

# Effect of AN363, a Novel GABA<sub>A</sub> α 2/3/5 Receptor Subtype Selective Positive Allosteric Modulator in Preclinical Models of Inflammatory and Neuropathic Pain

Dipak V. Amrutkar\*, Philip K. Ahring; Tino Dyhring, Karin S. Nielsen, Thomas A. Jacobsen and Janus S. Larsen. Saniona A/S, Baltorpevej 154, DK-2750 Denmark, \*[dva@saniona.com](mailto:dva@saniona.com)

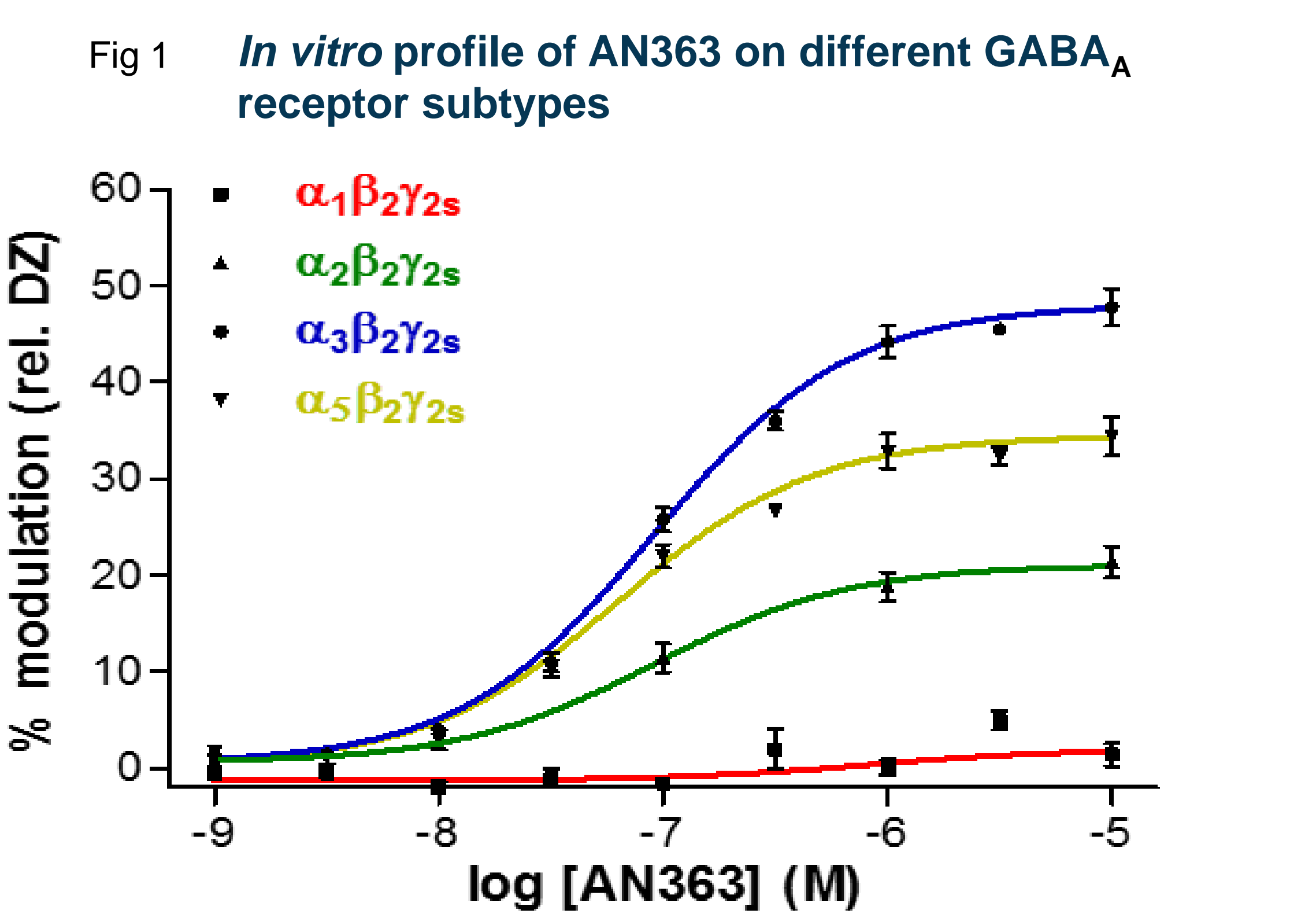
**Introduction:**  
GABA<sub>A</sub> receptors containing α2/3 subunits are current targets in the battle to develop new pain medications, as they are expressed in the spinal cord where increasing inhibitory drive should result in analgesia (1,2). Benzodiazepines, a class of positive allosteric modulators (PAMs) of GABA<sub>A</sub> receptors act through subtypes containing α1, α2, α3 or α5 subunits. Studies using point mutated GABA<sub>A</sub> receptor knock-in mice have shown that the anti-hyperalgesic effect of diazepam in the spinal cord is mediated by GABA<sub>A</sub> receptors containing α2 and α3 subunits, whereas sedative action was attributed to receptors containing α1 subunits (3). Based on this hypothesis, here we report analgesic activity of a novel selective GABA<sub>A</sub> α2/3/5 subtype receptor PAM, AN363 in preclinical models of inflammatory and neuropathic pain

**Conclusion:**  
AN363 is a selective positive modulator at α2, α3 and α5 containing GABA<sub>A</sub> receptors and devoid of activity at the α1 subtype. AN363 displays an attractive pharmacokinetic profile in rats with a long T<sub>1/2</sub> and bioavailability exceeding 80%. It readily penetrates the rat brain producing 82% receptor occupancy after oral administration of 10 mg/kg, resulting in an ED<sub>50</sub> of 4.6±2.8 mg/kg. AN363 shows robust analgesic effects in animal models of acute inflammatory and chronic neuropathic pain. It is highly stable in human hepatocytes with a T<sub>1/2</sub> exceeding 240 min. These properties makes AN363 an attractive clinical candidate and the compound is currently undergoing preclinical development.

**Methods**

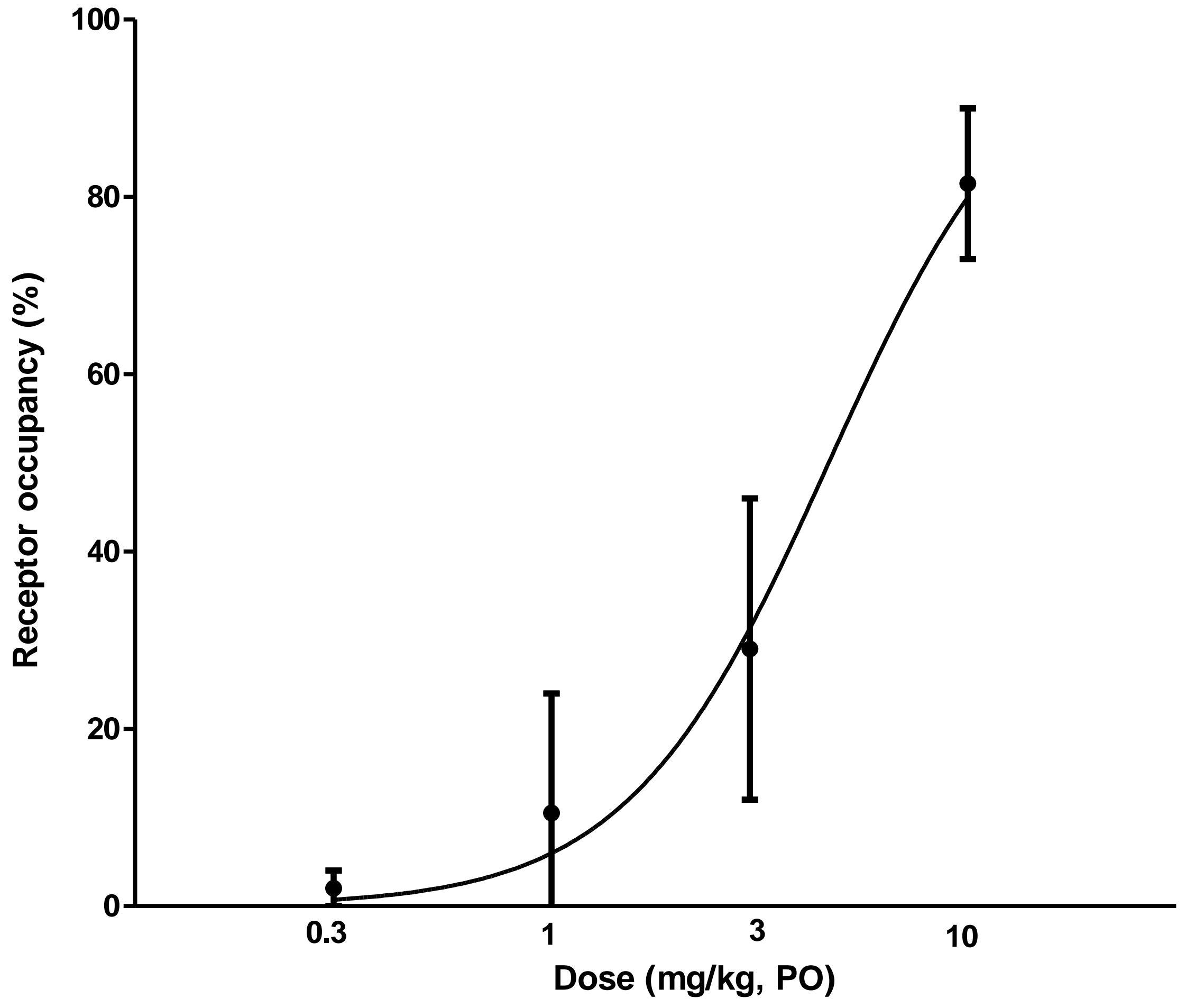
- 1) Electrophysiology:** Electrophysiological responses from *X. laevis* oocytes was measured using the two-electrode voltage clamp technique. For each experimental data set, GABA was dissolved in an oocyte ringer solution in a concentration giving rise to EC<sub>50</sub>-EC<sub>20</sub> elicited currents for a given GABA<sub>A</sub> receptor subtype combination (0.5-5 μM) and this solution was then used for controls as well as stock solution for dissolving the compounds to test in the experiment. Peak currents were read and normalized to a maximal effective concentration of diazepam, whereafter data points were fitted to the empirical Hill equation by non-linear regression. (Fig. 1)
- 2) AN363 stability in hepatocytes:** Performed by Cyprotex Discovery Ltd, UK, using standard protocol. (Table 1)
- 3) Pharmacokinetic parameters:** Sprague-Dawley (SD) male rats were dosed with AN363 at 10 mg/kg, per oral (po). Sampling and analysis was performed using standard methods as described previously (4). (Table 2)
- 4) Rat in vivo binding:** SD Rats were orally dosed with AN363 then after 30 min, 20.0 μCi of <sup>3</sup>H-L-655,708 was injected intravenously. Following 2 min of radioligand administration, rats were decapitated and the forebrain was rapidly excised. Brain tissue was homogenized in ice-cold Tris-citrate buffer. Aliquots were immediately filtered through GF/C glass fibre filters and washed with ice-cold buffer. The amount of radioactivity on the filters was determined by conventional scintillation counting. Groups of vehicle rats served as controls. To determine non-specific binding, groups of rats were administered clonazepam (10 mg/kg) i.p. 28 min before <sup>3</sup>H-L-655,708 injections. (Fig. 2)
- 5) Capsaicin induced flinches:** Capsaicin (1mg/ml) 10μl in 10% ethanol + 10%Tween80-saline, was injected intraplantarly in the left hind paw of SD male rats. Flinches were manually counted up to 30 minutes immediately after capsaicin injection. AN363, NS11394, vehicle and pregabalin were administered 60 minutes before capsaicin. NS11394 and pregabalin were used as reference. (Fig.3)
- 6) Formalin induced flinches:** Formalin (5% in saline) 50 μl, was injected subcutaneously into the dorsal surface of the left hind paw of male SD rats. Flinches were counted using automated nociception analyzer for 60 minutes immediately after formalin injection. AN363/vehicle was administered 60 minutes before formalin injection. (Fig.4)
- 7) Complete Freund's Adjuvant (CFA) induced thermal hyperalgesia :** CFA (1mg/ml) 100 μl, was injected intraplantarly into the left hind paw of male SD rats. Thermal hyperalgesia was developed after 24 h of CFA injection then rats were orally treated with vehicle, AN363 and diclofenac sodium. PWL was measured after 1h of drug/vehicle treatment. PWL was measured using Hargreaves apparatus at 50% IR intensity with cutoff latency of 30 seconds to avoid damage to the tissues. (Fig. 5)
- 8) Chronic constriction injury (CCI):** The Bennett and Xia model of CCI was used. Mechanical allodynia was assessed using von Frey filaments. Pregabalin 30 mg/kg, po was used as a reference. Mechanical allodynia was assessed at 30, 60 and 120 minutes post dose of vehicle, AN363 and pregabalin. Rats showing more than 6g of paw withdrawal threshold (PWT) post CCI were not included in the study and cutoff of 26g force was kept to avoid damage to the tissue. (Fig.6)

**References:**  
1. Munro, G. *et al.* (2008). *J Pharmacol Exp Ther* 327, 961-981  
2. Nickolls, S. *et al.* (2011). *Adv Pharm Sci* 728, 38-49  
3. Knabl, J. *et al.* (2008). *Nature* 451, 330-334  
4. Mirza, N. R. *et al.* (2008). *J Pharmacol Exp Ther* 327, 954-968



Electrophysiological responses from *X. laevis* oocytes was measured using the two-electrode voltage clamp technique. Peak currents were read and normalized to a maximal effective concentration of diazepam (N=9)

Fig 2 Receptor occupancy of AN363 in rat brain *in vivo* binding assay using <sup>3</sup>H-L-655,708



Receptor occupancy of AN363 as measured by displacement of <sup>3</sup>H-L-655,708, in rat brain. AN363 results in 82% receptor occupancy 30 minutes after oral administration of 10 mg/kg to rats, resulting in an ED<sub>50</sub> of 4.6 ± 2.8 mg/kg (n=2)

Table 1 Pharmacokinetic profile of AN363 at 10 mg/kg in rats

%F	T <sub>max</sub> / (h)	C <sub>max</sub> (ng/ml)	AUC(h*ng/ml)	B/P
83	2	2258	10747	0.5

AN363 was administered at 10 mg/kg, orally in 10ml/kg volume to SD male rats. Tongue blood and brain were collected at various time points post AN363 administration and samples were analysed for presence of AN363 (n=4). (B/P= blood/plasma ratio)

Table 2 Hepatocyte stability of AN363 at 5 μM concentration

Species	Left at 1h (%)	T <sub>1/2</sub> (min.)
Human	98.8	>240
Dog	67.1	160
Rat	100	>240

AN363 at 5 μM was incubated with cryopreserved hepatocytes from human, dog and rat. Samples were collected at various time point during incubation and analysed for the presence of AN363 (n=3)

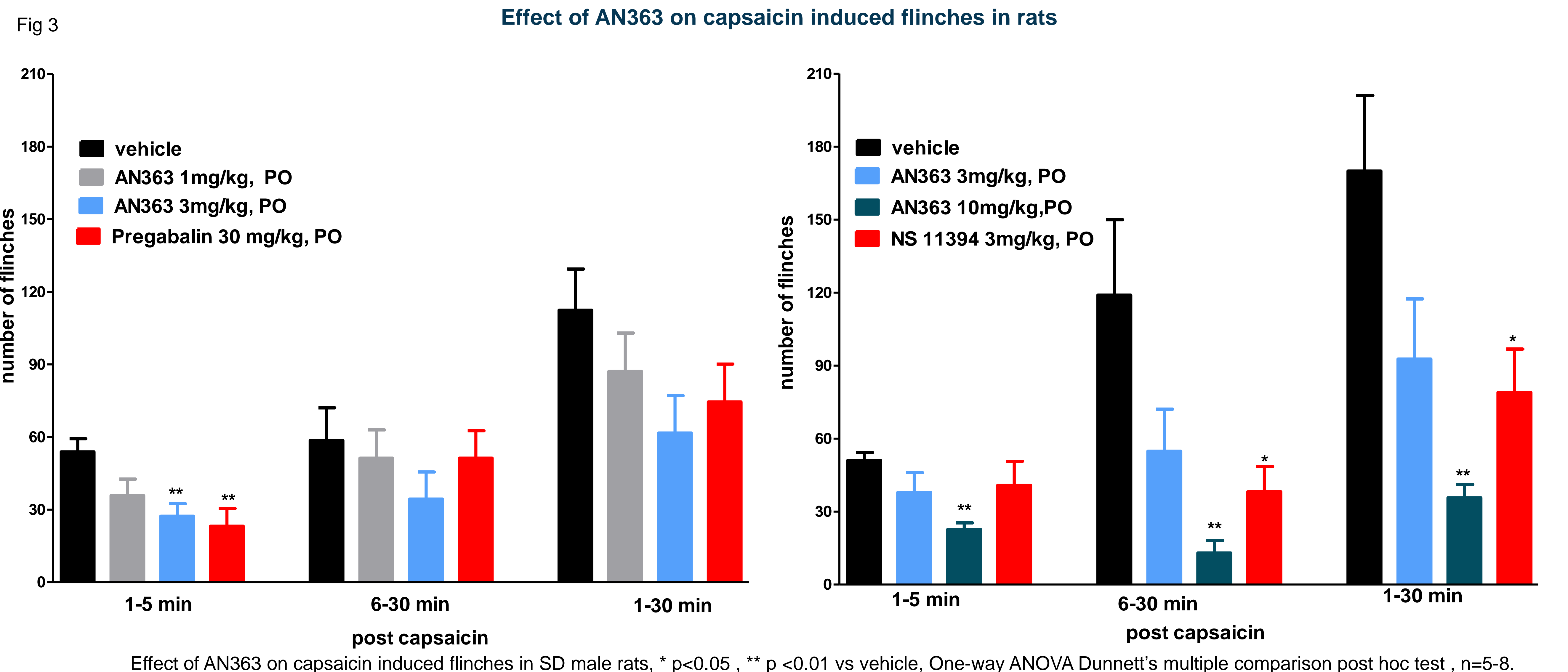
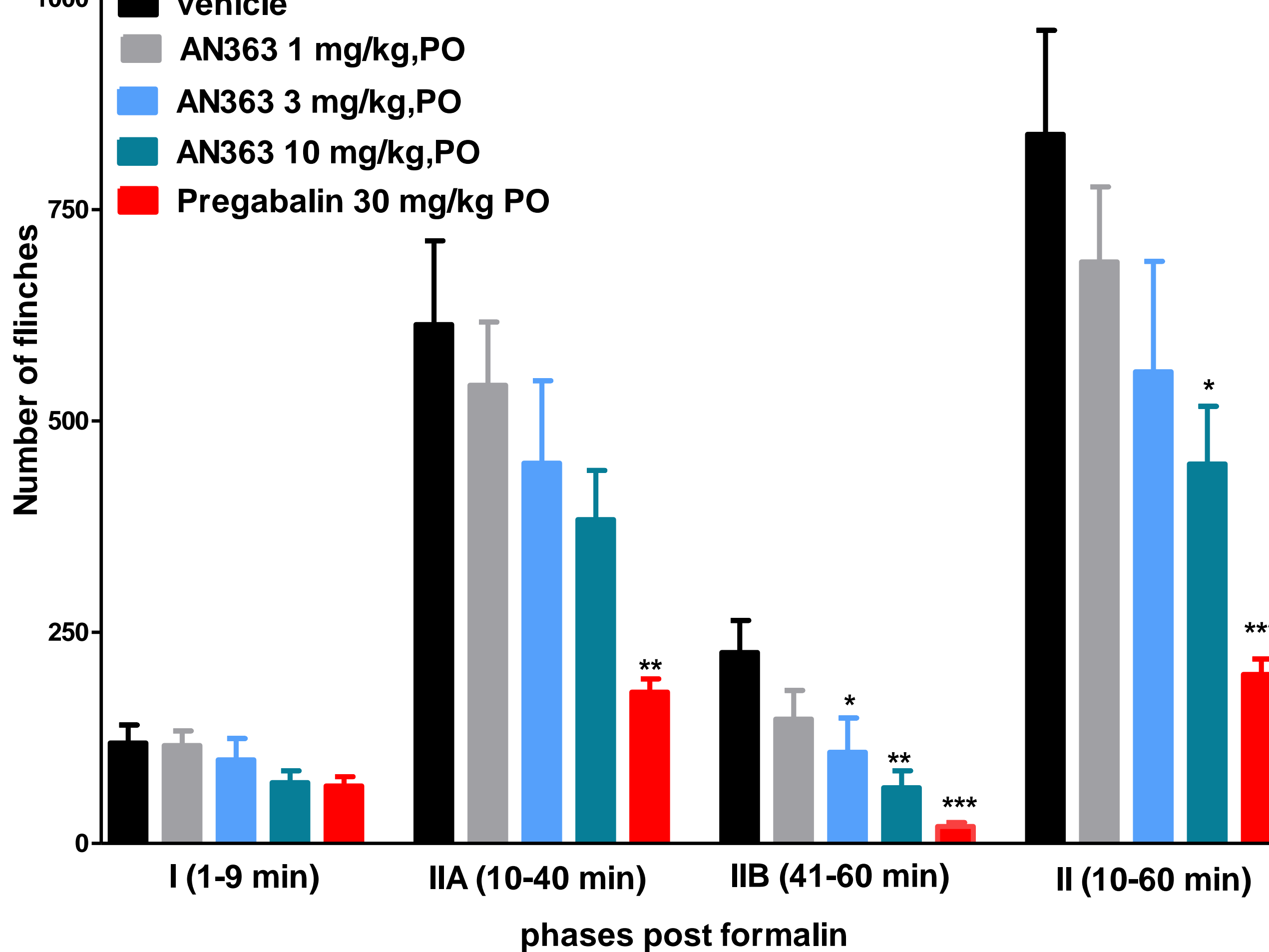
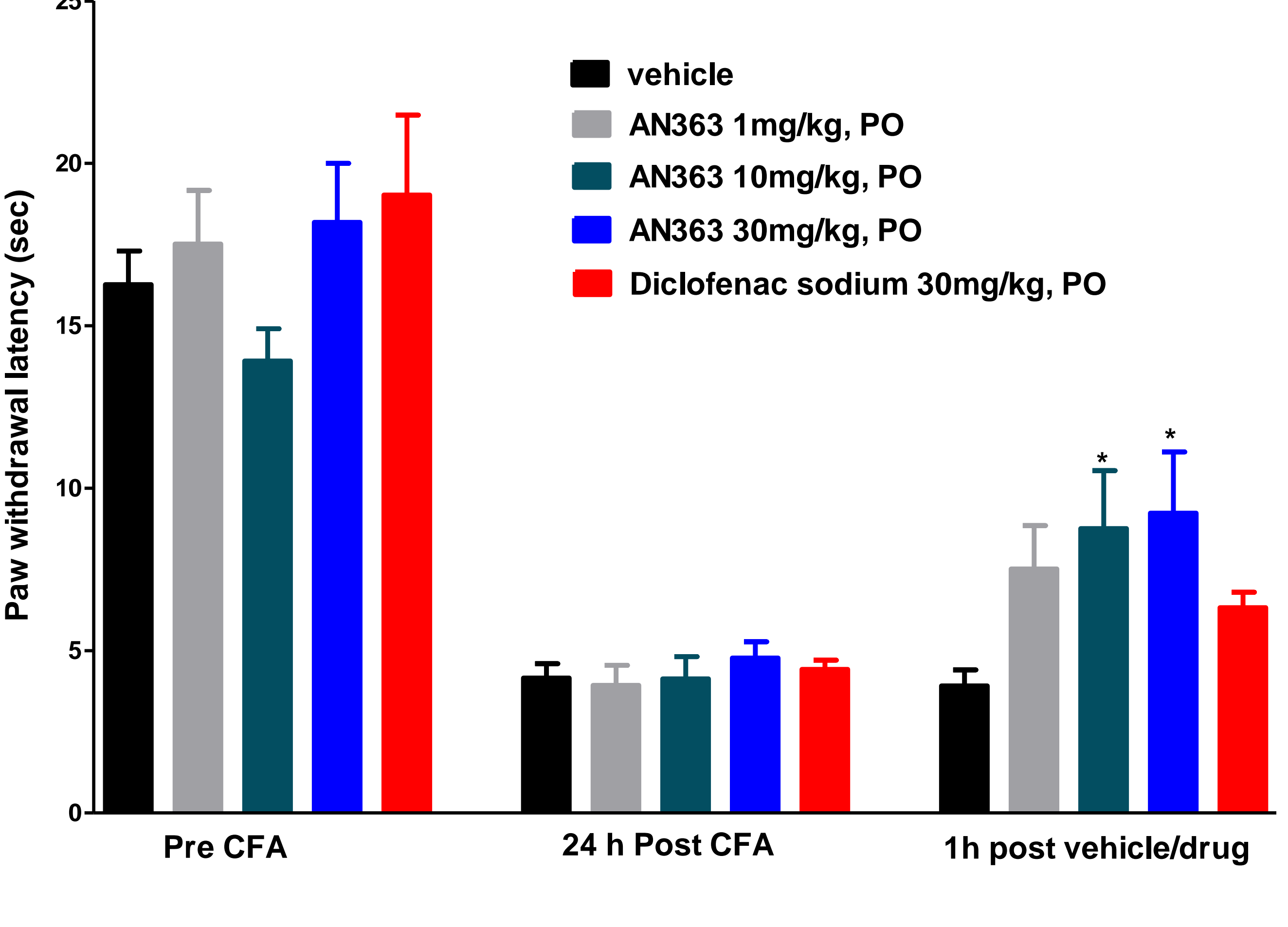


Fig 4 Effect of AN363 on formalin induced flinches in rats



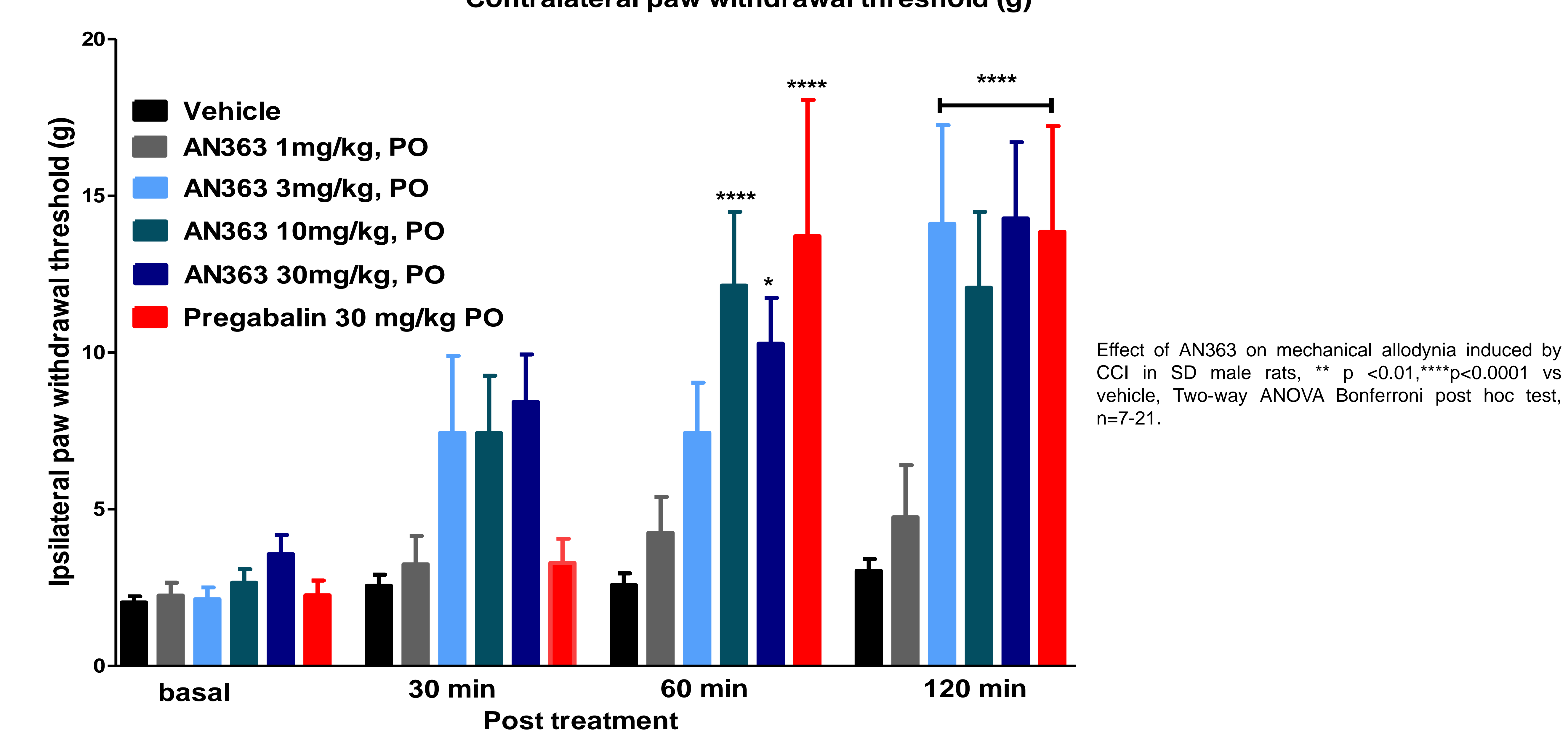
Effect of AN363 on formalin induced flinches in SD male rats, \* p<0.05, \*\* p<0.01, \*\*\*p<0.001 vs vehicle, One-way ANOVA Dunnett's multiple comparison post hoc test, n=7-8.

Fig 5 Effect of AN363 on CFA induced thermal hyperalgesia in rats



Effect of AN363 on thermal hyperalgesia induced by CFA in SD male rats, \* p<0.05, vs vehicle, One-way ANOVA Dunnett's multiple comparison post hoc test, n=6-8

Fig 6 Effect of AN363 on mechanical allodynia induced by CCI in rats



Effect of AN363 on mechanical allodynia induced by CCI in SD male rats, \*\* p<0.01, \*\*\*\*p<0.0001 vs vehicle, Two-way ANOVA Bonferroni post hoc test, n=7-21.

