Published in final edited form as:

Methods Find Exp Clin Pharmacol. 2007 December ; 29(10): 659–664. doi:10.1358/mf.2007.29.10.1147766.

Differential Anesthetic Activity of Ketamine and the GABAergic Neurosteroid Allopregnanolone in Mice Lacking Progesterone Receptor A and B subtypes

Doodipala S. Reddy* and Yong-Chun Zeng

Department of Molecular Biomedical Sciences, North Carolina State University College of Veterinary Medicine, Raleigh, NC 27606, USA.

Abstract

SUMMARY—Progesterone affects the function of the brain by multiple mechanisms. The physiological effects of progesterone are mediated by interaction of the hormone with progesterone receptors (PRs), which are widely expressed in the hypothalamus, hippocampus and limbic areas. The PR is composed of two protein isoforms, PR-A and PR-B, which are expressed from a single PR gene. In addition, progesterone influences neuronal activity through its conversion to allopregnanolone, a neurosteroid that acts as a positive allosteric modulator of GABA_A receptors. However, the role of PRs in the sedative-hypnotic action of neurosteroids is unclear. In this study, PR knockout (PRKO) mice were used as model to study the sedative-anesthetic actions of the progesterone-derived neurosteroid allopregnanolone and the non-competitive NMDA receptor antagonist ketamine. Mice were confirmed to be PR deficient by genotyping and immunohistochemistry of PR expression in the brain. Anesthetic potency was evaluated by the loss of the righting reflex paradigm. Allopregnanolone induced anesthetic activity was similar in PRKO mice and their wild-type littermates, suggesting that PRs are not involved in the anesthetic response to allopregnanolone. However, the non-competitive NMDA receptor antagonist ketamine has significantly reduced anesthetic potency in PRKO mice, suggesting a possible developmental plasticity of glutamate receptors. There was no marked gender-related difference to ketamine response in both genotypes. In conclusion, these results suggest that the neurosteroid allopregnanolone and ketamine produce differential anesthetic response in mice lacking PRs.

Keywords

Allopregnanolone; Anesthesia; Neurosteroid; Progesterone receptor

INTRODUCTION

Progesterone and progesterone-derived neurosteroids are endogenous neuromodulators with sedative, anxiolytic, and anesthetic activity (1-6). However, the molecular mechanisms underlying the central nervous system effects of progesterone remain unclear. Progesterone affects the function of the brain by at least two distinct mechanisms: binding to intracellular steroid receptors and metabolism to 5 α -reduced neurosteroids. The physiological effects of progesterone are mediated by intracellular progesterone receptors (PRs), which are nuclear transcription factors that mediate the action of progesterone in target cells (7,8). PRs are

*Address for correspondence: Doodipala S. Reddy, Ph.D. Department of Molecular Biomedical Sciences North Carolina State University College of Veterinary Medicine 4700 Hillsborough Street Raleigh, NC 27606 Tel: 919-513-6549 Fax: 919-513-6465 E-mail: samba_reddy@ncsu.edu.
Current address: Y-C Zeng, MPI Research Inc., Mattawan, Michigan, USA.

expressed in many brain regions in females including the hypothalamus, hippocampus, neocortex, and limbic areas (9-12). The PR is composed of two protein isoforms, PR-A and PR-B subtypes that are expressed from the same PR gene. In addition, current experimental evidence indicates that progesterone influences neuronal activity through its conversion to allopregnanolone, a neurosteroid that acts as a positive allosteric modulator of GABA_A receptors (13-16). However, the extent to which the sedative-hypnotic action of progesterone and allopregnanolone requires PRs is unclear.

To address the role of PRs in mediating the neuroendocrine effects of progesterone, we have used gene-targeting approaches to ablate the expression of PRs (7,17). Recently we have shown that the anxiolytic and anesthetic effects of progesterone occur as a result of its conversion to allopregnanolone (18,19). Using pharmacological and genetic approaches, we characterized the progesterone-induced anesthetic responses in mice lacking PRs (19), supporting the notion that the sedative-hypnotic actions of progesterone are not mediated by its interaction with PRs. Although PRs are expressed in many brain regions in males, the exact function of these PRs is unclear. Moreover, there are significant gender-related differences in the antiseizure activity of progesterone and allopregnanolone in female and male mice (17). Consistent with its allosteric activity at GABA_A receptors, allopregnanolone induces sleep in a benzodiazepine-like fashion (20,21). Although allopregnanolone binds to PRs with very low affinity, it may indirectly affect PRs by intracellular oxidation to 5 α -dihydroprogesterone, a moderately potent PR agonist (22,23).

In this study, PR knockout (PRKO) mice were used as a model to study the anesthetic actions of the neurosteroid allopregnanolone and the noncompetitive *N*-methyl-*D*-aspartate (NMDA) type glutamate receptor antagonist ketamine. The PRKO mouse model was developed by a targeted null mutation of the PR gene that abrogates the function of both PR-A and PR-B subtypes (7,17). Our results confirm that the PRs are not directly involved in the anesthetic activity of allopregnanolone and the PRKO mice had relative insensitivity to ketamine anesthesia.

MATERIALS AND METHODS

Animals

Adult female and male PRKO and wild-type (WT) mice (25–35 g) were used in the study. The development of PRKO mice has been described previously (7,17). WT and PRKO mice with a C57BL6/129SvEv hybrid background were housed separately, four to a cage, with food and water available *ad libitum*. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Genotyping

Each mouse was identified for the PR genotype using a validated protocol as described previously (18). Briefly, genomic DNA from mouse tails was extracted by the phenol/chloroform method and 1 μ l of DNA was subjected to PCR amplification using *taq* DNA polymerase (Invitrogen, Inc). PCR was performed by denaturing the DNA at 94 °C for 5 min, followed by 37 cycles of amplification: 94 °C for 1 min, 57 °C for 1 min, 72 °C for 1.5 min, and a final extension step at 72 °C for 10 min. The following PR-specific primers were used: P1 (5'-TAG ACA GTG TCT TAG ACT CGT TGT TG-3'), P3 (5'-GAT GGG CAC ATG GAT GAA ATC-3'), and a *neo* gene-specific primer, N2 (5'-GCA TGC TCC AGA CTG CCT TGG GAA A-3'). The presence of primer amplified PCR product was detected by agarose gels and visualized by ethidium bromide fluorescence using a DNA ladder of 100 bp as marker (MBI Fermentas, Inc).

Immunohistochemistry

An affinity purified anti-PR polyclonal antibody (DakoCytomation) was used for immunohistochemical identification of PR expression in the brain as described previously (18).

Loss of the righting reflex

Anesthetic effect of ketamine was evaluated using the loss of the righting reflex (LORR) method (13,19). Mice were given an intraperitoneal injection of ketamine (30–150 mg/kg) or subcutaneous injection of allopregnanolone (10–50 mg/kg) and placed on their backs. The time interval between loss and return of the righting reflex was then recorded in WT and PRKO mice. Animals were judged to have regained the reflex when they could right themselves three times within a 30-second interval. Each mouse was used only once in the LORR experiment. Thus, each group of animals received a single dose of either ketamine or allopregnanolone. Control group received vehicle (20% β -cyclodextrin solution). In the construction of dose-response curves, at least 6 mice were used at each dose point. Body temperature was not monitored and it's not clear if PR affects body temperature. After recording the onset and duration of LORR response, animals were promptly euthanized.

Drugs

Stock solutions of allopregnanolone was made in 20% hydroxypropyl- β -cyclodextrin (β -cyclodextrin) in water, and additional dilutions were made using normal saline. Ketamine was procured from Phoenix Pharmaceuticals (St. Joseph, MO). This product consists of a racemic mixture of ketamine. Drug solutions were administered s.c. or i.p. in a volume equaling 1% of the animal's body weight.

Data analysis

The effect of ketamine and allopregnanolone on the latency or duration of LORR response was evaluated in a dose-dependent fashion. The percent of LORR occurrence at each dose point was calculated from group of 6–8 mice. ED₅₀ values (the doses at which 50% of tested animals lost LORR) with 95% confidence limits were determined by log-probit analysis using the Litchfield and Wilcoxon procedure. In the construction of dose-response curves, at least 6 animals were tested at each dose. The significance of differences between ketamine dose-response curves in the percent LORR occurrence was assessed with the Litchfield and Wilcoxon X² test. Group comparisons in the percentage of animals exhibiting LORR response were made with the Fisher's exact probability test. To determine if PRKO mice had different latency or duration measures than WT mice, mean latency of LORR, and duration of LORR responses were analyzed by twoway ANOVA with genotype x dose or treatment as two variables, followed by Dunnett's *t*-test. In all tests, the criterion for statistical significance was $p < 0.05$.

RESULTS

Genotyping and PR expression in the brain

Genotyping and PR expression in the brain was confirmed by a validated protocol (18). For PR genotyping, genomic DNA was isolated from tails and analyzed by PCR procedure. The presence of primer amplified PCR product was detected by agarose gels and visualized by ethidium bromide fluorescence. We note the presence of 600 bp DNA band for wild-type (WT) (*P1/P3* primer mix), 480 bp band for homozygous (*P1/N2* primer mix) or both for mice heterozygous (Het) for PR gene. Consistent with PR genotyping, PR immunoreactivity was undetectable in the brains of PRKO mice, confirming the absence of PR expression in the brain section from PRKO mice (not shown).

Undiminished anesthetic activity of allopregnanolone in PRKO mice

To test the premise that the anesthetic activity of progesterone-derived neurosteroid allopregnanolone occurs independently of its actions via PRs, we examined the ability of allopregnanolone to induce anesthetic response in PRKO mice. The anesthetic activity of allopregnanolone was assessed by the LORR behavioral assay in female WT and PRKO mice. Allopregnanolone administration (10–50 mg/kg, s.c.) elicited a dose-dependent LORR response in WT mice (Fig. 1A). Allopregnanolone also induced LORR response in heterozygous and homozygous PRKO mice. In PRKO mice, the dose-response curve for LORR response of allopregnanolone was not significantly different from that of WT animals (Fig. 1A). The ED₅₀ values derived from these curves are listed in Table 1. The allopregnanolone (10–50 mg/kg)-induced duration of LORR in PRKO and WT mice was shown in Fig. 1B. Two-way ANOVA indicated that there was no significant difference in either latency of the LORR or duration of the LORR response with allopregnanolone in the PRKO mice than in WT mice. The time course for LORR response indicates that allopregnanolone (50 mg/kg) exhibited a slow onset of LORR (12 ± 2 min in WT; 10 ± 2 min in PRKO mice) and sustained duration of LORR (44 ± 4 min in WT; 55 ± 5 min in PRKO mice).

Gender-related differences in the anesthetic activity of allopregnanolone

To determine the potential gender-related differences in the anesthetic activity of allopregnanolone, the steroid was tested in male and female WT and PRKO mice. As shown in Fig. 1C, allopregnanolone (10–50 mg/kg, s.c.) administration induced a dose-dependent LORR occurrence in male and female WT mice. The ED₅₀ values derived from these curves are listed in Table 1. There were no significant gender-related differences in the anesthetic ED₅₀ values of allopregnanolone in WT or PRKO mice (Table 1). The allopregnanolone (10–50 mg/kg)-induced duration of LORR in male mice was similar to that of females (Fig. 1D). Although allopregnanolone appears to be little more potent in females, there was no significant gender-related difference in the anesthetic activity of allopregnanolone (Fig. 1C).

Reduced anesthetic activity of ketamine in PRKO mice

The anesthetic activity of ketamine was assessed by the LORR behavioral assay in WT and PRKO female mice. As shown in Fig. 2A, ketamine (30–150 mg/kg, i.p.) administration induced a dose-dependent LORR occurrence in female WT mice, indicating an anesthetic effect of ketamine. In PRKO mice, the dose-response curve for LORR response of ketamine was significantly ($p < 0.05$) shifted in a parallel manner to the right from that of WT animals (Fig. 2A), indicating that the ketamine induced anesthetic potency was significantly reduced in PRKO mice. The ED₅₀ values derived from these curves are listed in Table 1. Similarly, the duration of LORR response to ketamine (30–100 mg/kg) was significantly shorter in female PRKO mice as compared to WT controls (Fig. 2B). In contrast to allopregnanolone, ketamine (150 mg/kg)-induced latency of LORR was shorter (3.5 ± 1 min in WT; 3 ± 1 min in PRKO mice) and it diminished during the 40 min period after injection in both genotypes (Fig. 2B and 2D).

Gender-related differences in the anesthetic activity of ketamine

To determine the potential gender-related differences in the anesthetic response to ketamine, ketamine was tested in male and female mice belonging to both genotypes. Ketamine (30–150 mg/kg, i.p.) induced a dose-dependent LORR occurrence in male WT and PRKO mice (Fig. 2C). Male PRKO mice also exhibited similar relative insensitivity to ketamine anesthesia, as evident from decreased LORR occurrence (Fig. 2C) and shorter duration of LORR (Fig. 2D). The ED₅₀ values derived from these curves are listed in Table 1. The ketamine-induced LORR response was similar in female and male mice in both genotypes (Fig. 2A and C).

DISCUSSION

The key observation of this study is that the anesthetic activity of ketamine is reduced in PRKO mice, while the neurosteroid allopregnanolone has undiminished anesthetic activity in PRKO mice, which lack both forms of the PR. These results provide direct evidence that PRs appear to play a role in the differential anesthetic actions of neurosteroids and ketamine. In the present study, the anesthetic activity of ketamine was investigated in male and female WT and PRKO mice using the LORR as the behavioral model. However, the relationship between the LORR model to human sleep is not well defined and a variety of endogenous factors could influence the actions of anesthetic agents. Although this is a clear and straightforward study, there are certain caveats to the evidence that allopregnanolone and ketamine produce differential anesthetic responses in PRKO mice. First, the difference that occurred between allopregnanolone and ketamine was less than a two-fold shift. Second, only potency was affected for both drugs, while the efficacy was similar in both WT and PRKO mice. However, this differential response may not be related to the two different routes of drug administration that were utilized in the study.

In previous studies allopregnanolone has been demonstrated to induce sleep in animals and humans comparable to those of agonistic GABA_A receptor modulators (13,16,19,21). The GABA_A receptor is an important target for the neurosteroid allopregnanolone (14), but allopregnanolone is also capable of regulating gene expression via intracellular oxidation to dihydroprogesterone, which is a moderately potent PR agonist (22,23). In the present study, allopregnanolone has similar anesthetic activity in WT and PRKO mice, suggesting that PRs are not directly involved in the anesthetic activity of allopregnanolone. Thus, the anesthetic effects of allopregnanolone could be due to its interaction with GABA_A receptors.

Ketamine inhibits the NMDA type of glutamate receptors via non-competitive antagonism, resulting in dissociative anesthesia in animals. During a standard anesthetic procedure, we unexpectedly found that PRKO mice failed to respond to normal anesthetic doses of ketamine. This has led to further studies of ketamine in a dose-dependent fashion. Our results indicate that ketamine has a significantly reduced anesthetic activity in PRKO mice, indicating a modulatory role of PRs in ketamine actions. However, the mechanism underlying the ketamine insensitivity of PRKO mice is unknown. We believe that reduced ketamine responsiveness of PRKO mice is not related to pharmacokinetic factors because the latency of LORR response to ketamine in PRKO mice is similar to WT mice. This indicates that the reduced potency is attributable to alterations in target receptor function such as the potential developmental plasticity of NMDA type glutamate receptors. Future studies of gene expression profiles in the cortex of PRKO mice using DNA microarray techniques will identify changes in glutamate receptor subunits that could be relevant to ketamine actions.

There is considerable evidence indicating that males and females differ in their sensitivity to seizure stimuli or steroid potency. In animal seizure models, male mice are shown to be more seizure-prone than females. It has previously been observed that female mice and rats are more sensitive to the central nervous system actions of progesterone and allopregnanolone than are males (17,26,28). Moreover, female PRKO animals exhibited greater progesterone sensitivity (17), indicating that known differences in the density or distribution of the PR in the female and male brain are not responsible for the sexual dimorphism. In the present study, the anesthetic effects of ketamine and allopregnanolone are similar in male and female WT and PRKO mice, indicating the lack of gender-related difference in the actions of anesthetic neurosteroids. However, hormonal differences between females and males might still be responsible for gender-related effects of neurosteroids in other models.

In conclusion, the present study shows that ketamine and allopregnanolone produce differential anesthetic activity in PRKO mice, providing direct evidence that PRs may be linked to modulating responses to anesthetic agents. These results strongly confirm that neurosteroids such as allopregnanolone elicit anesthetic effects in mice lacking PR-A and PR-B isoforms.

Acknowledgements

This research was partly supported by Grants from the National Institute of Neurological Disorders and Stroke (R01 NS051398 to D.S.R.).

REFERENCES

1. Bitran D, Shiekh M, McLeod M. Anxiolytic effect of progesterone is mediated by the neurosteroid allopregnanolone at brain GABA_A receptors. *J Neuroendocrinol* 1995;7:171–177. [PubMed: 7606242]
2. Bixo M, Backstrom T. Regional distribution of progesterone and 5 α -pregnane-3,20-dione in rat brain during progesterone-induced anesthesia. *Psychoneuroendocrinology* 1990;15:159–162. [PubMed: 2359812]
3. Friess E, Tagaya H, Trachsel L, Holsboer F, Rupprecht R. Progesterone-induced changes in sleep in male subjects. *Am J Physiol* 1997;272:E885–E891. [PubMed: 9176190]
4. Merryman W, Boiman R, Barnes R, Rothchild I. Progesterone anesthesia in human subjects. *J Clin Endocrinol Met* 1959;14:1567–1569.
5. Reddy DS, Kulkarni SK. Differential anxiolytic effects of neurosteroids in the mirrored chamber behavior test in mice. *Brain Res* 1997;752:61–71. [PubMed: 9106441]
6. Selye H. Anaesthetic effects of steroid hormones. *Proc Soc Exp Biol* 1941;46:116–121.
7. Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Shyamala G, Conneely OM, O'Malley BW. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* 1995;9:2266–2278. [PubMed: 7557380]
8. Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* 2000;289:1751–1754. [PubMed: 10976068]
9. Conneely OM, Mulac-Jericevic B, Lydon JP, De Mayo F. Reproductive functions of the progesterone receptor isoforms: lessons from knock-out mice. *Mol Cell Endocrinol* 2001;179:97–103. [PubMed: 11420134]
10. Levine JE, Chappel PE, Schneider JS, Sleiter NC, Szabo M. Progesterone receptors as neuroendocrine integrators. *Front Neuroendocrinol* 2001;22:69–106. [PubMed: 11259133]
11. Mani SK, Blaustein JD, O'Malley BW. Progesterone receptor function from a behavioral perspective. *Horm Behav* 1997;31:244–55. [PubMed: 9213138]
12. Parsons B, Rainbow TC, MacLusky NJ, McEwen BS. Progesterone receptor levels in rat hypothalamic and limbic nuclei. *J Neurosci* 1982;2:1446–1452. [PubMed: 6889633]
13. Korneyev A, Costa E. Allopregnanolone (THP) mediates anesthetic effects of progesterone in rat brain. *Hormone Bevah* 1996;30:37–43.
14. Lambert JJ, Belelli D, Peden DR, Vardy AW, Peters JA. Neurosteroid modulation of GABA_A receptors. *Prog Neurobiol* 2003;71:67–80. [PubMed: 14611869]
15. Mok WM, Krieger NR. Evidence that 5 α -pregnane-3 α -ol-20-one is the metabolite responsible for progesterone anesthesia. *Brain Res* 1990;533:42–45. [PubMed: 2085732]
16. Soderpalm AH, Lindsey S, Purdy RH, Hauger R, Wit de H. Administration of progesterone produces mild sedative-like effects in men and women. *Psychoneuroendocrinology* 2004;29:339–354. [PubMed: 14644065]
17. Reddy DS, Castaneda DA, O'Malley BW, Rogawski MA. Anticonvulsant activity of progesterone and neurosteroids in progesterone receptor knockout mice. *J Pharmacol Exp Ther* 2004;310:230–239. [PubMed: 14982969]
18. Reddy DS, O'Malley BW, Rogawski MA. Anxiolytic activity of progesterone in progesterone receptor knockout mice. *Neuropharmacology* 2004;48:14–24. [PubMed: 15617723]

19. Reddy DS, Apanites L-A. Anesthetic effects of progesterone are undiminished in progesterone receptor knockout mice. *Brain Res* 2005;1033:96–101. [PubMed: 15680344]
20. Lancel M, Faulhaber J, Holsboer F, Rupprecht R. Progesterone induces changes in sleep comparable to those of agonist GABA_A receptor modulators. *Am J Physiol* 1996;34:E763–E772. [PubMed: 8897866]
21. Lancel M, Faulhaber J, Schifflholz T, Romeo E, Di Michele F, Holsboer F, Rupprecht R. Allopregnanolone affects sleep in a benzodiazepine-like fashion. *J Pharmacol Exp Ther* 1997;282:1213–1218. [PubMed: 9316828]
22. Rupprecht R, Reul JM, Trapp T, van Steensel B, Wetzel C, Damm K, Zieglansberger W, Holsboer F. Progesterone receptor-mediated effects of neuroactive steroids. *Neuron* 1993;11:523–530. [PubMed: 8398145]
23. Rupprecht R, Berning B, Hauser CAE, Holsboer F, Reul JM. Steroid receptor-mediated effects of neuroactive steroids: characterization of structure-activity relationship. *Eur J Pharmacol* 1996;303:227–234. [PubMed: 8813574]
24. Hsu SM, Raine L, Fanger H. The use of antiavidin antibody and avidin-biotin-peroxidase complex in immunoperoxidase technique. *Am J Clin Pathol* 1981;75:816–821. [PubMed: 6167159]
25. Moffatt CA, Rissman EF, Shupnik MA, Blaustein JD. Induction of progesterone receptors by estradiol in the forebrain of estrogen receptor- α gene-disrupted mice. *J Neurosci* 1998;18:9556–9563. [PubMed: 9801392]
26. Holmes GL, Weber DA. The effect of progesterone on kindling: a developmental study. *Brain Res* 1984;318:45–53. [PubMed: 6488053]
27. Finn DA, Gee KW. The estrous cycle, sensitivity to convulsants and the anticonvulsant effect of a neuroactive steroid. *J Pharmacol Exp Ther* 1994;271:164–170. [PubMed: 7965710]
28. Reddy DS, Kulkarni SK. Sex and estrous cycle-dependent changes in neurosteroid and benzodiazepine effects on food consumption and plus-maze learning behaviors in rats. *Pharmacol Biochem Behav* 1999;62:53–60. [PubMed: 9972845]

Fig. 1.

Allopregnanolone-induced anesthetic activity in male and female WT and PRKO mice. **A)** Dose-response relationship of allopregnanolone (10–50 mg/kg, s.c.)-induced LORR in female WT and PRKO mice. **B).** Duration of allopregnanolone-induced LORR in female WT and PRKO mice. **C)** Dose-response relationship of allopregnanolone (10–50 mg/kg, s.c.)-induced LORR occurrence in male and female WT mice. **D)** Duration of allopregnanolone (30–50 mg/kg, s.c.)-induced LORR in male and female WT mice. In upper graphs (A,C), each point represents the data from six to eight animals. In lower graphs (B,D), each point represents mean $\pm$ SEM of values from 6 to 8 mice.

Fig. 2.

Ketamine-induced anesthetic activity in male and female WT and PRKO mice. **A,C**) Dose-response relationship of ketamine (30–150 mg/kg, i.p.)-induced loss of the righting reflex (LORR) in male and female WT and PRKO mice. **B,D**) Duration of ketamine-induced LORR in male and female WT and PRKO mice. In upper graphs (**A,C**), each point represents the data from six to eight mice. In lower graphs (**B,D**), each point represents mean $\pm$ SEM of values from 6 to 8 mice. * $p < 0.05$ vs. WT control group (Dunnett's *t*-test).

TABLE 1Anesthetic ED₅₀ values (mg/kg) of ketamine and allopregnanolone in WT and PRKO mice.

Genotype	Ketamine	Allopregnanolone
Female		
WT (+/+)	64 (44–93)	24 (19–30)
PRKO (–/–)	87 (58–130) [*]	29 (21–40)
Male		
WT (+/+)	63 (50–78)	29 (20–42)
PRKO (–/–)	90 (78–103) [#]	24 (16–32)

Each value represents data from 4–6 groups (n = 6–8 mice per group).

Numbers in parentheses are 95% confidence limits.

[#] p<0.05 vs. WT (Litchfield and Wilcoxon X² test)

^{*} p<0.05 vs. WT at 100 mg/kg dose (Fisher's exact probability test).