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TITLE

The α_{2A}-adrenoceptor subtype plays a key role in the analgesic and sedative effects of xylazine

SHORT RUNNING TITLE

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

AUTHORS

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ABSTRACT

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

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

KEYWORDS

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

MAIN TEXT

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

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

on central and peripheral nerves. It is well known that I_1-imidazoline receptors partly mediate the hypotensive effect of α_2-AR agonists such as clonidine (Ernsberger, Meeley, Mann, & Reis, 1987). Furthermore, I_2-imidazoline receptors mediate an analgesic effect (Li & Zhang, 2011), and non-I_1/I_2-imidazoline receptors regulate the function of noradrenergic neurons (Göthert, Brüss, Bönisch, & Molderings, 1999; Ugedo, Pineda, Ruiz-Ortega, & Martín-Ruiz, 1998).

Xylazine, the first α_2-AR agonist to be used as an analgesic and sedative in veterinary medicine, has a lower potency and affinity for α_2-ARs than other agonists (Otsuguro, Yasutake, Ohta, & Ito, 2005; Virtanen, Savola, Saano, & Nyman, 1988); nevertheless, it is still used as an analgesic, sedative, and as part of anaesthetic protocols for large animals such as cattle and horses, as well as laboratory animals. It is unclear which receptor subtypes contribute to the clinical effects of xylazine. Previous study showed that the effects of systemically administered xylazine are inhibited by non-selective α_2-AR or preferential α_{2A}-AR antagonists, but not preferential $\alpha_{2B/C}$-AR antagonists (Millan et al., 1994); however, these findings are questionable due to the poor selectivity of the antagonists used (Gyires et al., 2009) and the lack of knowledge of their *in vivo* bioavailability. For example, these antagonists contain an imidazoline moiety, which enables them to bind to imidazoline receptors (Lowry & Brown, 2014; Renouard, Widdowson, & Cordi, 1993). Although it was widely believed that xylazine is unable to bind to imidazoline receptors because it lacks a typical imidazoline moiety in its chemical structure, Hikasa et al. (2013) demonstrated binding of this drug to I_1- and I_2-imidazoline receptors.

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

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

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

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

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

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

NA turnover was measured as described previously (Lakhlani et al., 1997). NA is metabolized to 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) after release from presynaptic nerve terminals (Kopin, 1985), and α_2-AR agonists reduce the MHPG/NA ratio by inhibiting presynaptic NA release via α_{2A}-ARs in some brain regions such as the hippocampus (Lakhlani et al., 1997; Lähdesmäki, Sallinen, MacDonald, Sirviö, & Scheinin, 2003). Mice were injected with xylazine (3 or 10 mg/kg i.p.), pentobarbital (50 mg/kg i.p., Kyoritsu Seiyaku, Tokyo, Japan) or saline (0.9% NaCl i.p.) in a volume of 20 ml/kg. The $GABA_A$ receptor agonist pentobarbital, which also inhibits NA release and reduces NA turnover (Mizuno, Ito, & Kimura, 1994; Nabeshima, Fujimori, & Ho, 1981), was used as a positive control. Thirty minutes after injection, the mice were euthanized by exposure to CO_2, and then their hippocampus was isolated immediately and placed in ice-cold Hanks' solution. The samples were homogenized and sonicated in 0.2 N perchloric acid (containing 100 μM

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

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

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

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

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

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

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

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

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

The inhibitory effect of xylazine on NA turnover was also abolished in D79N mice, 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,

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

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

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

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CONFLICT OF INTEREST

The authors have no conflict of interests to report.

AUTHORS' CONTRIBUTIONS

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

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FIGURE LEGENDS

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

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

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

Fig. 1.

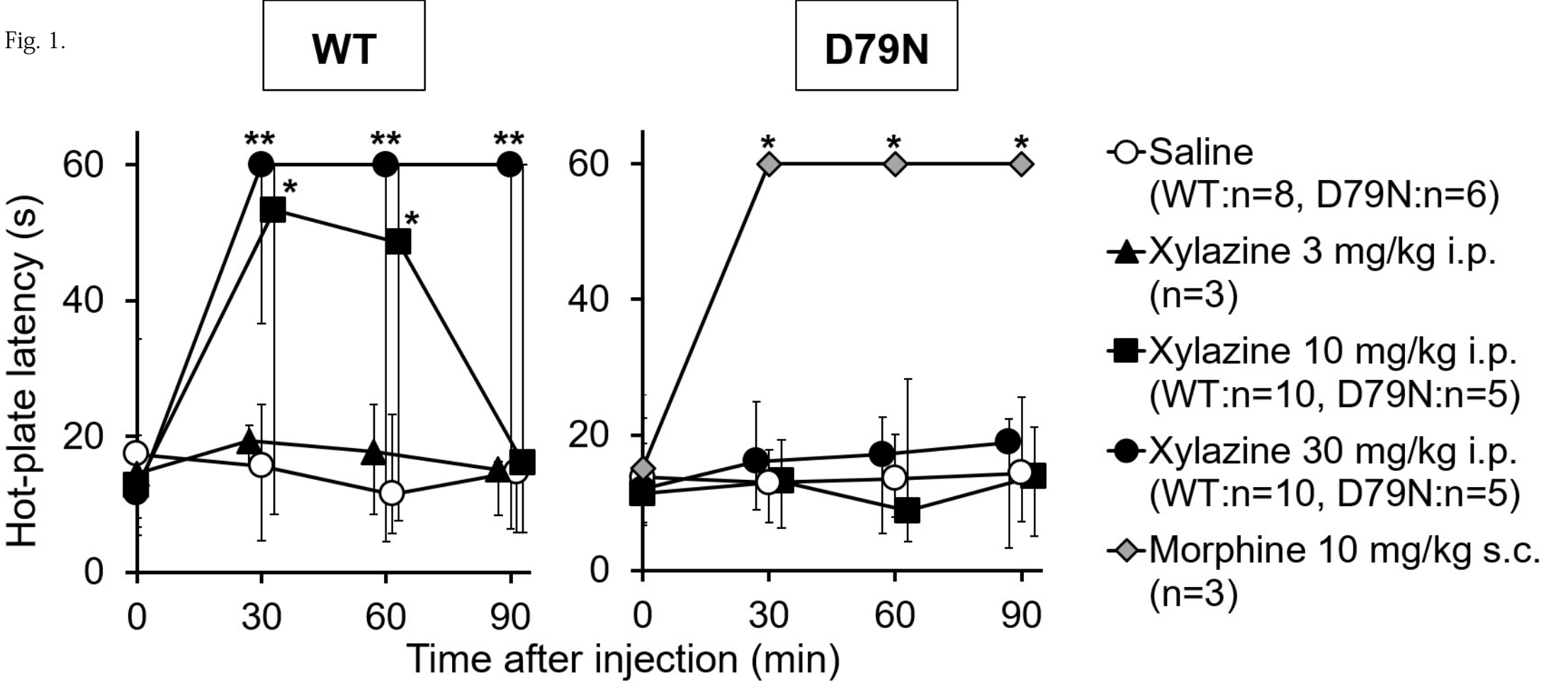

WT
D79N
Hot-plate latency (s)
60
40
20
0
0
30
60
90
Time after injection (min)
**
**
**
*
*
*
*
*
Saline
(WT:n=8, D79N:n=6)
Xylazine 3 mg/kg i.p.
(n=3)
Xylazine 10 mg/kg i.p.
(WT:n=10, D79N:n=5)
Xylazine 30 mg/kg i.p.
(WT:n=10, D79N:n=5)
Morphine 10 mg/kg s.c.
(n=3)

Fig. 2.

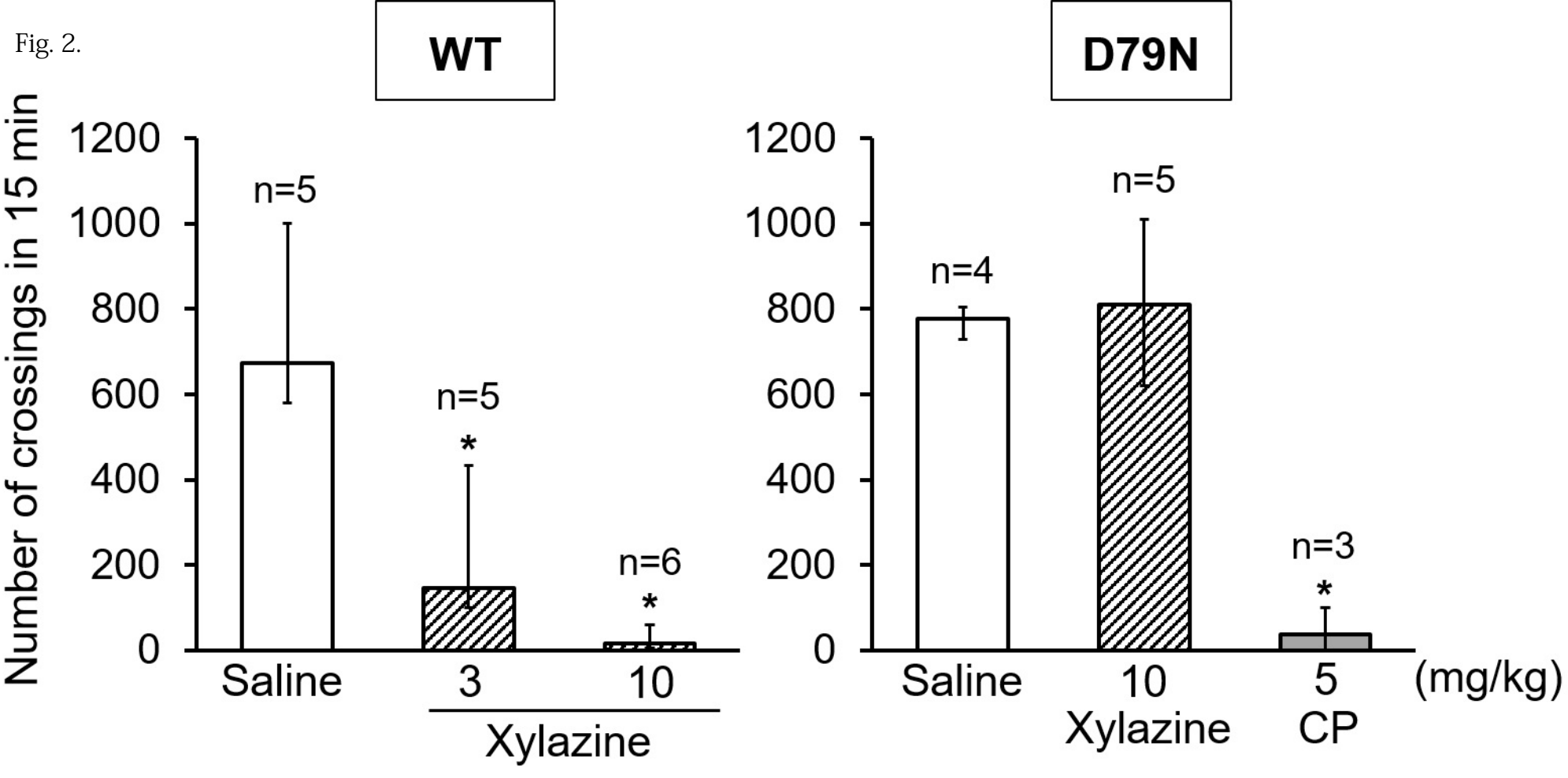
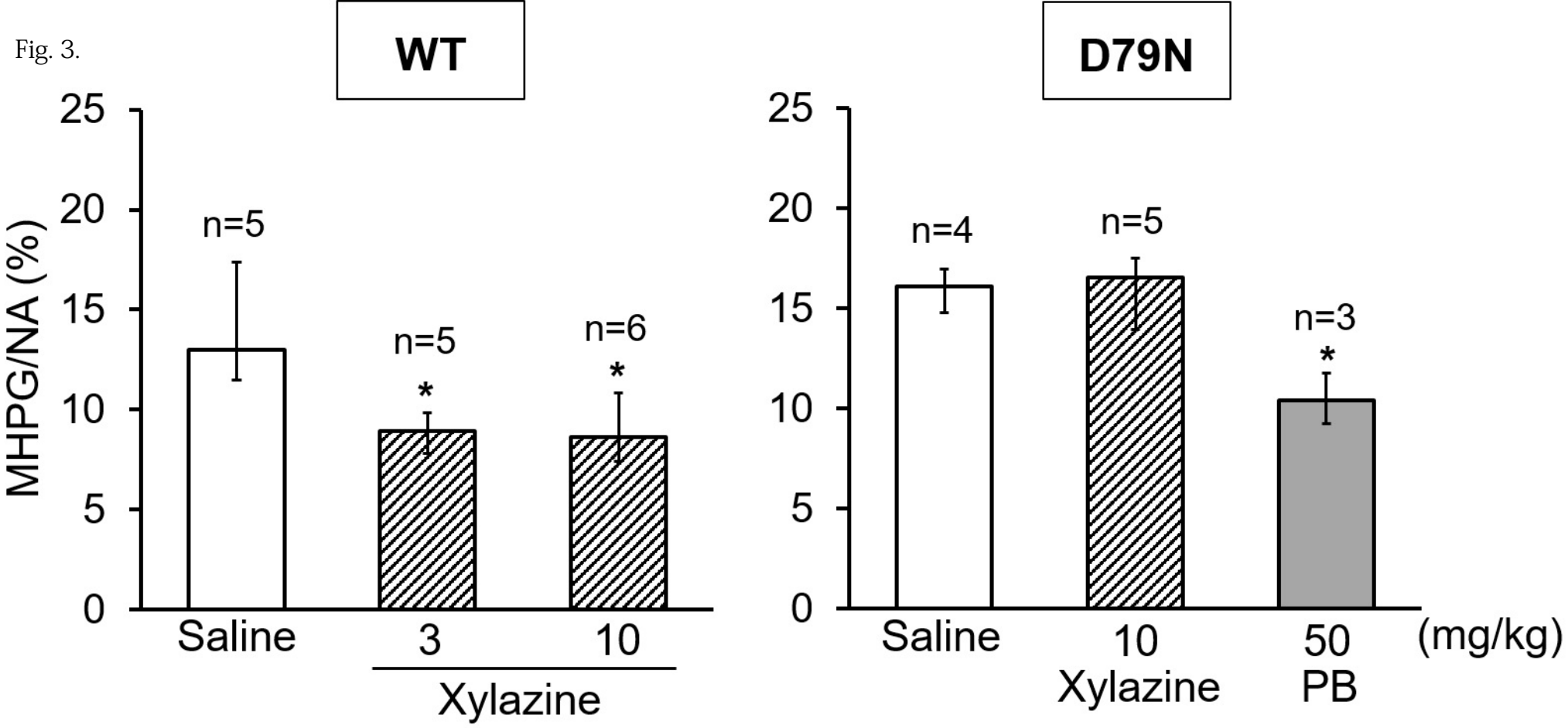

WT
D79N
Number of crossings in 15 min
1200
1000
800
600
400
200
0
n=5
n=5
*
n=6
*
n=4
n=5
n=3
*
Saline
3
10
Xylazine
Saline
10
Xylazine
5
CP
(mg/kg)

Fig. 3.


WT
D79N
MHPG/NA (%)
25
20
15
10
5
0
n=5
n=5
n=6
n=4
n=5
n=3
*
*
*
Saline
3
10
Saline
10
50
(mg/kg)
Xylazine
Xylazine
PB