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Inflammation-Induced Shift in the Valence of Spinal GABA-A Receptor Mediated Modulation of Nociception in the Adult Rat

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Abstract

The objective of this study was to assess the impact of persistent inflammation on spinal γ-aminobutyric acid-A (GABA-A) receptor mediated modulation of evoked nociceptive behavior in the adult rat. Nocifensive threshold was assessed with von Frey filaments applied to the dorsal surface of the hindpaw. The GABA-A receptor agonist muscimol, and antagonist gabazine, and the benzodiazepine receptor agonist midazolam and antagonists PK11195 and flumazenil, were administered spinally in the presence and absence of complete Freund's adjuvant (CFA)-induced inflammation. In naïve rats, muscimol increased, and gabazine decreased, nociceptive threshold. Following CFA, the effects of these compounds were reversed: low doses of muscimol exacerbated the inflammation-induced decrease in nociceptive threshold and gabazine increased nociceptive threshold. Midazolam increased nociceptive threshold both in the presence and absence of inflammation. Flumazenil, but not PK11195, blocked the analgesic effects of midazolam. These findings indicate inflammation-induced changes in GABA-A signaling are complex and are likely to involve several distinct mechanisms. Rectifying the changes in GABA-A signaling may provide effective relief from hypersensitivity observed in the presence of inflammation.

Perspective—An inflammation-induced shift in spinal GABA-A receptor signaling from inhibition to excitation appears to underlie inflammatory pain and hypersensitivity. Use of GABA-A receptor selective general anesthetics in association with therapeutic interventions may be contraindicated. More importantly, rectifying the changes in GABA-A signaling may provide effective relief from inflammatory hypersensitivity.

Keywords

Persistent pain; intrathecal; nociceptors sensitization; central sensitization; behavioral pharmacology

Introduction

γ-aminobutyric acid (GABA) is the major inhibitory neurotransmitter in the nervous system mediating its most rapid effects via the ionotropic GABA-A receptor. Via both descending and

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intrinsic GABAergic circuitry, this system appears to be critically involved in the modulation of nociception in the spinal cord dorsal horn 22. There is evidence for direct GABAergic input onto the central terminals of both low 21;31 and putative high-threshold 2;21 primary afferent neurons as well as projection neurons in lamina I 5;23 and V 33 thought to be involved in the transmission of nociceptive information to higher brain structures. Consistent with this distribution of GABA-A receptors, there is evidence that in the absence of tissue injury spinal administration of GABA-A receptor agonists are analgesic 9 (although this effect may be modality specific 14) while GABA-A receptor antagonists result in thermal and mechanical hypersensitivity (i.e., hyperalgesia and allodynia) 29;36. Electrophysiological data are consistent with these behavioral observations 1;25.

In the presence of inflammation, however, there appears to be changes in GABA signaling. Increases in excitability, as manifest by increases in spontaneous and evoked activity, in dorsal horn neurons (generally referred to as central sensitization) appears to reflect, at least in part, a decrease in GABA-A mediated inhibition of dorsal horn projection neurons 18. There is also evidence for the emergence of GABA-A mediated excitation of the central terminals of Aδ- and C-fibers, resulting in the generation of antidromically conducted action potentials, or a dorsal root reflex 11;35. While there is still debate over the exact mechanism underlying the generation of the dorsal root reflex 6;22;35, there is general agreement that this process plays a significant role in the neurogenic component of the inflammatory response associated with tissue injury. A natural prediction of these inflammation-induced changes in GABA-A mediated signaling is that in the presence of inflammation, GABA-A receptor agonists become pro-nociceptive and GABA-A receptor antagonists become antinociceptive. Surprisingly, we were unable to find evidence of a direct test of this prediction in the literature. Therefore, the purpose of the present study was to assess the impact of inflammation on spinal GABA-A receptor mediated changes in nociception. Changes in nociception were assessed with behavioral pharmacological experiments.

Materials and Methods

Male Sprague–Dawley rats (Harlan, Indianapolis, IN, USA) weighing 200–250g were housed in standard polypropylene cages at the Dental School animal facility until the day of the experiments. All procedures were performed in accordance with guidelines established by the International Association for the Study of Pain and National Institutes of Health and were approved by the University of Maryland Institutional Animal Care and Use Committee.

Nociceptive testing

Mechanical sensitivity was assessed with von Frey filaments as described previously 26. An ascending series of von Frey filaments was applied to the dorsolateral surface of the hindpaw. Filaments, ranging in bending force from 9 mg to 170.0 g, were applied 5 times, each, at the force necessary to induce filament bending for the duration of 1 second at a frequency of 2 seconds. The response frequency [(number of responses/number of stimuli) × 100%] was assessed and the ascending series was terminated when rats responded to each application of a filament. Response frequency data for each animal were fitted with a logistic equation of the form: Response frequency = $100/(1+\exp[(EF50 - VFH)/k])$, where Response frequency = the response frequency to each von Frey filament, VFH = the stimulus intensity associated with each von Frey filament, k = the slope factor and EF50 = force necessary to produce a 50% response frequency. EF50 was used to quantify changes in mechanical sensitivity. Mechanical sensitivity was assessed before and after injection of test compounds. The experimenter was blinded to the composition of the spinal injection.

Motor testing

To rule out gross motor effects associated with high doses of the GABA-A receptor agonist muscimol, performance on a rota-rod (Stoelting) was assessed following spinal injection of muscimol or saline in both naïve and CFA inflamed rats. Rats were placed on a stationary rod which was set to accelerate to a terminal rate of 10 rotations per minute in 30 second. Baseline data were collected from rats once per day for 3 days prior the day of testing. The latency to drop from the rod was automatically determined by a trigger in a platform below the rod.

Spinal injections

Rats were anesthetized with isoflurane in O_2/N_2 (30:70 v/v). Anesthesia was induced at 3% and then maintained with a nose cone at 3%. Anaesthetized rats were then placed on a cushioned flat surface. The lumbar part of the back of the animal was shaved. A round 1-inch vial placed underneath the abdomen was used to flex the vertebral column at the lumbar level, which helped widen the intervertebral spaces. A 30 gauge needle was inserted in the L4–L5 region. A microinjection pump (KD Scientific, KDS220) and Hamilton microsyringe were used to inject 20 µl of test solutions at a rate of 7 µl/min. A short flicking of the tail or a hindlimb was often observed in association with the insertion of the injection needle. In order to confirm the site of injections, this injection procedure was used to inject Evan's Blue (10%) in a group of 5 rats. Following spinal injections, rats were briefly returned to their home cage to recover from anesthesia. Behavioral testing was performed between 10 and 30 minutes following spinal injections.

Induction of Inflammation

Complete Freund's adjuvant (CFA, Sigma-Aldrich, St Louis MO), suspended in an oil/saline (1:1) emulsion, was used to induce persistent inflammation of the hindpaw. 0.1 µl/g CFA was injected subcutaneously into the lateral ventral surface of the hindpaw. All inflamed rats were studied 72 hours after the injection of CFA. Naive rats were used as a control in order to avoid potential confounds associated with needle prick needed for a saline injection.

Test solutions

The following compounds were used: the GABA-A receptor agonist, muscimol (0.01, 0.05, 0.1, 1 µg/20 µl 15); GABA-A antagonists, gabazine (SR-95531, 0.15 ng, 1.5 ng, 6.6 ng, 15 ng, 150 ng, 1.5 µg and 3.7 µg/20 µl; an allosteric inhibitor of GABA-A receptor channel opening 30); the benzodiazepine receptor agonist, midazolam (6, 20 and 60 ng/20 µl 37); the central benzodiazepine receptor antagonist, flumazenil (12 ng/20 µl 17); and the peripheral benzodiazepine antagonist, PK11195 (1.1 and 7.1 µg/20 µl 8;24). Saline was used as a control. Each rat was used for a single injection of a single concentration of a test agent or saline. The doses chosen in this study were based on results from previous studies suggesting that they are not associated with motor effects 3;8;9. All test compounds were obtained from Sigma-Aldrich.

Statistics

While von Frey filament data are generally analyzed with non-parametric statistics (given that the forces applied are discontinuous) transforming the data in order to generate an EF50 enabled us to employ two-way analyses of variance in order to assess the presence of main effects associated with the influence of test compounds or inflammation as well as an interaction between the two. Post hoc analysis (Holm-Sidak test) was performed if a statistically significant interaction was detected. $P < 0.05$ was considered statistically significant.

Results

Seventy hours after injection of CFA, there was a significant increase in mechanical sensitivity. This is illustrated by the leftward shift in the von Frey filament evoked stimulus response function in inflamed (closed symbols) relative to naïve (open symbols) rats (Figure 1). Epidural injection of saline had no significant influence on the stimulus-response function evoked in either CFA treated or untreated rats (Figure 1).

Inflammation-induced changes in GABA-A receptor signaling were assessed with muscimol, a GABA-A agonist. Stimulus-response function data were collected before and 10 minutes after muscimol (0.01, 0.05, 0.1, 1 μg/20 μl) was injected in the L4–L5 epidural space (Figure 2A). In naïve rats, muscimol induced a dose-dependent decrease in mechanical sensitivity as illustrated by an increase in EF50; the increase in EF50 observed following injection of even the lowest dose of muscimol employed, was statistically ($p < 0.01$) significant. In contrast to the shape of the dose-response curve obtained in naïve rats, epidural administration of muscimol in inflamed rats resulted in a “U-shaped” dose response curve, characterized by no detectable influence of 0.01 μg muscimol on nociceptive threshold, a significant ($p < 0.01$) exacerbation of the inflammation-induced decrease in EF50 with the 0.1 μg dose, and a significant increase in nociceptive threshold with the 1 μg dose (Figure 2B). While the concentrations of compounds used in the present study were used because they were below those previously shown to produce motor effects (i.e., 15), the roto-rod test was used to assess the influence of motor effects on the response to the highest dose of muscimol used in inflamed rats. The latency to drop from the rod was 151 ± 21 seconds ($n = 4$) and 140 ± 23 seconds ($n = 4$) for saline and muscimol (1 μg/20 μl) treated rats ($p > 0.05$, students t-test). The mean latency to drop following spinal injections was also not significantly ($p > 0.05$, paired t-test) different than baseline values obtained 2 hours prior to spinal injections: baseline values were 123 ± 26 seconds and 171 ± 5 seconds for saline and muscimol injected rats, respectively.

Inflammation-induced changes in GABA-A receptor signaling were next assessed with the GABA-A receptor antagonist, gabazine. In naïve rats, dose-dependent changes in EF50 were bidirectional (Figure 3A). Low concentrations (0.15 to 6.6 ng) of the antagonist were associated with a small but significant ($p < 0.01$) increase in EF50, while higher concentrations (.15 to 3.7 μg) were associated with a large ($p < 0.01$) decrease in EF50. In contrast, in CFA inflamed rats, gabazine was not only associated with a dose-dependent inhibition of the CFA-induced decrease in EF50, but the induction of analgesia (Figure 3B).

Because there is clinical evidence that spinal administration of midazolam, a benzodiazepine agonist, has analgesic efficacy in the presence of chronic pain 28, the impact of inflammation on GABA-A receptor signaling was further assessed with midazolam (Figure 4A). In naïve rats, spinal administration of midazolam was associated with a dose-dependent increase in EF50 which was significant ($p < 0.01$) at the lowest dose tested (6 ng/20 μl). Unlike the inflammation-induced change in the effects of muscimol and gabazine, however, spinal administration of midazolam to CFA inflamed rats was still associated with a dose-dependent increase in EF50. There appeared to be some loss of potency, however, because the lowest dose of midazolam (6 ng) had no detectable influence on EF50. Nevertheless, the 20 ng dose was associated with a significant reversal of hyperalgesia and the 60 ng dose was associated with analgesia (Figure 4B).

Because there is evidence that midazolam is an agonist for both central (i.e., on the GABA-A receptor 13;17) and peripheral 39 benzodiazepine receptors, we sought to determine which of these receptor subtypes was responsible for the antinociceptive effects observed following spinal administration of this compound. Selective antagonists of both peripheral (PK11195, 39) and central (flumazenil 13) benzodiazepine receptors were used for this purpose.

Experiments were run in a 2 × 2 design with one arm of the design with or without antagonist and the other arm of the design with or without midazolam. A dose of 20 ng midazolam was used in these studies because it produced submaximal effects both in the presence and absence of inflammation. Two doses of PK11195 were employed based on conflicting results in the literature 13. The effects of neither 1.1 μg nor 7.1 μg PK11195 were significantly different from vehicle in naïve rats (Figure 5A). Furthermore, neither dose antagonized the analgesic effects of midazolam (Figure 5A). In the presence of persistent inflammation, the combination of PK11195 (1.1 μg) and midazolam resulted in a significantly ($p < 0.05$) greater increase in nociceptive threshold than that induced by midazolam alone (Figure 5B).

Flumazenil (12 ng) alone also had no significant influence on nociceptive threshold in either the presence or absence of inflammation (Figure 6A and B). Furthermore, the combination of midazolam and flumazenil resulted in a reduction in the antinociceptive effects of midazolam both in the presence and absence of inflammation (Figure 6A and B, although the reduction in threshold in the absence of inflammation was not statistically significant with $p = 0.06$). Of note, the incomplete block of midazolam effects by flumazenil is consistent with binding affinity data suggesting that the affinity of both compounds for γ2 subunit containing GABA-A receptors, is similar 4. Taken together, these results suggest a central benzodiazepine receptor mediates the antinociceptive effects of midazolam.

Discussion

In the present study, we observed the following: 1) in naïve rats, spinal administration of muscimol dose-dependently decreased mechanical sensitivity while the dose-response for gabazine was associated with a decrease in mechanical sensitivity at low doses and an increase in mechanical sensitivity at higher doses; 2) in the presence of inflammation, spinal administration of low doses of muscimol exacerbated the inflammation-induced increase in mechanical sensitivity while spinal administration of gabazine dose-dependently decreased mechanical sensitivity; 3) spinal administration of midazolam dose-dependently decreased mechanical sensitivity both in the presence and absence of inflammation. 4) Central but not peripheral benzodiazepine receptor antagonists blocked midazolam effects.

Results with GABA-A receptor agonists and antagonists in naïve rats were consistent with previous electrophysiological 1;25 and behavioral results 9;29;36. However, there were a number of lines of evidence suggesting that a shift in GABA-A signaling observed in the present study would contribute to inflammatory hypersensitivity. First, intense noxious stimuli, such as that associated with an intradermal injection of capsaicin 19;32 or frank inflammation 35 result in an increase in dorsal root reflex activity in Aδ- and C-fibers. This activity appears to play a critical role in the neurogenic component of an inflammatory response 11;19;35. Importantly, this increase in activity is blocked by GABA-A receptor antagonists 19;35 and increased by manipulations leading to an increase in spinal GABA 32. Furthermore, pre-treatment with GABA-A receptor antagonists attenuates the swelling and hyperalgesia that develops in response to a peripheral injection of kaolin and carrageenan 35. While an increase in peripheral inflammation may by associated with an increase in pain and hyperalgesia, more relevant to the results of the present study is the possibility that dorsal root reflex activity in Aδ- and C-fibers may be associated with an increased input to spinal dorsal horn neurons 6. Second, sensitization of nociceptive specific dorsal horn neurons can be reversed with GABA-A receptor antagonists 12. Third, there is at least one report supporting the suggestion that inflammatory allodynia reflects a GABA-A receptor dependent activation of nociceptive afferents by low-threshold Aβ-fibers 11; although other investigators have failed to detect evidence in support of such a coupling between low-threshold and nociceptive afferents 32. Interestingly, the analgesia resulting from the spinal administration of gabazine in the presence of persistent inflammation suggests that there is not just an emergence of a role for GABA-A

signaling in inflammatory hyperalgesia, but rather, in the presence of inflammation, GABA-A receptor signaling appears to become a critical component of nociceptive signaling.

Our results with midazolam both in the presence and absence of inflammation are consistent with previous clinical 28 and pre-clinical 13;16;37 observations suggesting that this compound is analgesic both in the presence 16;28 and absence 13 of tissue injury. Recent evidence obtained with point-mutated knock-in mice suggests this analgesic efficacy is mediated via α2 and/or α3 subunit containing receptors 16. Our results with peripheral and central benzodiazepine receptor antagonists are consistent with these recent results and the suggestion that the antinociceptive effects of midazolam are mediated by central, but not peripheral benzodiazepine receptors both in the presence and absence of inflammation.

There is compelling evidence to suggest that injury-induced changes in GABA-A receptor signaling involve a depolarizing shift in the Cl^- equilibrium potential (E_{Cl}) 22. Such a change has been demonstrated most clearly in spinal dorsal horn neurons following peripheral nerve injury 7. The result is that GABA-A receptor activation may drive membrane depolarization and even spiking 7. While a similar mechanism could contribute to inflammation-induced changes in GABA-A receptor signaling 10, the changes in Cl^- co-transporter expression described 20 were not necessarily predictive of an increase in nociceptive signaling. Furthermore, our results with midazolam suggest that a change in E_{Cl} alone, is insufficient to account for the inflammation-induced shift in the actions of muscimol and gabazine. If midazolam is acting through a central benzodiazepine receptor, then it should facilitate GABA-A mediated neurotransmission. Thus, in the face of an inflammation-induced depolarization of E_{Cl}, midazolam should have exacerbated hyperalgesia. This is clearly not what was observed suggesting that there are at least two changes in GABA-A receptor signaling in the spinal cord in the presence of inflammation.

The observation that the antinociceptive actions of benzodiazepines contain α2 and/or α3 subunits and that both subunits are present in primary afferent and dorsal horn neurons 16 could be used to support the suggestion that pro- and anti-nociceptive effects of GABA-receptor activation are mediated by different populations of neurons. However, another possibility is that GABA-A receptor subtypes are differentially coupled to local changes in E_{Cl}. The benzodiazepine receptor is formed between α and γ subunits 34, and γ subunit containing GABA-A receptors are generally associated with synapses 27. Consequently, in the absence of a change in E_{Cl} at the synapse in the presence of inflammation, midazolam would retain its antinociceptive efficacy. The implication of this line of reasoning is that a depolarizing shift in E_{Cl} around extrasynaptic receptors would have to underlie the pro-nociceptive actions of GABA-A receptor activation observed in the presence of inflammation. Consistent with this suggestion, recent evidence suggests that the potency of muscimol may be higher at extrasynaptic GABA-A receptors 38, which would account for the “U-shaped” dose response curve obtained with muscimol in the presence of inflammation: low doses of the agonist preferentially activated the pro-nociceptive extrasynaptic receptors, while higher concentrations ultimately activated the antinociceptive synaptic receptors.

In summary, we have described inflammation induced changes in the impact of spinally administered gabazine and muscimol, but not midazolam, on mechanical nociceptive threshold. Taken together, these observations suggest that inflammation-induced changes in spinal GABA-A signaling must involve more than simple shifts in E_{Cl} and/or sensitization of central terminals of nociceptive afferents. Importantly, rectifying the changes in GABA-A signaling may provide effective relief from hypersensitivity observed in the presence of inflammation.

Abbreviations

CFA, complete Freund's Adjuvant; EF50, measure of mechanical threshold represented by the force effective in producing a withdrawal response 50% of the time it is applied; GABA, gamma-aminobutyric acid; GABA-A, the "A" subtype of the GABA receptor.

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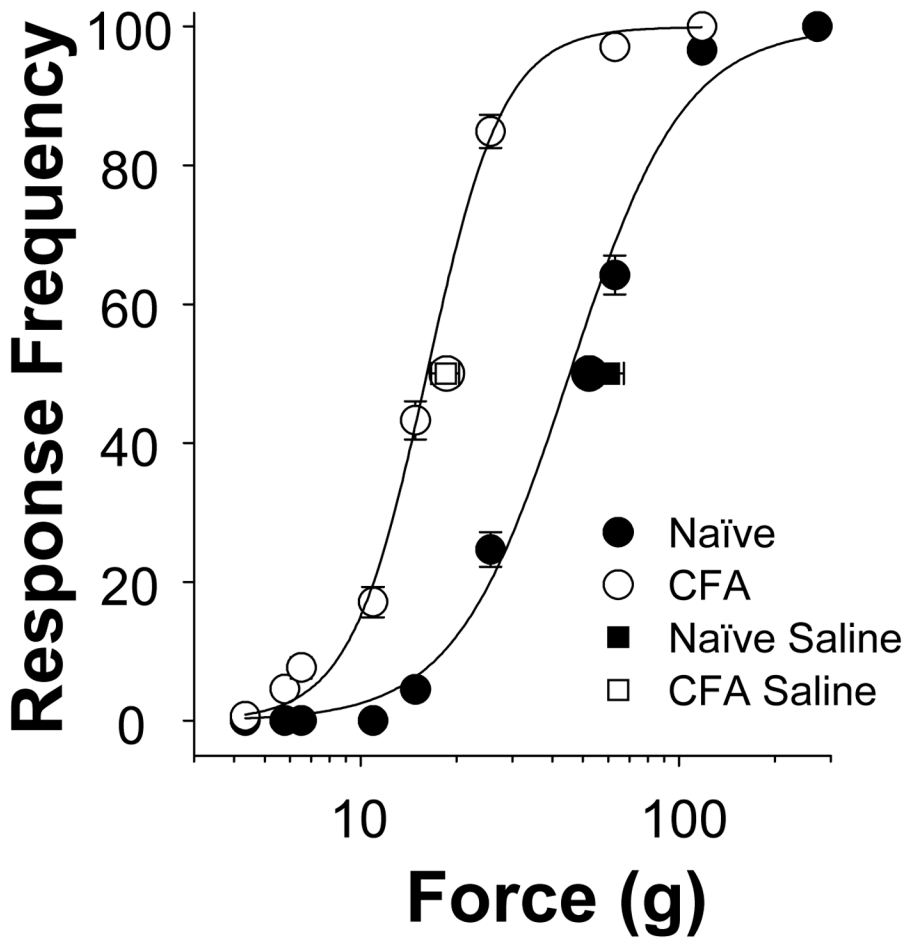

Figure 1.
Stimulus–response functions obtained from naïve rats (open symbols) and from rats (closed symbols) in which inflammation of the hindpaw had been induced with an injection of CFA, 3 days before nociceptive testing: Data are mean ± SEM. The mean fitted EF50 from naïve and CFA rats is plotted in larger symbols. The mean EF50 from naïve and CFA rats after spinal saline injection (squares) is also plotted for comparison. Each rat was stimulated with an ascending series of von Frey filaments and the data from rats in each group were pooled. Note that inflammation was associated with a leftward shift in stimulus response function while spinal administration of saline had little effect in either CFA inflamed or naïve rats. The number of rats studied in each group was 129, 123, 18 and 15 for Baseline Naïve, Baseline CFA, Saline Naïve and Saline CFA, respectively.

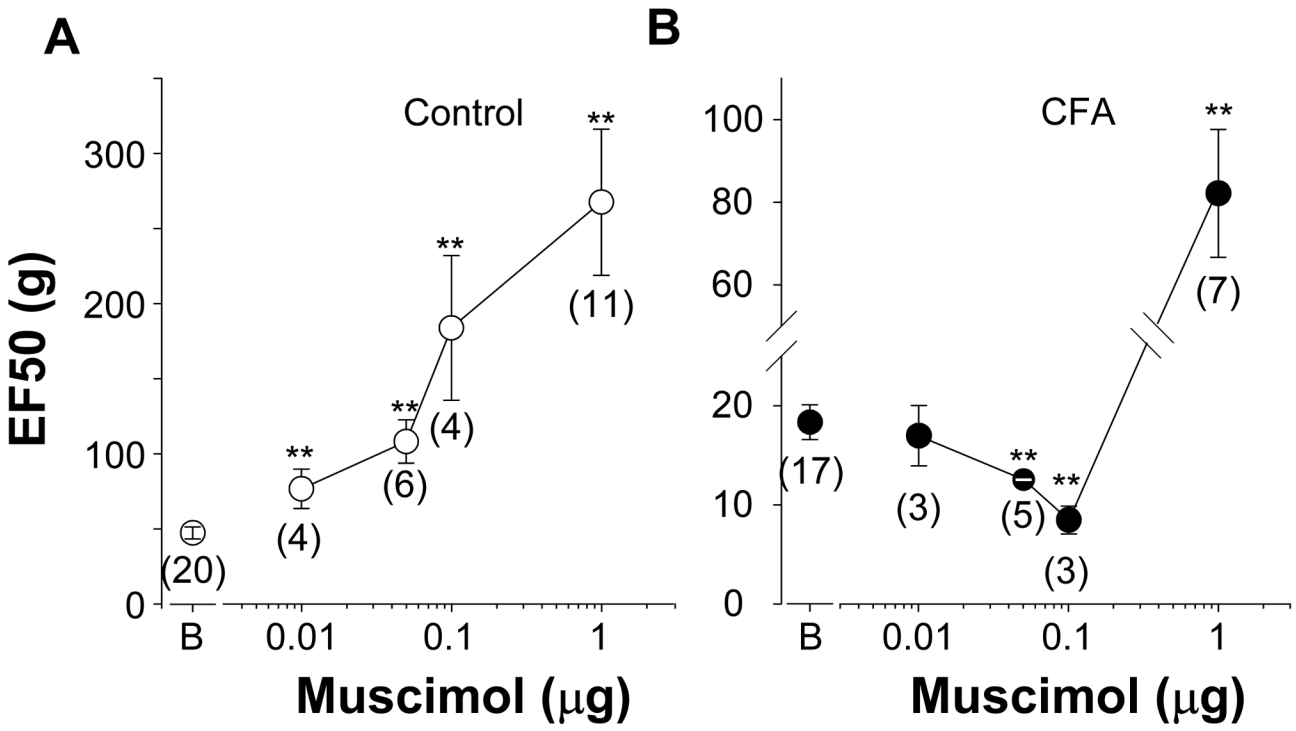

Figure 2.

A. Spinal injection of the GABA-A receptor agonist, muscimol in naïve rats resulted in a dose dependent increase in EF50 which was significant at the lowest dose tested (ANOVA, $p<0.01$). Baseline data (B) for the group of naïve rats treated with muscimol, collected prior to the injection of muscimol are plotted for comparison. **B**. 72 hrs after the induction of inflammation with CFA, spinal muscimol resulted in a "u-shaped" dose response curve reflecting a significant decrease in EF50 at 0.1 μg and an increase in EF50 at 1 μg. Baseline data (B) for the group of inflamed rats treated with muscimol, collected prior to the injection of muscimol are plotted for comparison. The number of rats pooled for each data point is indicated in parentheses. Error bars are smaller than the symbols for several data points. ** is $p < 0.01$, Holm-Sidak post-hoc test.

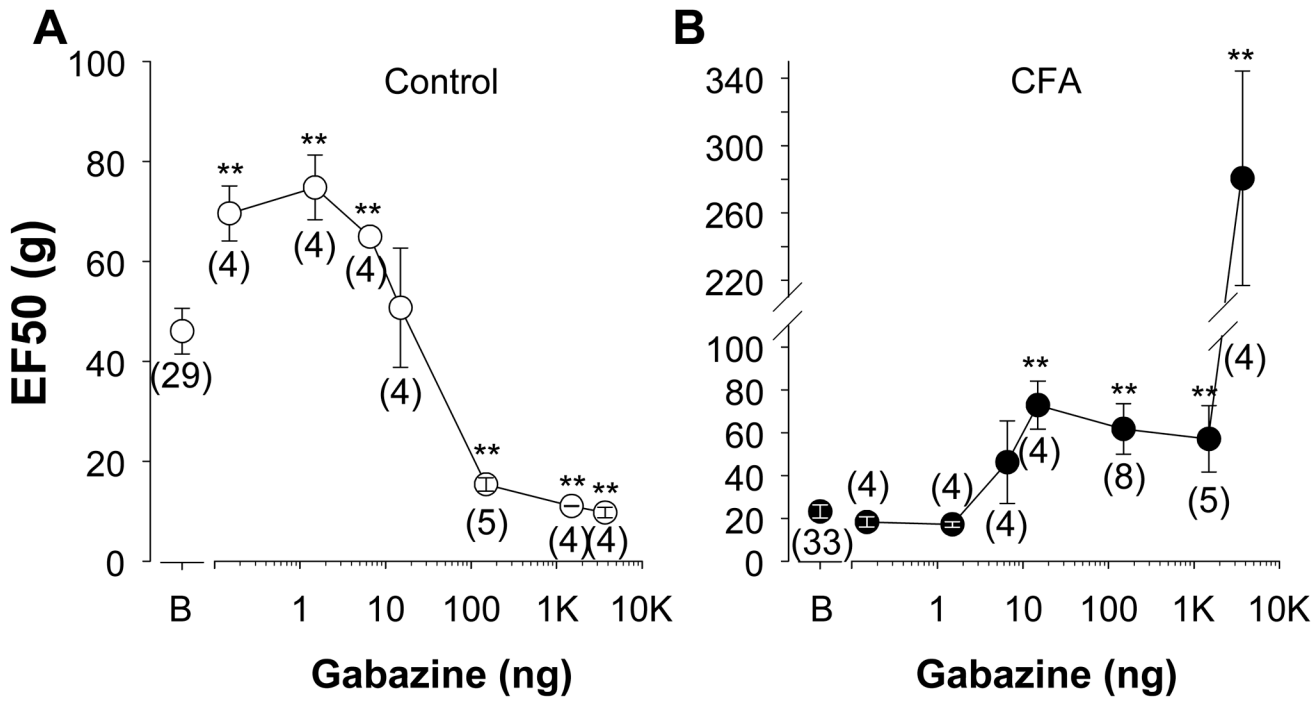

Figure 3.

A. Spinal injection of the GABA-A receptor antagonist, gabazine, in naïve rats resulted in a bi-modal dose-response curve which was associated with an elevation in the EF50 at low doses and a decrease in EF50 at higher doses. Baseline data (B) for the group of naïve rats treated with gabazine, collected prior to the injection of muscimol are plotted for comparison. **B**. Spinal injection of gabazine 72 hrs after CFA-induced inflammation resulted in a significant increase in EF50 (ANOVA; $p<0.01$). Baseline data (B) for the group of inflamed rats treated with gabazine, collected prior to the injection of muscimol are plotted for comparison. Note, data are plotted on different Y-axis scales in order to more clearly illustrate changes in EF50. The number of rats pooled for each data point is indicated in parentheses. Error bars are smaller than the symbols for several data points. ** is $p < 0.01$, Holm-Sidak post-hoc test.

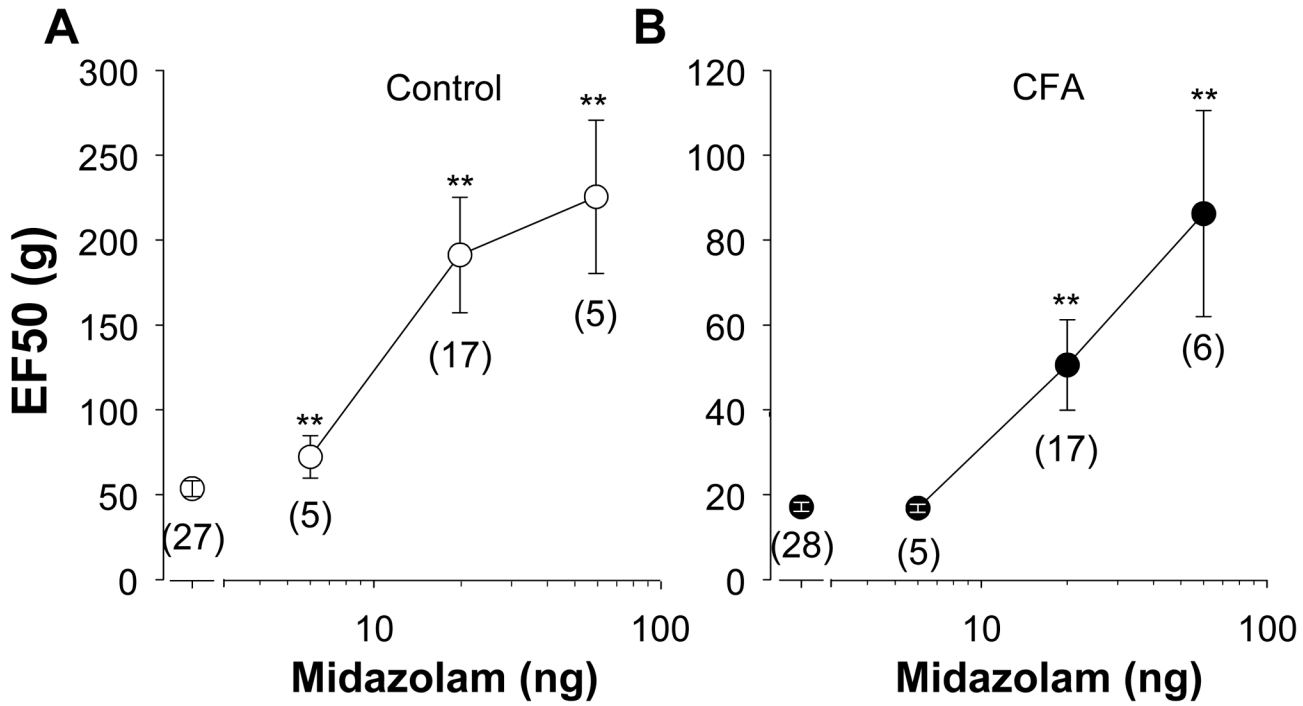

Figure 4.

A. Spinal injection of the benzodiazepine agonist, midazolam, in naïve rats resulted in a dose-dependent increase in EF50, which was significant at 6 ng, the lowest concentration tested (ANOVA; $p<0.05$). Baseline data (B) for the group of naïve rats treated with midazolam, collected prior to the injection of muscimol are plotted for comparison. **B**. Spinal injection of midazolam 72 hrs after CFA-induced inflammation resulted also in a significant increase in EF50 (ANOVA; $p<0.5$). Baseline data (B) for the group of inflamed rats treated with midazolam, collected prior to the injection of muscimol are plotted for comparison. Data are plotted on different Y-axis scales in order to more clearly illustrate changes in EF50. The number of rats pooled for each data point is indicated in parentheses. Error bars are smaller than the symbol for one data point. ** is $p < 0.01$, Holm-Sidak post-hoc test.

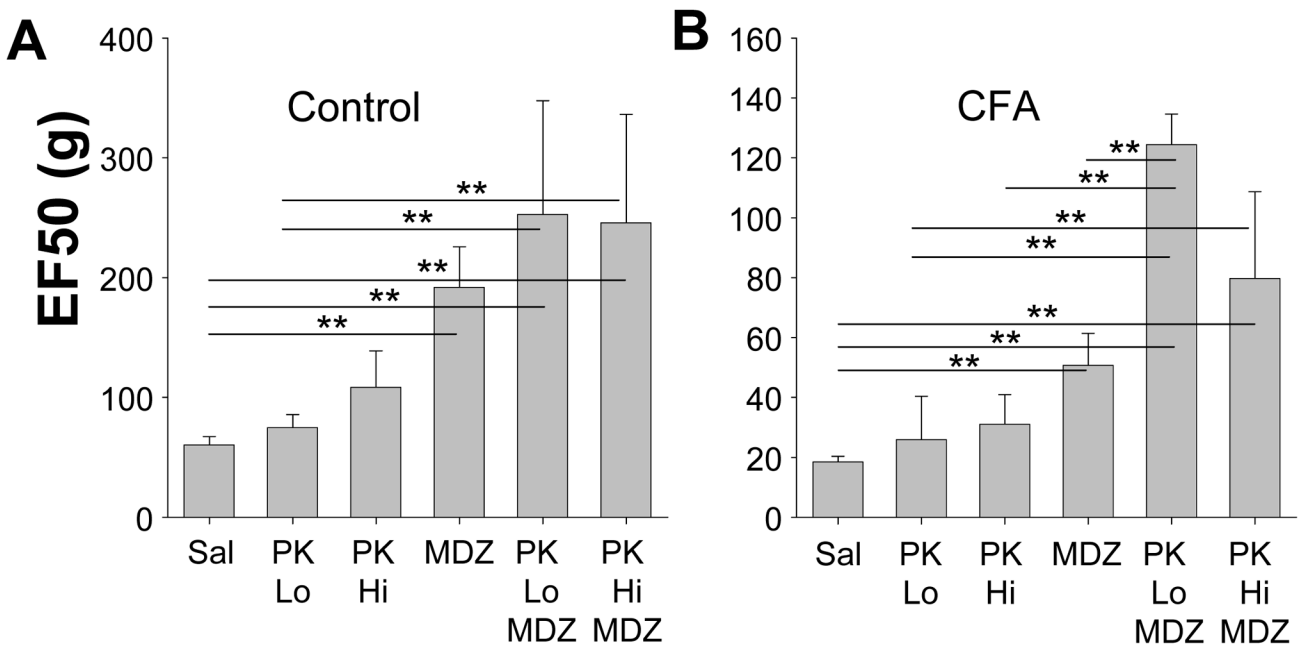

Figure 5.

A. Spinal injections of peripheral benzodiazepine receptor antagonist, PK11195 (PK where PK Lo is 1.1 μg (n = 4) and PK Hi is 7.1 μg (n = 4)), in naïve rats did not produce a shift in the EF50 relative to saline (sal) injected rats (n = 18). The combination of either 1.1 μg (n = 5) or 7.1 μg (n = 4) PK11195 with 20 ng midazolam (MDZ) resulted in an increase in EF50 that was not significantly different than that induced by 20 ng midazolam alone (17). **B**. 72 hours after CFA injection neither 1.1 μg (PK Lo, n = 3) nor 7.1 μg (PK Hi, n = 3) attenuated the increase in nociceptive threshold induced by 20 ng midazolam. Rather, this combination resulted in a further increase in nociceptive threshold that was significantly larger at the 1.1 μg dose than midazolam alone (n = 17). When given alone, neither the 1.1 μg dose (n = 4) nor the 7.1 μg dose (n = 3) had effects different from saline (n = 21). The start and end of each horizontal bar indicates groups that are significantly different based on post-hoc (Holm-Sidak) comparisons. ** is $p < 0.01$.

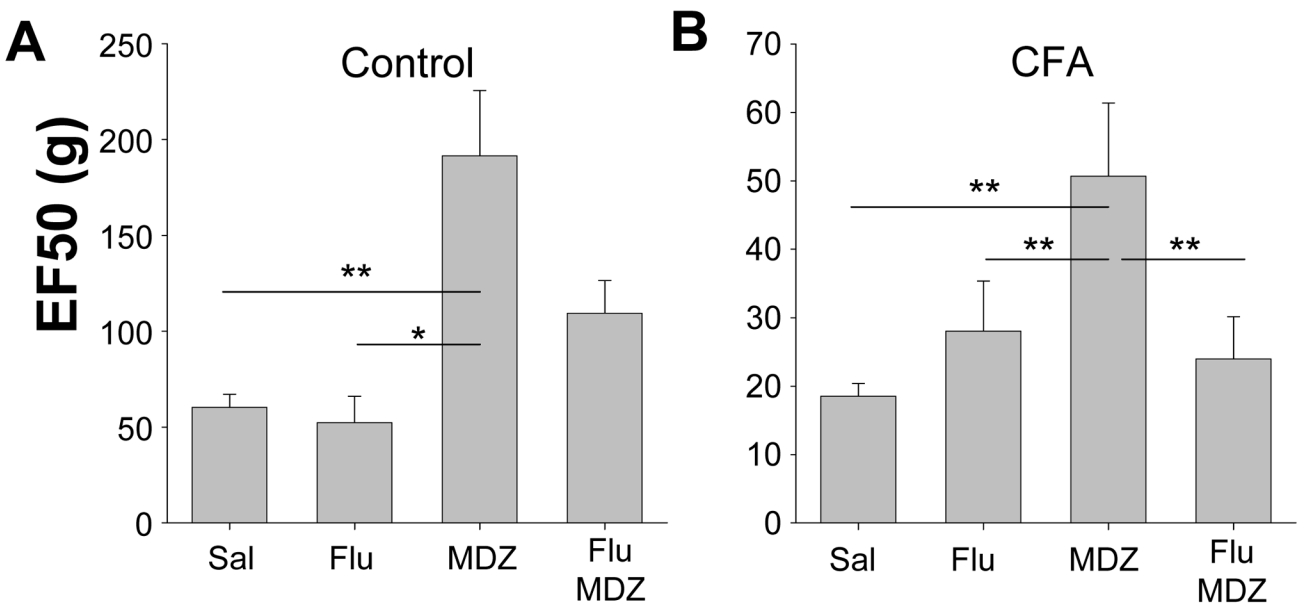

Figure 6.

A. In naïve rats, spinal injection of the central benzodiazepine receptor antagonist, flumazenil (12 ng, Flu, n = 6) alone, or in combination with midazolam (20 ng, n = 6) had an influence on EF50 that was not significantly different from that produced by saline (Sal, n = 18). The increase in EF50 induced by midazolam alone (20 ng, n = 17) was significantly larger than that induced by saline or flumazenil alone. **D**. 72 hours following CFA injection, spinal injection of flumazenil (12 ng) with midazolam (Flu/MDZ, n = 6) significantly attenuated the increase in EF50 associated with midazolam (20 ng) alone (n = 17). Flumazenil (12 ng) alone (n = 7) had an effect on EF50 that was not significantly different from that produced by saline (n = 18). The start and end of each horizontal bar indicates groups that are significantly different based on post-hoc (Holm-Sidak) comparisons. * = $p < 0.05$, ** = $p < 0.01$.