



Title	The alpha(2A)-adrenoceptor subtype plays a key role in the analgesic and sedative effects of xylazine
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Citation	Journal of veterinary pharmacology and therapeutics, 42(2), 243-247 https://doi.org/10.1111/jvp.12724
Issue Date	2019-03
Doc URL	http://hdl.handle.net/2115/76848
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Type	article (author version)
File Information	Journal of veterinary pharmacology and therapeutics42(2)_243-247.pdf



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1 **TITLE**

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3 xylazine

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5 **SHORT RUNNING TITLE**

6 The α_{2A} -adrenoceptors play a key role in the effects of xylazine

7

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12

13 **ABSTRACT**

14 Xylazine, the classical α_2 -adrenoceptor (α_2 -AR) agonist, is still used as an analgesic and
15 sedative in veterinary medicine, despite its low potency and affinity for α_2 -ARs. Previous
16 pharmacological studies suggested that the α_{2A} -AR subtype plays a role in mediating the
17 clinical effects of xylazine; however, these studies were hampered by the poor subtype-
18 selectivity of the antagonists used and a lack of knowledge of their bioavailability *in vivo*.
19 Here, we attempted to elucidate the role of the α_{2A} -AR subtype in mediating the clinical
20 effects of xylazine by comparing the analgesic and sedative effects of this drug in wild-type
21 mice with those in α_{2A} -AR functional knockout mice using the hot-plate and open field tests,
22 respectively. Hippocampal noradrenaline turnover in both mice was also measured to
23 evaluate the contribution of α_{2A} -AR subtype to the inhibitory effect of xylazine on presynaptic
24 noradrenaline release. In wild-type mice, xylazine (10 or 30 mg/kg) increased the hot-plate

25 latency. Furthermore, xylazine (3 or 10 mg/kg) inhibited the open field locomotor activity, and
26 decreased hippocampal noradrenaline turnover. By contrast, all of these effects were
27 abolished in α_{2A} -AR functional knockout mice. These results indicate that the α_{2A} -AR subtype
28 is mainly responsible for the clinical effects of xylazine.

29

30 **KEYWORDS**

31 α_{2A} -Adrenoceptor, analgesics, sedatives, xylazine

32

33 **MAIN TEXT**

34 α_2 -Adrenoceptors (α_2 -ARs) are GTP-binding protein (G-protein) coupled receptors that are
35 expressed on central and peripheral nerves (Gyires, Zádori, Török, & Mátyus, 2009).
36 Activation of α_2 -ARs produces intracellular inhibitory signals via inhibitory G-proteins ($G_{i/o}$)
37 and regulates various neuronal functions by inhibiting neurotransmitter release (presynaptic
38 inhibition) and/or hyperpolarization of the neuronal cells (postsynaptic inhibition) (Khan,
39 Ferguson, & Jones, 1999). α_2 -AR agonists are widely used as analgesics and sedatives.

40 α_2 -ARs are genetically classified into three subtypes, namely, α_{2A} , α_{2B} and α_{2C}
41 (Bylund et al., 1994). Behavioral analyses of knockout mice revealed that the α_{2A} -AR subtype
42 is mainly responsible for mediating the analgesic and sedative effects of several α_2 -AR
43 agonists, including dexmedetomidine, UK-14,304, and clonidine (Fairbanks & Wilcox, 1999;
44 Hunter et al., 1997; Lakhani et al., 1997). On the other hand, the analgesic effect of I_1 -
45 imidazoline receptor/ α_2 -AR agonist moxonidine is diminished in the α_{2C} -AR knockout mice
46 (Fairbanks et al., 2002), suggesting agonist-specific differences in the contributions of
47 various α_2 -AR subtypes to their effects. In addition, because most α_2 -AR agonists contain
48 an imidazoline moiety, their effects can also be mediated via imidazoline receptors expressed

49 on central and peripheral nerves. It is well known that I₁-imidazoline receptors partly mediate
50 the hypotensive effect of α_2 -AR agonists such as clonidine (Ernsberger, Meeley, Mann, &
51 Reis, 1987). Furthermore, I₂-imidazoline receptors mediate an analgesic effect (Li & Zhang,
52 2011), and non-I₁/I₂-imidazoline receptors regulate the function of noradrenergic neurons
53 (Göthert, Brüss, Bönisch, & Molderings, 1999; Ugedo, Pineda, Ruiz-Ortega, & Martín-Ruiz,
54 1998).

55 Xylazine, the first α_2 -AR agonist to be used as an analgesic and sedative in
56 veterinary medicine, has a lower potency and affinity for α_2 -ARs than other agonists
57 (Otsuguro, Yasutake, Ohta, & Ito, 2005; Virtanen, Savola, Saano, & Nyman, 1988);
58 nevertheless, it is still used as an analgesic, sedative, and as part of anaesthetic protocols
59 for large animals such as cattle and horses, as well as laboratory animals. It is unclear which
60 receptor subtypes contribute to the clinical effects of xylazine. Previous study showed that
61 the effects of systemically administered xylazine are inhibited by non-selective α_2 -AR or
62 preferential α_{2A} -AR antagonists, but not preferential $\alpha_{2B/C}$ -AR antagonists (Millan et al.,
63 1994); however, these findings are questionable due to the poor selectivity of the antagonists
64 used (Gyires et al., 2009) and the lack of knowledge of their *in vivo* bioavailability. For
65 example, these antagonists contain an imidazoline moiety, which enables them to bind to
66 imidazoline receptors (Lowry & Brown, 2014; Renouard, Widdowson, & Cordi, 1993).
67 Although it was widely believed that xylazine is unable to bind to imidazoline receptors
68 because it lacks a typical imidazoline moiety in its chemical structure, Hikasa et al. (2013)
69 demonstrated binding of this drug to I₁- and I₂-imidazoline receptors.

70 The aim of this study was to evaluate the contribution of the α_{2A} -AR subtype to the
71 clinical effects of xylazine. We examined the analgesic and sedative effects of
72 intraperitoneally injected xylazine, as well as its ability to inhibit hippocampal noradrenaline

73 (NA) turnover, in wild-type (WT) and α_{2A} -AR functional knockout mice. Our hypothesis is that
74 these effects of xylazine are observed in WT mice, but not α_{2A} -AR functional knockout mice.

75 All animal care and experimental protocols were approved by the Animal Care and
76 Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University.
77 Breeding pairs of B6.129S2-*Adra2a*^{tm1Lel}/J mice, which are heterozygous ($\alpha_{2A}^{\text{WT/D79N}}$) for a
78 point mutation (D79N) in α_{2A} -ARs (MacMillan, Hein, Smith, Piascik, & Limbird, 1996) were
79 purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred to obtain WT
80 ($\alpha_{2A}^{\text{WT/WT}}$) and functional α_{2A} -AR knockout mice (D79N, $\alpha_{2A}^{\text{D79N/D79N}}$). The mice were
81 genotyped by PCR amplification of the gene region encoding the α_{2A} -AR and subsequent
82 digestion of the product with the restriction enzyme NheI. The mice were fed ad libitum in the
83 room kept on a 12-hr light-dark cycle at $22 \pm 4^\circ\text{C}$. Male WT and D79N mice (7–8 weeks old)
84 were used in all experiments. Behavioural tests were done in the enclosed room kept at 22
85 $\pm 4^\circ\text{C}$. The researchers performing and assessing the behavioural tests were not blinded to
86 the type of mice and treatment assignment. Xylazine (10 mg/ml, Sigma-Aldrich, St. Louis,
87 MO, USA) and morphine (1 mg/ml, Daiichi Sankyo, Tokyo, Japan) were dissolved in distilled
88 water, and stored at -20°C . All drugs were diluted in saline (0.9% NaCl) immediately before
89 the experiments as needed.

90 The analgesic effect of xylazine was evaluated using the hot-plate test by T.
91 Kobayashi. Mice were injected with xylazine (3, 10 or 30 mg/kg i.p.), morphine (10 mg/kg
92 s.c.), or saline (0.9% NaCl i.p.) in a volume of 10 ml/kg. The μ -opioid receptor agonist
93 morphine, which causes analgesia via a similar signaling mechanism to that used by α_2 -AR
94 agonists (Paddleford & Harvey, 1999), was used as a positive control of analgesic effect for
95 D79N mice and injected subcutaneously to avoid first pass effect. Before (0 min) and after
96 injection (30, 60 and 90 min), each mouse was placed on a hot-plate (Hot/Cold Plate 35100,

97 Ugo Basile, Lombardia, Italy) that was preheated to 53°C. When a nociceptive reaction
98 (licking a hind paw or jumping) was observed, the mouse was removed from the hot-plate
99 immediately and the latency period was recorded. To prevent tissue damage caused by heat,
100 the cut-off time was set at 60 s.

101 The sedative effect of xylazine was evaluated using the open field test by T. Kitano.
102 Mice were injected with xylazine (3 or 10 mg/kg i.p.), chlorpromazine (5 mg/kg i.p.,
103 Yoshitomiya, Osaka, Japan) or saline (0.9% NaCl i.p.) in a volume of 20 ml/kg. The
104 dopamine D2 receptor antagonist chlorpromazine, which is another sedative drug used
105 clinically, was used as a positive control of sedative effect for D79N mice. Fifteen minutes
106 after injection, each mouse was placed at the center of a 70 cm square open field that was
107 divided into a 10 cm square grid, and the number of crossings between divisions was counted
108 for 15 min by the researcher on the spot.

109 NA turnover was measured as described previously (Lakhlani et al., 1997). NA is
110 metabolized to 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) after release from
111 presynaptic nerve terminals (Kopin, 1985), and α_2 -AR agonists reduce the MHPG/NA ratio
112 by inhibiting presynaptic NA release via α_{2A} -ARs in some brain regions such as the
113 hippocampus (Lakhlani et al., 1997; Lähdesmäki, Sallinen, MacDonald, Sirviö, & Scheinin,
114 2003). Mice were injected with xylazine (3 or 10 mg/kg i.p.), pentobarbital (50 mg/kg i.p.,
115 Kyoritsu Seiyaku, Tokyo, Japan) or saline (0.9% NaCl i.p.) in a volume of 20 ml/kg. The
116 GABA_A receptor agonist pentobarbital, which also inhibits NA release and reduces NA
117 turnover (Mizuno, Ito, & Kimura, 1994; Nabeshima, Fujimori, & Ho, 1981), was used as a
118 positive control. Thirty minutes after injection, the mice were euthanized by exposure to CO₂,
119 and then their hippocampus was isolated immediately and placed in ice-cold Hanks' solution.
120 The samples were homogenized and sonicated in 0.2 N perchloric acid (containing 100 μ M

121 EDTA-2Na), incubated on ice for 30 min, and then centrifuged at 20,000 × *g* for 15 min. The
122 supernatants were collected and filtered to remove tissue debris. The levels of NA and MHPG
123 in the supernatants were analyzed using a high-performance liquid chromatography system
124 equipped with an electrochemical detector. The mobile phase, consisted of a citric acid buffer
125 (0.1 M citric acid, 0.1 M sodium acetate; pH 3.5), 13% methanol, 5 mg/l EDTA-2Na, and
126 190 mg/l 1-octanesulfonic acid sodium salt, was degassed and perfused at a rate of
127 0.5 ml/min, and the supernatant samples (20 µl) were injected using an autosampler (Model
128 33, System Instruments, Tokyo, Japan). NA and MHPG in the samples were separated on
129 an octadecylsilane column (EICOMPAK SC-5ODS, 3.0 Ø × 150 mm, EICOM, Kyoto, Japan)
130 at 30°C, and detected at +500 mV with an electrochemical detector (ECD-300, EICOM,
131 Kyoto, Japan). The ratio of MHPG to NA (MHPG/NA) was calculated from the area under
132 each peak. The detection limits for NA and MHPG were about 0.05 and 0.1 pmol, respectively.
133 The inter- and intra-assay coefficients of variation for both detection were 8% or less.

134 Multiple comparisons were performed using the Kruskal-Wallis test followed by the
135 Steel post-hoc test (Ekuseru-Toukei 2008, Social Survey Research Information Co., Ltd.,
136 Tokyo, Japan). A *p*-value less than 0.05 was considered statistically significant.

137 In WT mice, xylazine (10 or 30 mg/kg i.p.) significantly increased the hot-plate
138 latency from 30 min after injection, while a lower dose of xylazine (3 mg/kg i.p.) had little
139 effect (Fig. 1). In mice given xylazine (10 mg/kg), this analgesic effect disappeared at 90 min
140 post-injection, whereas the higher dose of xylazine (30 mg/kg) caused a sustained analgesic
141 effect that lasted until the 90 min time-point. By contrast, neither dose of xylazine had an
142 analgesic effect in the D79N mice. Morphine (10 mg/kg s.c.) increased the hot-plate latency
143 in D79N mice at least for 90 min.

144 Additionally, in WT mice, xylazine (3 or 10 mg/kg i.p.) significantly decreased the

145 number of crossings between divisions on the field (Fig. 2). Especially, the higher dose of
146 xylazine (10 mg/kg) almost abolished the locomotor activity of the mice. By contrast, this
147 sedative effect of xylazine was not seen in D79N mice, even at the higher dose (10 mg/kg).
148 Chlorpromazine (5 mg/kg i.p.) decreased the number of crossings in the D79N mice to
149 approximately the same level as that in WT mice injected with xylazine (10 mg/kg).

150 Xylazine (3 or 10 mg/kg i.p.) also significantly reduced the MHPG/NA ratio in the
151 hippocampus of WT mice, but not that of D79N mice (Fig. 3). Pentobarbital (50 mg/kg i.p.)
152 decreased the MHPG/NA ratio in the hippocampus of D79N mice.

153 These results show that analgesia, sedation and reduction of NA turnover caused
154 by intraperitoneally injected xylazine disappear in mice by the functional knockout of α_{2A} -ARs.

155 In the current study, the lower dose of xylazine (3 mg/kg) exerted the sedative effect,
156 but not the analgesic effect, on WT mice. Previous report showed that the sedative effect of
157 clonidine (0.025 mg/kg i.v.) on horses appears more rapidly and lasts longer than analgesic
158 effect (Dirikolu et al., 2006).

159 The functional density of α_{2A} -ARs in D79N mice is 80% lower than that in WT mice,
160 even though the expression levels of the mRNA encoding the receptor are comparable in the
161 two strains (MacMillan et al., 1996). In addition, the D79N mutation causes substantial
162 attenuation of α_{2A} -AR/G-protein coupling both *in vivo* and *in vitro* (Chabre, Conklin, Brandon,
163 Bourne, & Limbird, 1994; Lakhiani et al., 1997), resulting in elimination of the function of α_{2A} -
164 ARs in D79N mice. In the current study, the analgesic and sedative effects of xylazine were
165 not observed in D79N mice, indicating that the α_{2A} -AR subtype plays a crucial role in the
166 clinical effects of therapeutic doses of xylazine.

167 The inhibitory effect of xylazine on NA turnover was also abolished in D79N mice,
168 indicating that α_{2A} -ARs contribute substantially to the inhibitory effect of xylazine on

169 presynaptic NA release, a mechanism that underlies its analgesic and sedative effects. This
170 finding is in agreement with the fact that α_{2A} -ARs play a predominant role in the presynaptic
171 inhibition of neurotransmitter release (Gyires et al., 2009), and supports our conclusion that
172 the clinical effects of xylazine are mediated mainly by α_{2A} -ARs. On the other hand, the
173 inhibitory effect of dexmedetomidine, another α_2 -AR agonist, on NA turnover is reduced but
174 not abolished in D79N mice (Lakhlani et al., 1997). One possible explanation for this drug-
175 specific finding is the involvement of imidazoline receptors. There are some reports indicating
176 the existence of presynaptic imidazoline receptors and their inhibitory effect on NA release
177 from sympathetic nerve endings (Chung et al., 2010; Göthert et al., 1999). Dexmedetomidine
178 contains a typical imidazoline moiety and has higher affinity for imidazoline receptors than
179 xylazine (Hikasa et al., 2013), and may therefore inhibit NA release in D79N mice by binding
180 to imidazoline receptors. Another possible explanation for that is the involvement of other α_2 -
181 AR subtypes. Neither xylazine nor medetomidine displays selectivity for the α_2 -AR subtypes
182 (Schwartz & Clark, 1998), and α_{2C} -ARs also play a role in the presynaptic inhibition of
183 neurotransmitter release in the central nervous system (Bücheler, Hadamek, & Hein, 2002).
184 Further studies are needed to address this issue.

185 There are several reports indicating the α_{2A} -AR-independent effects of xylazine. For
186 example, intraplantar injection of xylazine exerts a peripheral analgesic effect via α_{2C} -ARs
187 (Romero, de Castro Perez, de Francischi, & Gama Duarte, 2009). In addition, xylazine has
188 an α_2 -AR-independent inhibitory effect on the spontaneous firing of cortical neurons
189 (O'Regan, 1989). Our previous study using electrophysiological approaches also showed
190 that the inhibitory effect of high concentrations of xylazine on nociceptive synaptic
191 transmission is retained in isolated spinal cords of D79N mice, and that xylazine inhibits the
192 spinal nerve conduction of action potentials in an α_{2A} -AR-independent manner. (Kobayashi,

193 Otsuguro, Yamaguchi, & Ito, 2015). These α_{2A} -AR-independent mechanisms seem to have
194 less contribution to the clinical effects, at least, by intraperitoneally injected xylazine, since
195 xylazine had no effects on D79N mice.

196 In this study, behavioural analyses were done in a non-blind fashion, which may
197 limit potentially the usefulness of our results. However, since the effects of xylazine in D79N
198 mice were clearly different from those in WT mice, it is unlikely that our conclusions are
199 changed by this potential bias. On the other hand, the present study may have other
200 limitations. We investigated the effects of intraperitoneally injected xylazine on mice.
201 However, xylazine is often used for large animals by not only systemic administration but
202 also local administration such as intrathecal injection. In addition, it is well known that the
203 potency of xylazine is highly species-dependent. The contribution of α_{2A} -ARs to the effects
204 of xylazine in the different species and/or those by the different administration route should
205 be investigated in the future.

206 In conclusion, systemically administered xylazine exerts its analgesic and sedative
207 effects via α_{2A} -ARs. Xylazine also inhibits presynaptic NA release mainly via α_{2A} -ARs.

208

209 **ACKNOWLEDGMENTS**

210 This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society
211 for the Promotion of Science (JSPS) (No.26450440 to K.O).

212

213 **CONFLICT OF INTEREST**

214 The authors have no conflict of interests to report.

215

216 **AUTHORS' CONTRIBUTIONS**

217 T. Kitano contributed to the study design, performed the experiments and data analysis, and
218 drafted the manuscript. T. Kobayashi performed the experiments and data analysis. S. Y.
219 contributed to the experiments and assisted with drafting the manuscript. K. O. contributed
220 to the study design and experiments, and drafted the manuscript. All authors have read and
221 approved the final manuscript.

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357

358 **FIGURE LEGENDS**

359 Fig. 1. Analgesic effect of xylazine in WT and D79N mice. A hot-plate test was performed
360 before (0 min) and 30, 60, and 90 min after injection of mice (n = 3–10) with xylazine (3, 10
361 or 30 mg/kg i.p.), morphine (10 mg/kg s.c.), or saline. Data are expressed as the median and
362 range. **p* < 0.05 and ***p* < 0.01 vs. saline (Steel test).

363

364 Fig. 2. Sedative effect of xylazine in WT and D79N mice. An open field test was performed
365 15 min after injection of mice (n = 3–6) with xylazine (3 or 10 mg/kg i.p.), chlorpromazine (CP,
366 5 mg/kg i.p.), or saline. The number of crossings between divisions in the open field was
367 counted for 15 min. Data are expressed as the median and range. **p* < 0.05 vs. saline (Steel
368 test).

369

370 Fig. 3. Inhibitory effect of xylazine on NA turnover in the hippocampus of WT and D79N mice.
371 NA turnover in the hippocampus was quantified as the MHPG/NA ratio (%) 30 min after
372 injection of WT and D79N mice (n = 3–6) with xylazine (3 or 10 mg/kg i.p.), pentobarbital (PB,
373 50 mg/kg i.p.), or saline. Data are expressed as the median and range. **p* < 0.05 vs. saline
374 (Steel test).

375

376

Fig. 1.

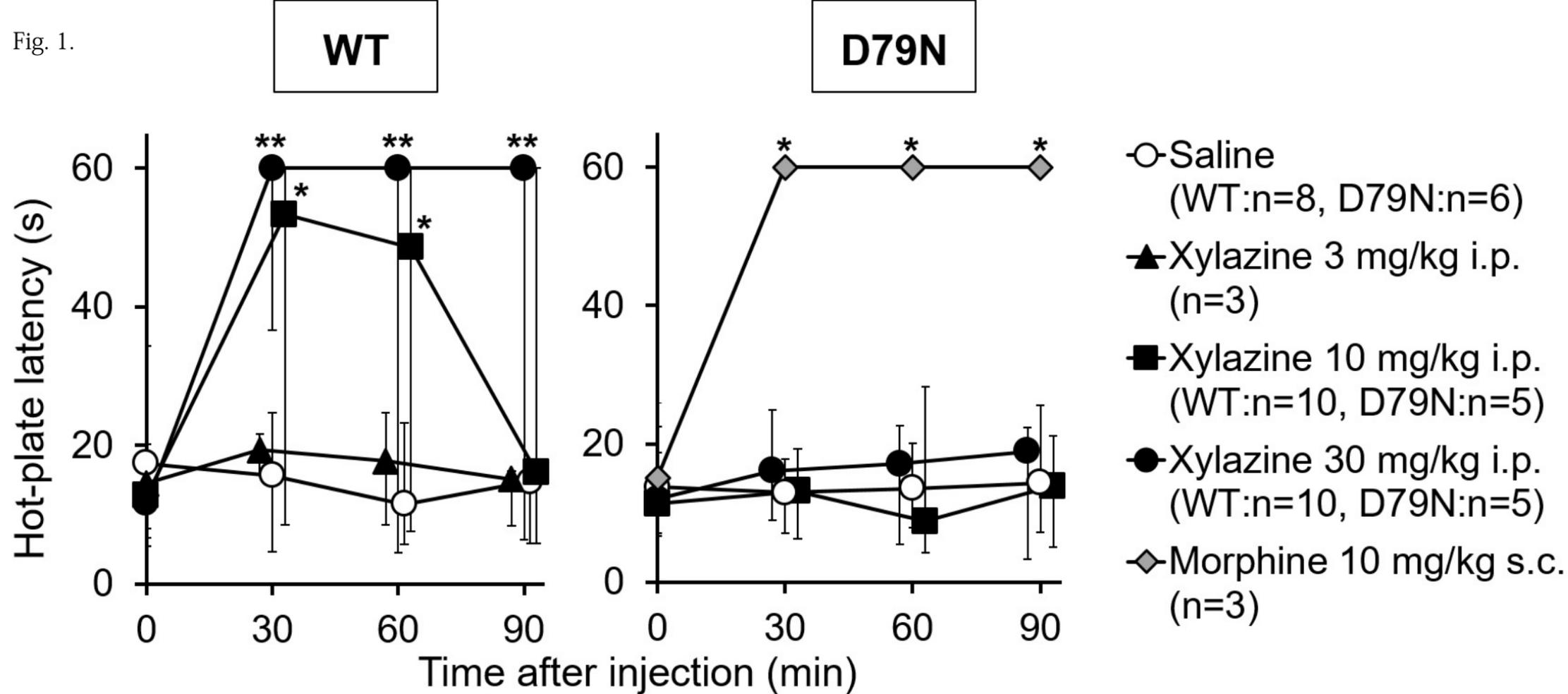


Fig. 2.

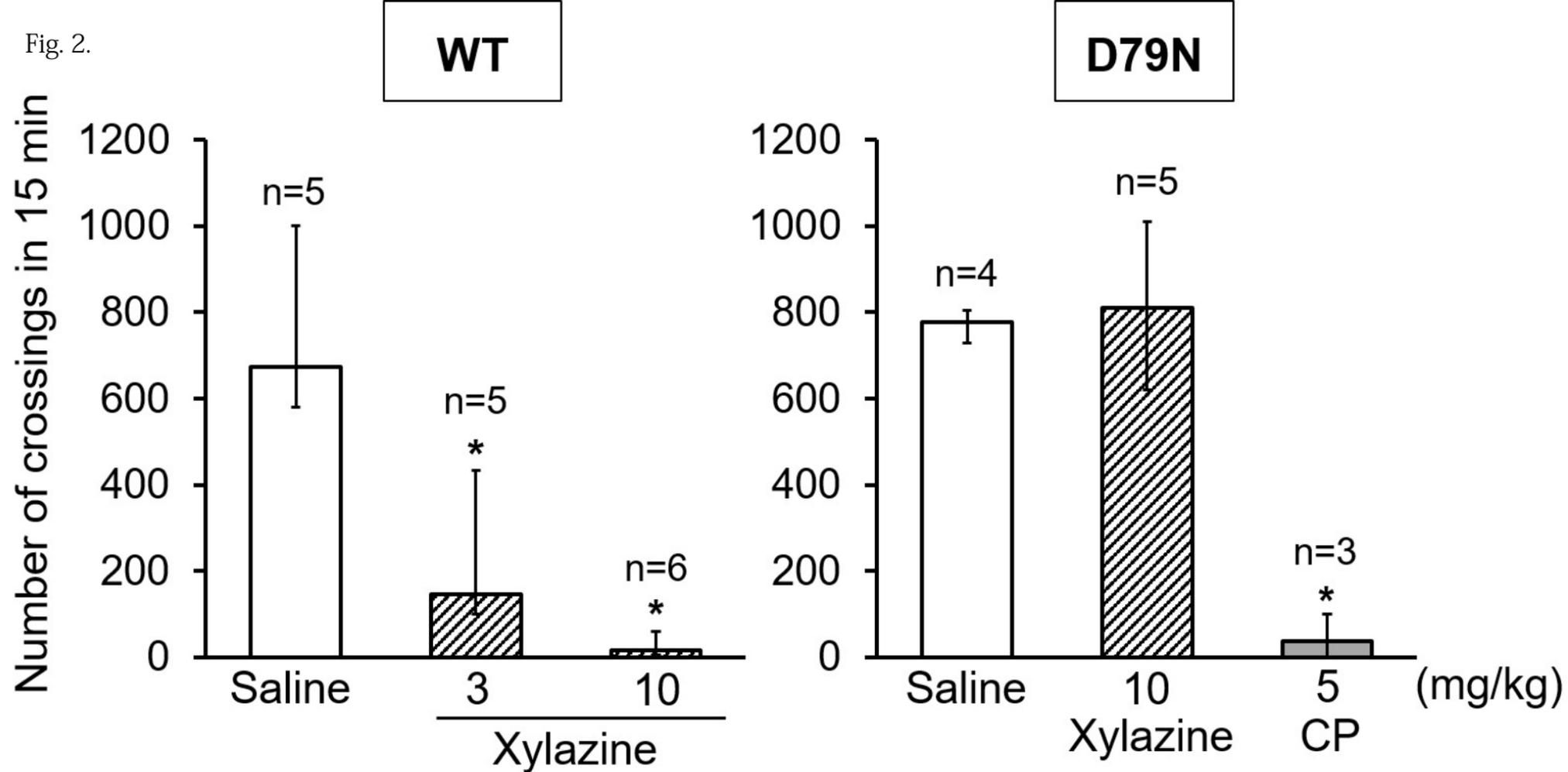


Fig. 3.

WT

D79N

