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

The $\alpha_{2A}$-adrenoceptors play a key role in the effects of xylazine.

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Xylazine, the classical $\alpha_2$-adrenoceptor ($\alpha_2$-AR) agonist, is still used as an analgesic and sedative in veterinary medicine, despite its low potency and affinity for $\alpha_2$-ARs. Previous pharmacological studies suggested that the $\alpha_{2A}$-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 $\alpha_{2A}$-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 $\alpha_{2A}$-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 $\alpha_{2A}$-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 α2A-AR functional knockout mice. These results indicate that the α2A-AR subtype is mainly responsible for the clinical effects of xylazine.

KEYWORDS
α2A-Adrenoceptor, analgesics, sedatives, xylazine

MAIN TEXT
α2-Adrenoceptors (α2-ARs) are GTP-binding protein (G-protein) coupled receptors that are expressed on central and peripheral nerves (Gyires, Zádori, Török, & Mátyus, 2009). Activation of α2-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). α2-AR agonists are widely used as analgesics and sedatives. α2-ARs are genetically classified into three subtypes, namely, α2A, α2B and α2C (Bylund et al., 1994). Behavioral analyses of knockout mice revealed that the α2A-AR subtype is mainly responsible for mediating the analgesic and sedative effects of several α2-AR agonists, including dexmedetomidine, UK-14,304, and clonidine (Fairbanks & Wilcox, 1999; Hunter et al., 1997; Lakhani et al., 1997). On the other hand, the analgesic effect of I1-imidazoline receptor/α2-AR agonist moxonidine is diminished in the α2C-AR knockout mice (Fairbanks et al., 2002), suggesting agonist-specific differences in the contributions of various α2-AR subtypes to their effects. In addition, because most α2-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 I1-imidazoline receptors partly mediate the hypotensive effect of α2-AR agonists such as clonidine (Ernsberger, Meeley, Mann, & Reis, 1987). Furthermore, I2-imidazoline receptors mediate an analgesic effect (Li & Zhang, 2011), and non-I1/I2-imidazoline receptors regulate the function of noradrenergic neurons (Göthert, Brüss, Bönisch, & Molderings, 1999; Ugedo, Pineda, Ruiz-Ortega, & Martín-Ruiz, 1998).

Xylazine, the first α2-AR agonist to be used as an analgesic and sedative in veterinary medicine, has a lower potency and affinity for α2-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 α2-AR or preferential α2A-AR antagonists, but not preferential α2B/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 I1- and I2-imidazoline receptors.

The aim of this study was to evaluate the contribution of the α2A-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 α2A-AR functional knockout mice. Our hypothesis is that these effects of xylazine are observed in WT mice, but not α2A-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-Adra2α^{tm1Lei}/J mice, which are heterozygous (α2A^{WT/D79N}) for a point mutation (D79N) in α2A-ARs (MacMillan, Hein, Smith, Piascik, & Limbird, 1996) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and bred to obtain WT (α2A^{WT/WT}) and functional α2A-AR knockout mice (D79N, α2A^{D79N/D79N}). The mice were genotyped by PCR amplification of the gene region encoding the α2A-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 α2-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 (Lakhlini et al., 1997). NA is metabolized to 3-methoxy-4-hydroxyphenylethylene glycol (MHPG) after release from presynaptic nerve terminals (Kopin, 1985), and α2-AR agonists reduce the MHPG/NA ratio by inhibiting presynaptic NA release via α2A-ARs in some brain regions such as the hippocampus (Lakhlini 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 CO2, 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 $\alpha_2A$-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 $\alpha_2A$-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 $\alpha_2A$-AR/G-protein coupling both in vivo and in vitro (Chabre, Conklin, Brandon, Bourne, & Limbird, 1994; Lakhiani et al., 1997), resulting in elimination of the function of $\alpha_2A$-ARs in D79N mice. In the current study, the analgesic and sedative effects of xylazine were not observed in D79N mice, indicating that the $\alpha_2A$-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 $\alpha_2A$-ARs contribute substantially to the inhibitory effect of xylazine on
presynaptic NA release, a mechanism that underlies its analgesic and sedative effects. This finding is in agreement with the fact that $\alpha_{2A}$-ARs play a predominant role in the presynaptic inhibition of neurotransmitter release (Gyires et al., 2009), and supports our conclusion that the clinical effects of xylazine are mediated mainly by $\alpha_{2A}$-ARs. On the other hand, the inhibitory effect of dexmedetomidine, another $\alpha_2$-AR agonist, on NA turnover is reduced but not abolished in D79N mice (Lakhiani et al., 1997). One possible explanation for this drug-specific finding is the involvement of imidazoline receptors. There are some reports indicating the existence of presynaptic imidazoline receptors and their inhibitory effect on NA release from sympathetic nerve endings (Chung et al., 2010; Göthert et al., 1999). Dexmedetomidine contains a typical imidazoline moiety and has higher affinity for imidazoline receptors than xylazine (Hikasa et al., 2013), and may therefore inhibit NA release in D79N mice by binding to imidazoline receptors. Another possible explanation for that is the involvement of other $\alpha_2$-AR subtypes. Neither xylazine nor medetomidine displays selectivity for the $\alpha_2$-AR subtypes (Schwartz & Clark, 1998), and $\alpha_{2C}$-ARs also play a role in the presynaptic inhibition of neurotransmitter release in the central nervous system (Bücheler, Hadamek, & Hein, 2002).

Further studies are needed to address this issue.

There are several reports indicating the $\alpha_{2A}$-AR-independent effects of xylazine. For example, intraplantar injection of xylazine exerts a peripheral analgesic effect via $\alpha_{2C}$-ARs (Romero, de Castro Perez, de Francischi, & Gama Duarte, 2009). In addition, xylazine has an $\alpha_2$-AR-independent inhibitory effect on the spontaneous firing of cortical neurons (O’Regan, 1989). Our previous study using electrophysiological approaches also showed that the inhibitory effect of high concentrations of xylazine on nociceptive synaptic transmission is retained in isolated spinal cords of D79N mice, and that xylazine inhibits the spinal nerve conduction of action potentials in an $\alpha_{2A}$-AR-independent manner.
These $\alpha_{2A}$-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 $\alpha_{2A}$-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 $\alpha_{2A}$-ARs. Xylazine also inhibits presynaptic NA release mainly via $\alpha_{2A}$-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.

REFERENCES


**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)**

- **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)

**Time after injection (min)**

0 30 60 90
Fig. 2.

**WT**

Number of crossings in 15 min

- Saline: n=5
- 3 mg/kg Xylazine: n=6
- 10 mg/kg Xylazine: n=5

**D79N**

Number of crossings in 15 min

- Saline: n=5
- 10 mg/kg Xylazine: n=4
- 5 mg/kg CP: n=3

(mg/kg)
Fig. 3.

WT

D79N

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<th>Dose (mg/kg)</th>
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* Indicates significant difference.