that a comparable pharmacotherapeutic approach should be considered in the clinical management of acute and chronic itch. Most importantly, as sedation is the major cause for discontinuing baclofen treatment in patients,[42] it is particularly significant that a synergistic combination of very low doses of baclofen and subthreshold doses of other GABA agonists retain profound antipruritic effects, without concomitant sedation.

Clinical implications: Preclinical data encourage the design of human studies to explore the use of GABA-agonists, such as US Food and Drug Administration–approved baclofen in the management of acute and chronic itch.

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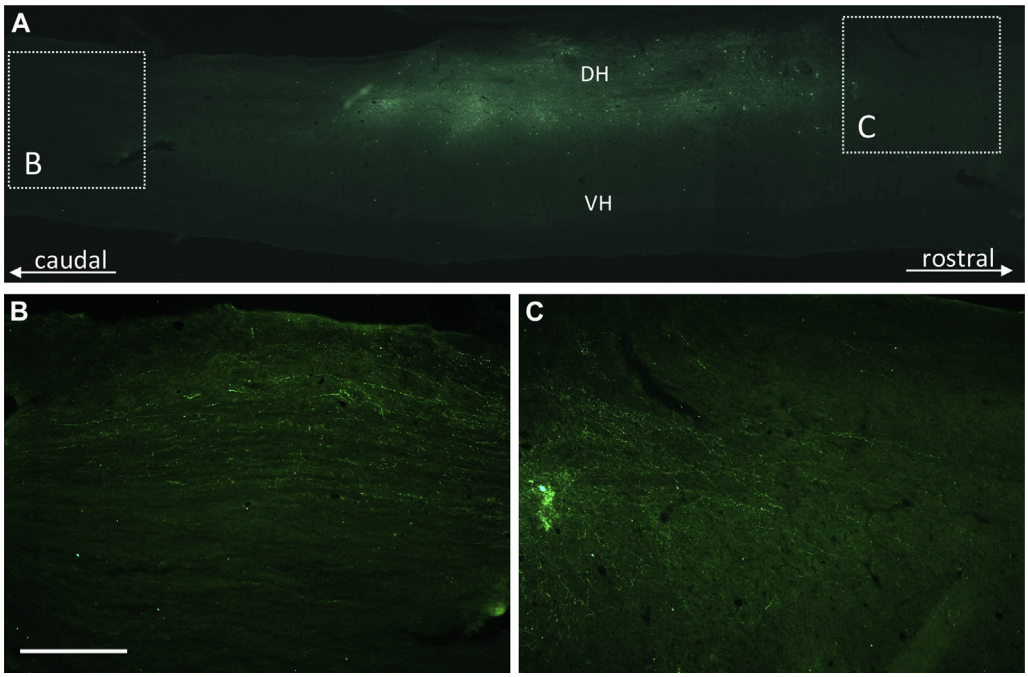

FIG E1. Transplanted GFP-expressing MGE cells (*green*) extend long processes **(A)**, both caudal **(B)** and rostral **(C)** to and well beyond the injection sites. *DH*, Dorsal horn; *VH*, ventral horn. Bar = 300 μm in **A** and 100 μm in **B** and **C**.

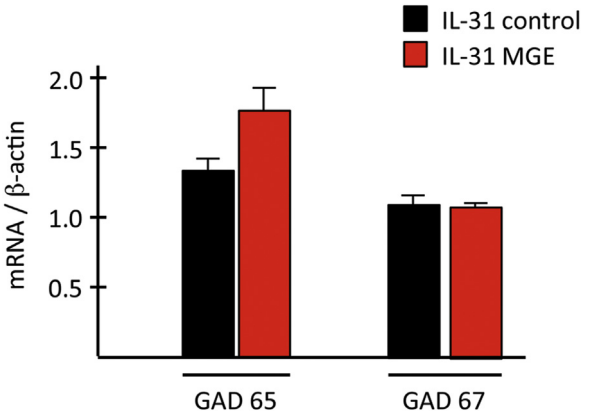

FIG E2. There is no difference in GAD65 or GAD67 expression levels between MGE- and medium-injected IL-31Tg mice (n = 5; Student *t* test).