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α-1-Adrenergic Receptor Agonist Activity of Clinical α-Adrenergic Receptor Agonists Interferes with α-2-Mediated Analgesia

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Background: **The use of α-2 adrenergic agonists for analgesia is limited due to a narrow therapeutic window. Definition of the role of alpha receptor subtypes in alpha agonist mediated analgesia may identify strategies to separate the analgesic from sedative and cardiovascular effects.**

Methods: **Analgesic activity of brimonidine, clonidine, and tizanidine was investigated in wild-type C57B/6, α-2A, and α-2C knockout mice with allodynia induced by *N*-methyl-*D*-aspartate or sulprostone. The alpha receptor selectivity of the alpha agonists was assessed using functional *in vitro* recombinant assays.**

Results: **Brimonidine, clonidine, and tizanidine reduced *N*-methyl-*D*-aspartate- and sulprostone-induced allodynia in wild-type mice, but not α-2A knockout mice. In α-2C knockout mice, brimonidine and tizanidine reduced allodynia in both models, whereas clonidine only reduced *N*-methyl-*D*-aspartate-induced allodynia. *In vitro*, clonidine and tizanidine activated α-1 and α-2 receptors with similar potencies, whereas brimonidine was selective for α-2 receptors. In α-2C knockout mice with sulprostone-induced allodynia, blockade of clonidine's α-1 receptor agonist activity restored clonidine's analgesic efficacy. In wild-type mice, the analgesic potency of intrathecal clonidine and tizanidine was increased 3- to 10-fold by coadministration with the α-1A-selective antagonist 5-methylurapidil without affecting sedation. Following intraperitoneal administration, the therapeutic window was negligible for clonidine and tizanidine, but greater for brimonidine. 5-Methylurapidil enhanced the therapeutic window of intraperitoneal clonidine and tizanidine approximately 10-fold.**

Conclusions: **α-1A receptor agonist activity can counterbalance α-2 receptor agonist-induced analgesia. Greater α-2 selectivity may enhance the therapeutic window of α-2 agonists in the treatment of pain.**

α-2-ADRENOCEPTORS are known to mediate analgesia, and α-2-adrenoceptor agonists (such as clonidine) have proven effective in the treatment of pain, such as intractable cancer pain,[1,2] postsurgical pain,[3,4] and sympathetically maintained pain.[5] However, the systemic use of α-2 agonists for long-term analgesia has been limited by a narrow therapeutic window due to the potential for sedation and cardiovascular depression.

Clonidine and other α-2 agonists used clinically (tizanidine, brimonidine and dexmedetomidine) activate to varying degrees three structurally distinct subtypes of the α-2 receptor: α-2A, α-2B, and α-2C.[6] Studies in knockout mice have demonstrated that pain modulation in the spinal cord is mediated primarily by the α-2A receptor and also by α-2C receptors; however, the α-2A subtype has also been associated with dose-dependent sedation and decreased blood pressure, thus narrowing the therapeutic window of α-2 agonists when used clinically for analgesic activity.[7–10] The studies to date in α-2 knockout mice have either utilized noxious stimuli such as heat and spinal substance P injection or tactile stimuli following nerve transection. Further research to define the role of various alpha receptor subtypes is needed to identify strategies for separating the analgesic and sedative effects of α-2 agonists.

We investigated the α-adrenergic pharmacology of analgesia using models of tactile hypersensitivity induced with chemical stimuli that sensitize pain pathways at spinal or peripheral sites. The α-2A receptor was necessary for the analgesic activity of brimonidine, clonidine, and tizanidine, but a surprising difference identified in the activity of the α-2 agonists in α-2C knockout mice led to investigation of the roles of α-1 and α-2 receptor activation in pain modulation.

Materials and Methods

α-2A knockout and α-2C knockout mice on a C57B/6 background were provided by Brian Kobilka, Ph.D. (Professor, Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Palo Alto, California) and bred by Charles River Labs (Wilmington, MA) by homozygous matings. Age-matched wild-type mice on the same C57B/6 background were obtained from Charles River Labs. All experimental animals were housed in standard plastic cages designed to allow easy access to food and water. All animals were kept in controlled temperature chambers (24 ± 1°C) on a 12:12 light-dark cycle (light on 6:00 AM–6:00 PM). All experiments were done in compliance with protocols approved by the Allergan Institutional Animal Care and Use Committee (Irvine, CA).

Intrathecal Injection

Intrathecal injections were done according to the method devised by Hylden and Wilcox.[11] A sterile 30-

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gauge ½ inch needle attached to a microsyringe was inserted between the L5 and L6 vertebrae and a 5 μl volume slowly injected in the subarachnoid space.

Mouse Allodynia Model

Male mice, weighing approximately 25 g, were administered 100 ng intrathecal *N*-methyl-*D*-aspartate (NMDA; Sigma Chemical Company, St. Louis, MO), 200 ng intrathecal or 300 ng/kg (in a 1 ml/kg volume) intraperitoneal sulprostone (Cayman Chemical Company, Ann Arbor, MI) to induce tactile hypersensitivity through different pain pathways as described in Gil *et al.*[12] The alpha agonists brimonidine (UK 14,304 tartrate salt; Allergan, Inc.; 0.4 μg), clonidine (Sigma; 0.4 μg for the sulprostone model and 1 μg for the NMDA model) and tizanidine (Sigma; 3 μg) were coadministered intrathecally with the allodynic agent. In some experiments, various doses of α-2 agonists brimonidine, clonidine, and tizanidine were administered intraperitoneally 15 min before intrathecal NMDA or intraperitoneal sulprostone. The α-1 antagonists prazosin (Sigma; 100 ng/kg) or 5-methylurapidil (5-MU; Sigma; 30 μg/kg) were administered intraperitoneally 15 min before the allodynic agent. NMDA and sulprostone were dissolved in 100% dimethyl sulfoxide (DMSO; Sigma), whereas brimonidine, clonidine, tizanidine, prazosin, and 5-MU were dissolved in distilled water.

Mice were assessed for tactile sensitivity by light stroking of the hind flank with a small paint brush every 5 min during a 35-min period. Measurements started at least 15 min after injection of the allodynic agent since the mice do not exhibit acute pain behaviors at these time points. Responses were scored as 0 (no response), 1 (avoidance), or 2 (vigorous avoidance), and scores were added to give a total pain score (maximum of 16 for combined scores from each of the eight time points).

Mouse Exploratory Activity

Thirty minutes following drug dosing, locomotor activity was determined using an automated exploratory chamber (Omnitech Electronics, Dartmouth, Nova Scotia, Canada). This system has motion-sensitive photobeams that can measure movement as horizontal activity. Beam interruptions were analyzed using the accompanying computer software to determine total activity over a 5-min time period.

Fluorometric Imaging Plate Reader Calcium Assay

Intracellular calcium response was measured in HEK293 cells stably expressing bovine α-1A (B_{max} = 3.8 pmol receptor/mg protein) and rat α-1B (B_{max} = 3.4 pmol receptor/mg protein) receptors, or coexpressing with G_{16} or G_{qi5} protein[13] human α-2A (B_{max} = 7.8 pmol receptor/mg protein), mouse α-2B (B_{max} = 1.4 pmol receptor/mg protein) and human α-2C (B_{max} = 6.8 pmol receptor/mg protein) receptors. Cell lines were maintained in Dulbecco's Modified Eagle's Medium, 10% fetal bovine serum, 1 × penicillin/streptomycin/amphotericin B, and 0.25 μg/ml puromycin. Cells were plated in 384 well plates at the following densities: 20,000 cells/well for HEK293 α-2A/G_{qi5}; 25,000 cells/well for HEK293 α-1A and HEK293 α-1B; 30,000 cells/well for HEK293 α-2B/G_{16}; and 40,000 cells/well for HEK293 α-2C/G_{qi5}. Before assay, cells were washed twice with fluorometric imaging plate reader buffer (Hanks balanced salt solution with 20 mM HEPES), pH 7.4, then dye loaded at 37°C for 40 min with fluo-4, 4 μM. Excess dye was removed by washing the cells four times with fluorometric imaging plate reader buffer. The cells were

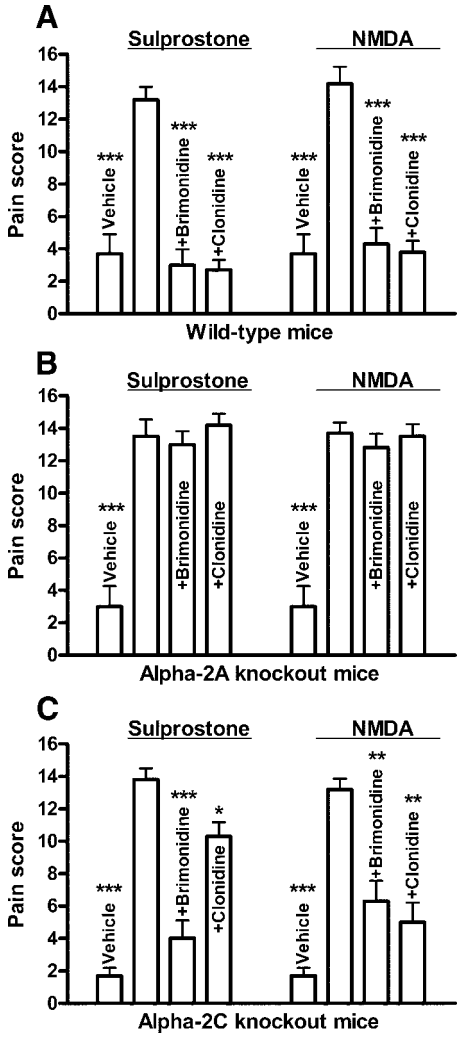

Fig. 1. Effects of the α-2 agonists brimonidine and clonidine on sulprostone- or NMDA-induced allodynia in wild-type, α-2A knockout, and α-2C knockout mice. Total pain scores after administration of vehicle (DMSO), an allodynic agent (sulprostone or NMDA), or the allodynic agent following brimonidine or clonidine pretreatment, in (*A*) wild-type mice, (*B*) α-2A knockout mice, and (*C*) α-2C knockout mice. The α-2 agonists were inactive in α-2A knockout mice. Clonidine was also inactive in α-2C knockout mice with sulprostone-induced allodynia. DMSO = dimethyl sulfoxide; NMDA = *N*-methyl-*D*-aspartate. * $P < 0.05$, ** $P \leq 0.01$, *** $P < 0.001$ *versus* allodynic agent, N = 6 mice per group.

Table 1. Effect of Tizanidine on Allodynia in α-2 Wild-type and Knockout Mice

Treatment	Wild-type Mice	α-2A Knockout Mice	α-2C Knockout Mice
Sulprostone model			
Vehicle	5.0 ± 0.6*	4.8 ± 0.6*	4.7 ± 0.7*
Sulprostone	12.5 ± 0.6	12.7 ± 0.4	13.0 ± 0.6
Tizanidine + sulprostone	5.5 ± 0.3*	13.8 ± 0.8	4.8 ± 0.8*
N-methyl-*D*-aspartate model			
Vehicle	5.0 ± 0.6*	4.8 ± 0.6*	4.7 ± 0.7*
Sulprostone	12.8 ± 0.7	13.3 ± 0.6	12.8 ± 0.6
Tizanidine + sulprostone	5.0 ± 1.0*	13.7 ± 0.7	4.8 ± 0.6*

* $P < 0.001$ *versus* sulprostone alone.

incubated at 37°C for 3 min before loading in the fluorometric imaging plate reader system (FLIPR$^{TETRA®}$, Molecular Devices, Sunnyvale, CA).

Assays were carried out under the following conditions: 37°C; excitation filter 470–495 nm; emission filter 515–575 nm; typical gain 170; typical excitation intensity 90; and exposure time 0.4 s. Test compounds were added in triplicate, and fluorescence was read for 230 s. Norepinephrine, a natural full agonist for alpha adrenergic receptors, was used to determine the maximum calcium signal; mean peak responses for each study drug concentration were expressed as a percentage of the norepinephrine response, and sigmoidal concentration-response curves were obtained by nonlinear least squares curve fitting to the following equation:

$$Y = m1 + (m2 - m1)/(1 + [m0/m3]);$$

where m1 = maximum response, m2 = minimum response, m0 = drug concentration, and m3 = EC_{50} (concentration where 50% of the maximum effect was observed).

Data Analysis and Statistical Procedures

Data were compiled and analyzed using Statistical Package for the Social Sciences (SPSS, Chicago, IL), Microsoft® Office Excel (Redmond, WA), and/or KaleidaGraph® (Synergy Software, Reading, PA). Data were expressed as mean ± SE (SEM), and comparisons between groups were made using a one-way analysis of variance, followed by a Dunnett's test; the significance value was set at $P < 0.05$. Pain score comparisons were between groups treated with the allodynic agent in the absence and presence of drug. The sedative effect of alpha agonists was compared to vehicle control.

Results

The allodynia-inducing chemicals NMDA and sulprostone, a noninflammatory selective prostaglandin receptor agonist, cause tactile hypersensitivity. They act by distinct mechanisms as evidenced by primarily spinal (NMDA) and peripheral (sulprostone) activity and their sensitivity to pharmacological antagonism.[12] In wild-type mice, intrathecal brimonidine, clonidine, and tizanidine significantly reduced sulprostone- and NMDA-induced allodynia to vehicle levels (fig. 1A, table 1). The α-2 agonists were inactive in both allodynia models in α-2A knockout mice (fig. 1B, table 1). In α-2C knockout mice, the agonists were effective against NMDA-induced allodynia, but only brimonidine and tizanidine exhibited strong activity in the sulprostone model (fig. 1C, table 1).

The lack of clonidine analgesic activity in the sulprostone-induced allodynia model in α-2C knockout mice prompted an investigation into the *in vitro* functional selectivity of the clinically used α-2 agonists clonidine, brimonidine, and tizanidine. In the *in vitro*, cell-based assay of intracellular calcium elevation, clonidine and tizanidine exhibited agonist activity at α-1A (fig. 2A) and α-1B receptors (fig. 2B). Clonidine was a potent α-1A agonist with an EC_{50} (47 nM) similar to the α-1 agonist phenylephrine (56 nM; table 2). Brimonidine was significantly less potent and efficacious at either α-1 receptor subtype. Further analysis of α-1A, α-2A, α-2B, and α-2C

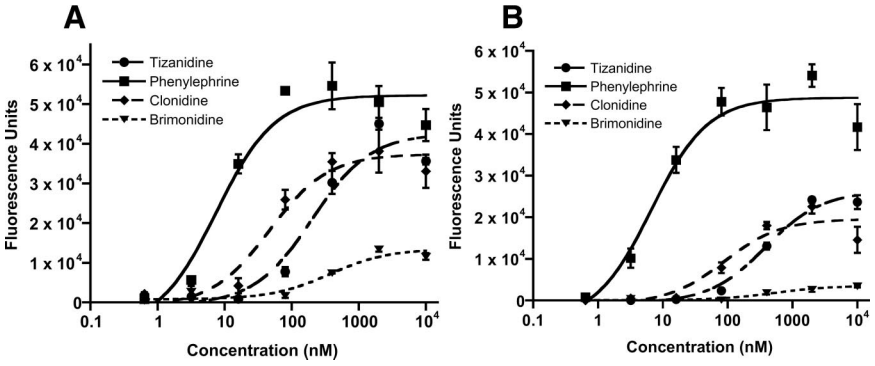

Fig. 2. Effects of α-2 agonists in α-1A and α-1B fluorometric imaging plate reader assay. Intracellular calcium concentration in HEK293 cells expressing (*A*) bovine α-1A receptors and (*B*) rat α-1B receptors. Clonidine and tizanidine are strong α-1A agonists and partial α-1B agonists, but brimonidine is a weak agonist compared with the α-1 agonist, phenylephrine. Data are a representative experiment in triplicate.

Table 2. Functional Activity of α-adrenergic Agonists in Fluorometric Imaging Plate Reader Assay

	EC_{50} (nM)				
Compound	1A	1B	2A	2B	2C
Brimonidine	1466 ± 350 (0.4) N = 4	NA N = 4	3.2 ± 1.0 (0.9) N = 8	39.4 ± 5.7 (0.7) N = 16	10.5 ± 3.4 (1.0) N = 8
Clonidine	46.7 ± 6.2 (0.9) N = 11	1287 ± 198 (0.2) N = 4	40.5 ± 4.6 (0.7) N = 10	89.6 ± 26.3 (0.4) N = 6	55.4 ± 8.6 (0.8) N = 7
Tizanidine	472 ± 42.6 (1.0) N = 3	2097 ± 324 (0.2) N = 3	490 ± 36.4 (0.8) N = 3	NA N = 3	896 ± 79.5 (0.7) N = 3
Norepinephrine	3.5 ± 1.1 (1.0) N = 17	1.4 ± 0.2 (1.0) N = 6	8.9 ± 3.1 (1.0) N = 17	3.6 ± 1.7 (1.0) N = 11	2.8 ± 0.8 (0.9) N = 12
Phenylephrine	56.0 ± 9.2 (1.0) N = 10	27.4 ± 4.9 (1.0) N = 42			

Values are the mean ± standard error of at least 3 independent experiments.

Values in parentheses are efficacy relative to the reference full agonist norepinephrine.

EC_{50} = concentration where 50% of the maximum effect is observed; NA = not active.

receptor subtype activity demonstrated that clonidine and tizanidine are equipotent agonists of α-1 and α-2 receptors, with an α-1A/α-2A agonist selectivity ratio of 1.2 and 1.0, respectively (table 2). In contrast to clonidine, brimonidine demonstrated marked α-2 receptor selectivity, with an α-1A/α-2A selectivity ratio of 458 (table 1).

To determine if the α-1 agonist activity of clonidine contributed to the loss of clonidine efficacy in the α-2C receptor knockout mice, these mice were coadministered clonidine and the α-1 antagonist prazosin (to block clonidine's α-1 agonist activity). Although prazosin was inactive by itself, the combination of the two drugs restored the analgesic effect of clonidine in the sulprostone-induced allodynic response in α-2C knockout mice (fig. 3). These data suggest that the α-1 agonist activity of clonidine reduces the compound's analgesic efficacy.

We further investigated whether the α-1 activity of clonidine and tizanidine was a liability in wild-type mice.

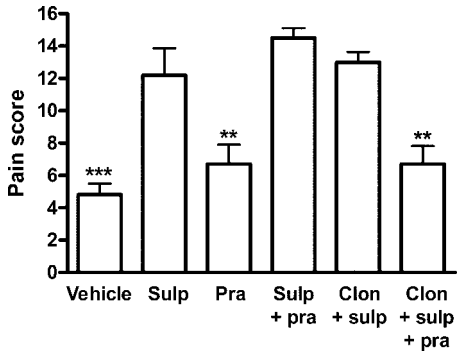

Fig. 3. The α-1 antagonist prazosin restores clonidine analgesia in α-2C knockout mice. Total pain scores in α-2C knockout mice after administration of vehicle (DMSO), sulprostone, prazosin 100 ng/kg intraperitoneally, sulprostone plus prazosin, and sulprostone following clonidine pretreatment, with or without prazosin. The effect of prazosin alone was not significantly different from vehicle. α-1 blockade by prazosin restored clonidine analgesia in α-2C knockout mice with sulprostone-induced allodynia. Clon = clonidine; DMSO = dimethyl sulfoxide; Pra = prazosin; Sulp = sulprostone. ** $P < 0.01$, *** $P < 0.001$ *versus* sulprostone, N = 6 mice per group.

Intrathecal dosing with clonidine resulted in a 10-fold therapeutic window between a strongly analgesic dose (0.4 μg in the sulprostone model, fig. 4A; 1 μg in the NMDA model, fig. 4B) and a sedative dose (10 μg, fig. 4C). There was a minimal therapeutic window with intrathecal tizanidine, since a 3 μg dose that resulted in a strong analgesic effect in both the sulprostone and NMDA models (fig. 4D, E) also caused significant sedation (fig. 4F). Intraperitoneal administration of the α-1A-selective antagonist 5-MU in wild-type mice increased the potency of clonidine-mediated analgesia 10-fold in both the sulprostone and NMDA models (fig. 4A, B). 5-MU also enhanced the potency of tizanidine threefold (fig. 4D, E). Administration of 5-MU alone had no effect on sulprostone- or NMDA-induced allodynia (fig. 4G). Clonidine- and tizanidine-induced sedation was not affected by 5-MU (fig. 4C, F); thus, treatment with the α-1A antagonist enhanced the therapeutic window of intrathecal clonidine and tizanidine.

The α-2 agonists clonidine and tizanidine are often prescribed for systemic use. Following systemic (intraperitoneal) administration to wild-type mice with sulprostone-induced allodynia, the therapeutic window between analgesic efficacy and sedation is virtually negligible for clonidine and tizanidine. However, brimonidine, which has little effect on α-1 receptors (table 2), had an approximately 10-fold greater therapeutic window than clonidine and tizanidine (fig. 5A). Cotreatment with intraperitoneal 5-MU enhanced the analgesic potency of intraperitoneal clonidine and tizanidine by approximately 10-fold without affecting the sedative potency, resulting in a therapeutic window similar to that of brimonidine (fig. 5B, C).

Discussion

Previous studies have demonstrated that the α-2A receptor plays a predominant role in α-2 agonist-induced analgesia and sedation.[7–9] Since the α-2A receptor is required for

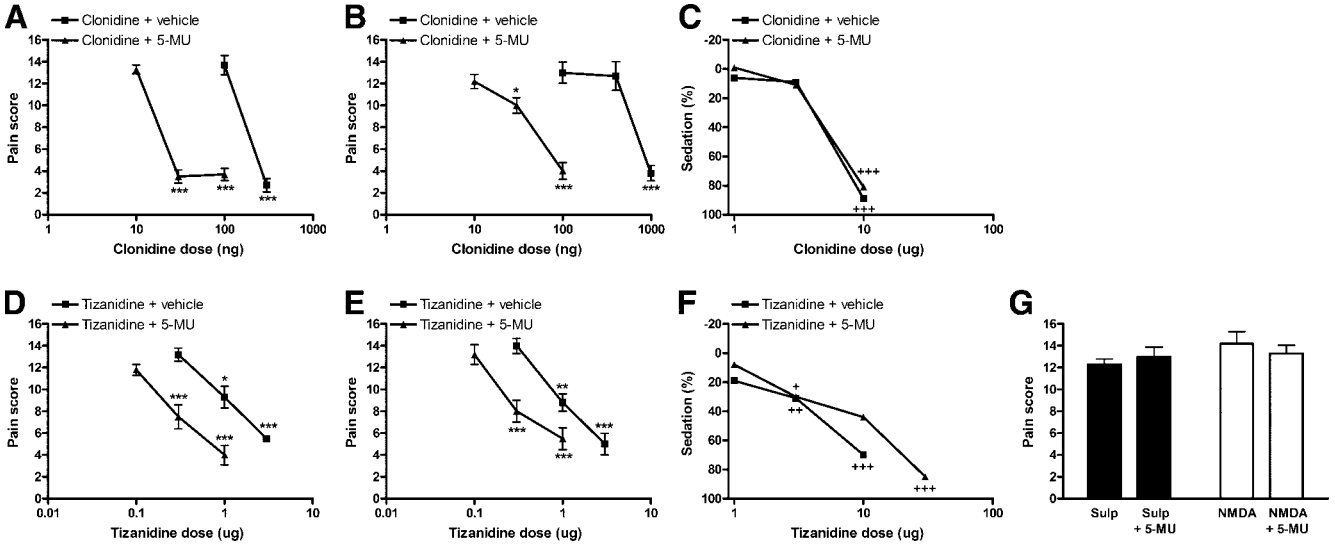

Fig. 4. α-1A-selective antagonist enhances the therapeutic window between analgesia and sedation for intrathecal clonidine and tizanidine in wild-type mice. (*A–C*) Effect of intrathecal clonidine pretreatment, with (▲) or without (■) 30 μg/kg intraperitoneal 5-methylurapidil (5-MU) on total pain scores in wild-type mice with (*A*) sulprostone-induced allodynia and (*B*) NMDA-induced allodynia, and on (*C*) activity counts in an exploratory chamber, expressed as percent sedation. (*D–F*) Effect of intrathecal tizanidine pretreatment, with (▲) or without (■) 30 μg/kg intraperitoneal (5-MU) on total pain scores in wild-type mice with (*D*) sulprostone-induced allodynia and (*E*) NMDA-induced allodynia, and on (*F*) activity counts in an exploratory chamber, expressed as percent sedation. (*G*) Lack of effect of 30 μg/kg intraperitoneal 5-MU alone on total pain scores in wild-type mice with sulprostone- or NMDA-induced allodynia. 5-MU = 5-methylurapidil; NMDA = *N*-methyl-*D*-aspartate; Sulp = sulprostone. * $P < 0.05$; ** $P < 0.01$, *** $P < 0.001$ *versus* allodynic agent alone for pain score, + $P < 0.05$, ++ $P < 0.01$, +++ $P < 0.001$ *versus* vehicle for sedation; N = 6 mice per group.

both actions, it is difficult to conceive a strategy to decrease the side effects of α-2 agonists without losing the efficacy. The results of our studies provide insight into the negative influence of α-1 receptor agonist activity on the α-2 agonist-mediated analgesia. These findings highlight a potential therapeutic strategy for increasing the therapeutic window of current α-2 agonists.

The α-2A receptor is also required for α-2 agonist-induced analgesia in the chemical models of tactile hypersensitivity, as the ability of brimonidine, clonidine, and tizanidine to alleviate NMDA- and sulprostone-induced allodynia (fig. 1A, table 1) was absent in α-2A knockout mice (fig. 1B, table 1). Unexpectedly, we observed a selective reduction of clonidine activity in the peripheral sulprostone sensitization model in the α-2C knockout mice (fig. 1C). The ability in the chemical allodynia models to titrate the dose of allodynia agent to the sensitization threshold and separately explore peripheral sensitization may have revealed this selective effect. Previous studies have assessed the role of α-2 receptors in acute nociceptive pain models, such as hot plate, tail flick, and substance P-induced behavior assays.[7–9] One previous study also investigated Von Frey hair tactile sensitivity,[14] but it was conducted in mice that had undergone tibial nerve transection, and such nerve injuries are thought to lead to altered expression of α-2 receptors.[15,16]

The activity of drugs *in vivo* can be influenced by their bioavailability, metabolism, protein binding, and access to tissues, which may explain why brimonidine is less potent than clonidine following intraperitoneal dosing (fig. 5A) but more or equipotent than clonidine following intrathecal dosing (fig. 1A). However, these factors cannot explain the selective reduction of clonidine activity in only the sulprostone model in α-2C knockout mice. Potential pharmacological differences between the agonists were investigated in an *in vitro* cellular calcium readout of α-2 agonist activity. Brimonidine is a more potent α-2 agonist, particularly at the α-2A receptor (table 2). Clonidine and tizanidine exhibit α-1A receptor activity, with clonidine being more potent, and brimonidine is minimally active at the α-1A receptor (table 2, fig. 2). When comparing the EC_{50} values for activating the α-2A and α-1A receptors (table 2), clonidine and tizanidine are equipotent, while brimonidine is 458-fold selective for the α-2A receptor. While these selectivity ratios are dependent on the assays used, the rank order of selectivity is unlikely to change.

The relevance of clonidine's and tizanidine's low *in vitro* α-2/α-1 selectivity to *in vivo* activity depends on tissue-specific factors such as α-1 receptor expression, receptor reserve and signal transduction. The impact of α-1 activity was tested by *in vivo* α-1 receptor blockade. Figure 3 illustrates that the elimination of clonidine's α-1 activity, by cotreatment with the α-1 antagonist prazosin, restored clonidine's analgesic activity in α-2C knockout mice rendered allodynic with sulprostone. Prazosin is 30- to 100-fold selective for the α-1 receptors (K_i values ≈0.1 nM [17]) relative to the α-2B and α-2C receptors (K_i values ≈10 nM [18]), so it likely acts selectively to block α-1 receptors at the low dose (100 ng/kg) that was used.

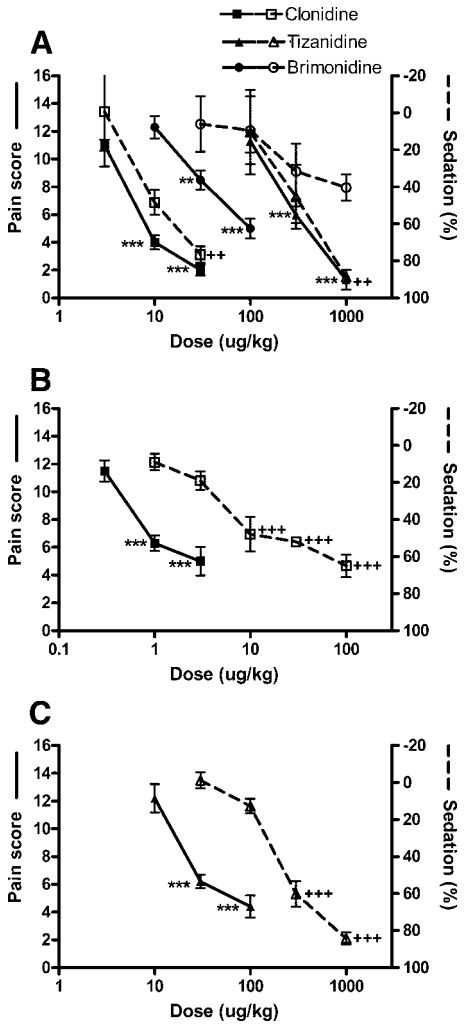

Fig. 5. Greater α-2 selectivity enhances the therapeutic window between analgesia and sedation after intraperitoneal dosing in wild-type mice. Superimposed dose responses for reduction of total pain scores in wild-type mice with sulprostone-induced allodynia (solid lines; N = 6) and reduction of activity counts (dashed lines; N = 4): (*A*) Following treatment with intraperitoneal clonidine (■, □), tizanidine (▲, △) or brimonidine (●, ○); a therapeutic window was seen only with the highly α-2-selective agonist brimonidine. (*B*) Following treatment with intraperitoneal clonidine plus intraperitoneal 5-methylurapidil (5-MU); cotreatment with 5-MU, a selective α-1A antagonist, enhanced the analgesic potency of clonidine by approximately 10-fold without affecting sedative potency (N = 6 mice per group). (*C*) Following treatment with intraperitoneal tizanidine plus intraperitoneal 5-MU; cotreatment with 5-MU also enhanced the analgesic potency of tizanidine by approximately 10-fold without affecting sedative potency (N = 5 mice per group). ** *P* < 0.01, * *P* < 0.001 *versus* vehicle for pain score. ++ *P* < 0.01, +++ *P* < 0.001 *versus* vehicle for sedation.**

Previous work has demonstrated that α-1 and α-2 receptors differentially modulate pain processing.[19,20] We hypothesized that clonidine's activation of α-1 receptors counterbalanced the α-2 receptor-mediated analgesia in α-2C knockout mice. The α-2C knockout mice have increased peripheral sympathetic norepinephrine outflow under low frequency stimulation conditions due to the absence of normal feedback inhibition of norepinephrine release.[21] This could explain the increased sensitivity of the α-2C knockout mice to the α-1 activity of clonidine and why it becomes apparent in the peripheral sulprostone allodynia model. There was no reduction of tizanidine analgesic activity in the α-2C knockout mice (table 1) even though it is equipotent at the α-1A and α-2A receptors like clonidine (table 2). This may be due to the sedation observed with 3 μg tizanidine, which likely masks the level of its analgesic activity.

For this finding to be of broader significance, there should be evidence of α-1 activity counteracting α-2 analgesia in the wild-type mice. Since the *in vitro* data demonstrated that clonidine and tizanidine are efficacious α-1A receptor agonists (fig. 2A, table 2), and based on our previous finding[12] that phenylephrine sensitization is inhibited by the α-1A receptor antagonist 5-MU,[22] wild-type mice were cotreated with 5-MU and clonidine or tizanidine. The cotreatment resulted in increased potency (3-fold for tizanidine and 10-fold for clonidine) to alleviate NMDA- or sulprostone-induced allodynia (fig. 4A, B, D, E). Since analgesia and sedation are mediated by α-2 receptors in different tissues, we also investigated the impact of clonidine and tizanidine α-1 activity on α-2 sedation. Cotreatment with 5-MU did not alter the sedative potency of clonidine and tizanidine (fig. 4C, F); thus, the therapeutic window between analgesic and sedative effects was substantially increased in the presence of 5-MU. These results suggest that the α-1 activity of α-2 agonists such as clonidine and tizanidine may reduce their analgesic potency.

α-2 Agonists including clonidine and tizanidine are used systemically, but they could be more widely used if there was a therapeutic margin between analgesia and the dose-limiting effects (*i.e.*, sedation) following systemic dosing. In wild-type mice with sulprostone-induced allodynia, there is no therapeutic window for clonidine and tizanidine, but there is a 10-fold window for the more α-2 selective brimonidine (fig. 5A). Similar to the findings with intrathecal dosing, there was an approximately 10-fold enhancement of the analgesic potency of intraperitoneal clonidine and intraperitoneal tizanidine and no effect on their sedative potency following treatment with the α-1 antagonist 5-MU (fig. 5B, C).

Although commonly referred to as "α-2" agonists, the classic α-2 agonists used to treat pain, such as clonidine and tizanidine, have little selectivity between the alpha receptors. Clonidine and other old alpha agonists, including UK 14,304, medetomidine and xylazine, were initially characterized in perfused tissue and binding assays using heterogenous tissues that can contain an assortment of receptors and result in misleading pharmacology.[23–25] Overall, our experiments suggest that 1) α-2 adrenergic receptor agonists with minimal activity at α-1 adrenergic receptors need to be tested to determine whether they are more effective for the treatment of

neuropathic pain, and 2) decreasing the α-1 agonist activity of current α-2 agonists, *via* coadministration with an α-1 antagonist, may enhance their *in vivo* analgesic potency. Because α-1 receptor activity seems to impact α-2 analgesia more than α-2 sedation, these strategies may enhance the therapeutic window of α-2 agonists and potentially overcome problematic adverse effects of current nonselective α-2 agonists in clinical use.

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