


Article

The Characteristics and Distribution of α 2D-, α 2B- and α 2C-Adrenoceptor Subtypes in Goats

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Simple Summary: α 2-Adrenergic receptors mediate many diverse biological effects of the endogenous catecholamines epinephrine and norepinephrine. Three distinct subtypes of α 2-adrenergic receptors, α 2B, α 2C and α 2D, have been identified in goats; however, the characteristics and distribution of α 2-adrenoceptors in goats remain unclear. Therefore, the aim of our study was to assess the characteristics and distribution of α 2-adrenoceptor subtypes in goats. Our study highlights the wide but uneven distribution of α 2-adrenoceptor subtypes in goats. Additionally, our study showed that α 2D-ceptor has a better analgesic effect in goats than α 2B- and α 2C-adrenoceptor, whereas α 2C-adrenoceptor plays a more important role in thermoregulation than α 2B- and α 2D-adrenoceptors.

Abstract: α 2-Adrenergic receptors (α 2Rs) are important presynaptic modulators of central noradrenergic function (auto receptors) and postsynaptic mediators of many of the widespread effects of catecholamines and related drugs. Studies have shown that ruminants (such as goats and cattle) express special α 2DR subtypes in addition to α 2BR and α 2CR. Real-time quantitative PCR and Western blotting were used to investigate the distribution and density of α 2R in different nuclei of the goat central nervous system, selected regions of the spinal cord (L4-L6), and in various peripheral tissues. α 2-AR subtype-specific antibodies were injected intrathecally and intracerebroventricularly into the tested goats to block the corresponding subtype of receptors. Pain threshold and physiological parameters were evaluated to explore the functional characteristics of α 2BR, α 2CR and α 2DR in goats. Our results suggest that the expression of the mRNAs and proteins of all three α 2R subtypes are widely but unevenly distributed in the goat CNS and peripheral tissues. Furthermore, α 2DR plays a more important role in α 2R-mediated analgesia in goats than α 2BR and α 2CR, whereas α 2CR activation exerts a greater effect on body temperature than α 2BR and α 2DR.

Keywords: α 2 adrenoceptor subtypes; intracerebroventricular injection; intrathecal injection; analgesia; goat



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1. Introduction

α 2-Adrenergic receptor (α 2R) agonists are some of the most commonly used sedative analgesic drugs in veterinary medicine due to their availability, reversibility and rapid onset [1–3]. This class of drugs is commonly used as a premedication to sedate animals or as an anesthetic adjuvant to reduce the side effects of other drugs. In ruminants, α 2R agonists are often used alone in minor procedures, such as rectal exams, biopsies, or radiographs [4]. The combination of an α 2R agonist with a local anesthetic is typically used in more painful procedures, such as flank surgery, castration, or wound management [5]. The side effects of α 2R agonists include hypothermia, decreased heart rate and biphasic effects on blood pressure, which manifest as transiently increased blood pressure at an early stage and decreased blood pressure at a later stage [6].

Mechanistically, $\alpha 2R$ agonists exert their effects by reacting with presynaptic $\alpha 2R$ in the central nervous system and reducing neurotransmitter release through a negative feedback mechanism [7]. Previous studies have implicated the locus coeruleus, a major nucleus in the central nervous system, in the control of the vigilance and initiation of behavioral responses [8]. The sedative effect of $\alpha 2R$ agonists results from the suppression of neuronal signals from the locus coeruleus [9]. The analgesic effect of $\alpha 2R$ agonists was reported to be achieved through interference with ascending nociceptive pathways in the dorsal horn of the spinal cord [10]. The $\alpha 2R$ agonist significantly decreases glutamate and substance p release from the dorsal horn of the spinal cord and thereby reduces nociceptive transmission. Supraspinal sites are also involved in $\alpha 2R$ agonist-mediated analgesic effects; Peng et al. indicated that $\alpha 2R$ in the periventricular gray is important for suppressing pain using the cell clamp technique [11]. Herbert et al. detected abundant $\alpha 2R$ in the parabrachial nucleus [12]. As the parabrachial nucleus is a relay center for pain signal transmission, it was postulated to be an important target that mediates $\alpha 2R$ agonist-induced analgesic effects. In addition, $\alpha 2R$ agonists also activate postsynaptic $\alpha 2R$ in effector organs and are mainly responsible for its cardiovascular effects [13]. Kästner et al. also reported that an $\alpha 2R$ agonist activates pulmonary intravascular macrophages (PIMs), a specialized group of macrophages found in ruminants, which are capable of producing a range of vasoactive mediators and cytokines [14]. One study showed the rapid onset of arterial hypoxemia and its complete reversal by $\alpha 2$ antagonists given before or within the first 5 min after xylazine administration [15], indicating that functional changes such as vasoconstriction and the redistribution of blood flow are responsible for the first stage of impaired oxygenation. It was suggested that alterations in pulmonary hemodynamics with significantly increased mean pulmonary artery pressure, pulmonary arterial occlusion pressure and estimated capillary pressure constitute the initial stage of $\alpha 2R$ agonist-induced pulmonary edema. The increased pressure may facilitate PIM activation and then lead to pulmonary edema and hypoxia [16].

$\alpha 2Rs$ are a group of G protein-coupled receptors located on the cell membranes of both neural and nonneural tissues. Researchers have confirmed that $\alpha 2Rs$ are heterogeneous receptors that consist of $\alpha 2A$, $\alpha 2B$, $\alpha 2C$ and $\alpha 2D$ subtypes, according to their pharmacological characteristics [14]. Ruminants express a special $\alpha 2DR$ subtype, which is the orthologue of $\alpha 2A$ subtypes in other species [15]. Only 45–55% similarity in the amino acid sequences of the $\alpha 2A/D$, $\alpha 2B$ and $\alpha 2C$ receptor subtypes has been observed. In addition, species differences in the expression and distribution of $\alpha 2R$ subtypes have been identified; for example, the human spinal cord expresses both $\alpha 2BR$ and $\alpha 2CR$ [17], while the rat spinal cord mainly expresses $\alpha 2CR$ [18]. Interestingly, the dosage of $\alpha 2R$ agonist in ruminants is only one-tenth of that in other animals; however, a low dose of $\alpha 2R$ agonist still induces significant side effects in ruminants, including a 50% decrease in cardiac output, hypothermia and hypotension [19]. Researchers have not yet determined whether this superior sedative and analgesic effect of $\alpha 2R$ agonists on ruminants is attributed to the distribution and density of the $\alpha 2R$ subtypes.

To the best of our knowledge, no scientific reports have documented the distribution and functional characteristics of $\alpha 2R$ subtypes in ruminants. A previous study used a radiolabeled $\alpha 2R$ agonist to investigate the distribution of $\alpha 2R$ in ruminants; however, these $\alpha 2R$ agonists are not truly subtype selective and do not differentiate subtypes. In the present study, we described the distribution, density and functional characters of $\alpha 2R$ subtypes in goats, which would help develop $\alpha 2R$ subtype-specific agonists that are more effective in analgesia and sedation, and exert fewer side effects on ruminants.

2. Materials and Methods

2.1. Animal Preparation

One- to two-year-old healthy local male goats (Yichang White Goat) weighing 23–28 kg were purchased from the Laboratory Animal Research Institute in Wuhan, China. All goats had free access to food and water and were dewormed and adapted to the environment for

7 days. The study was conducted under the ethical guidelines approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University (HZAUGO-2019-03) and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

2.2. Experimental Design

Thirty goats were used in this study. The goats were randomly divided into 5 groups ($n = 6$): nonspecific immunoglobulin (IgG) (Frdbio, Wuhan, China), yohimbine (Yoh) (Absin, Shanghai, China), $\alpha 2BR$ antibody ($\alpha 2BR$ Ab), $\alpha 2CR$ antibody ($\alpha 2CR$ Ab) and $\alpha 2DR$ antibody ($\alpha 2DR$ Ab) groups. Catheters were placed in the third ventricle and intrathecal space for 14 days. After 7 days of intubation, the same doses of $\alpha 2DR$ -, $\alpha 2BR$ - and $\alpha 2CR$ -specific neutralizing antibody (30 $\mu\text{g}/\text{kg}$) (Frdbio, Wuhan, China); nonspecific control immunoglobulin (30 $\mu\text{g}/\text{kg}$); and the $\alpha 2R$ nonselective antagonist yohimbine (3 $\mu\text{g}/\text{kg}$) were injected intrathecally or intracerebroventricularly in each group. After 30 min of injection, a low dose of xylazine (Absin, Shanghai, China) (0.05 mg/kg) was injected intramuscularly, and the nociception threshold at different time points after the xylazine injection was statistically analyzed.

2.3. Catheter Implantation Procedure

The goats were anesthetized with isoflurane and placed in a stereotaxic frame. A stainless-steel cannula (RWD, Shenzhen, China) was stereotaxically introduced into the third ventricle of each goat, as described by Qiu et al. [20]. The position of the cannula tip was confirmed in the third ventricle when CSF flowed out. The cannula was then fixed on the skull with acrylic dental cement. A plastic cap was then placed on the top of the cannula to prevent backflow of the CNS fluid when injecting $\alpha 2DR$ -, $\alpha 2BR$ - and $\alpha 2CR$ -specific neutralizing antibodies. The cap was removed, and a catheter was introduced into the cannulas for injection. For intrathecal catheter implant placement, stainless-steel cannulas were slowly inserted perpendicularly into the spinal column. Then, a catheter (RWD, Shenzhen, China) was slowly inserted approximately 10 cm into the intrathecal space through the lumen of the cannula. The cannula was then removed. The implanted catheter remained in place with 10 cm left outside the animal and stitched onto the animal. After catheter implant placement, the goats were administered 7000 U/kg penicillin via intramuscular injection daily for 4 consecutive days and then rested for two weeks.

2.4. Antibody Preparation

The gene sequences or corresponding deduced amino acid sequences of $\alpha 2DR$ (XM_018041717.1, XP_017897206.1 α), $\alpha 2BR$ (XM_018054838.1, XP_017910327.1 α) and $\alpha 2CR$ (XM_013964836.2, XP_013820290.2 α) were used as queries to search the National Center for Biotechnology Information database (<https://www.ncbi.nlm.nih.gov/>, accessed on 18 December 2018). The $\alpha 2R$ subtypes from goats were aligned, and each subtype from human, mouse, cattle and goat was compared, as demonstrated in Figures S1–S4. The extracellular domains of the $\alpha 2DR$, $\alpha 2BR$ and $\alpha 2CR$ proteins were predicted with TMHMM Server V. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM/>, accessed on 27 February 2022). The immunogenicity of the extracellular domain of the proteins was analyzed using DNASTar software (DNASTar Inc, Madison, WI, USA). The extracellular amino acid sequence that exhibited high immunogenicity was then determined to generate neutralizing antibodies. The sequences of anti- $\alpha 2DR$, anti- $\alpha 2BR$ and anti- $\alpha 2CR$ peptides were as follows: FRREQPLAEGSFAPMGSLQPDAGC, DHQEPYSVQATAAC and GAPWGP PPGQYSAGC, respectively. The peptides were synthesized and purified by Frdbio (Wuhan, China). Synthetic antigenic peptides were conjugated to keyhole limpet hemocyanin (KLH) protein, and each was used to immunize rabbits. Male New Zealand White rabbits (3 to 4 kg) were immunized subcutaneously with 500 μg of the conjugated protein in Freund's complete adjuvant (Sigma-Aldrich Inc, St. Louis, MO, USA). Three booster injections were administered subcutaneously at 2-week intervals with the same amount of conjugated protein emulsified

in Freund's incomplete adjuvant. The rabbits were bled after the fourth immunization. The polyclonal antibodies against $\alpha 2DR$, $\alpha 2BR$ and $\alpha 2CR$ were then purified using Protein A affinity chromatography (Frdbio, Wuhan, China) for use in subsequent experiments.

2.5. Nociception Threshold Determination

The basic nociception threshold was measured before the intracerebroventricular or intrathecal injection. Goats were lightly tied to a padded rack. The goats were standing during drug injections and pain threshold testing. Changes in the nociception threshold were measured every 10 min after injections by recording potassium iontophoresis and the hindlimb withdrawal threshold (HWT) [21,22]. Before the measurement, the skin in the central area of the left abdomen of the goat was shaved, washed with soap and 75% alcohol, degreased and disinfected. When measuring the nociception threshold with the DL-ZII DC induction electrotherapy machine (Shantou Medical Equipment Factory Co., Ltd., Shantou, China), two acupuncture probe electrodes dipped in a saturated potassium chloride solution were adhered tightly to the left abdomen skin with an interval of approximately 2–3 cm. The voltage intensity was gradually increased and stopped when the tested skin and muscles started to tremble and shrink. The voltage intensity at this time point was measured as the goat nociception threshold.

The HWT method was used to measure the reflex nociception threshold. Before the measurement, the tension sensor (connected to the tactile measurement kit) (ZS Dichuang Science and Technology Development Co., Ltd., Beijing, China) was calibrated with a 30 g standard weight. A tactile measurement kit was used to stimulate the right hind leg of the goat slowly with the intensity increasing from light to strong. When the goat showed a retraction reflex, mechanical stimulation stopped automatically. At this time point, the intensity value measured by the tension sensor was determined as the nociception threshold.

After the xylazine injection, the nociception threshold was measured every 10 min and recorded as the nociception thresholds T_n and V_n at the time point (each method was repeated 3 times, with an interval of 2 min). Before the antibody injection, the mechanical nociception threshold and potassium iontophoresis nociception threshold were repeatedly measured three times as the basic values T_0 and V_0 . A statistical analysis was performed to evaluate the nociception threshold at different time points after the xylazine injection.

2.6. Measurement of Physiological Indices

The basic physiological indicators of goat were measured before the intraventricular (intrathecal) drug injection and recorded every 10 min after the antibody was injected. Xylazine was injected 40 min after the antibody. We recorded the parameters for 1 h after the xylazine injection, i.e., the measurement in the whole process of the environment lasted 100 min. The physiological indicators, including body temperature, heart rate, respiratory rate, blood oxygen saturation and blood pressure, were measured 3 times, and the average values were calculated as the results for each time point. Blood oxygen saturation was monitored with a Mindray PM-9000 portable multiparameter ECG monitor (Mindray Biomedical Electronics Co., Ltd., Shenzhen, China). Heart rates were monitored with an electrocardiogram monitoring unit. Blood pressure was monitored with a blood pressure monitoring unit and a suitable size cuff, which connected the cuff vent and wrapped around the upper left knee joint of the goat. The sensor was placed in the position corresponding to the femoral artery of the goat. Blood pressure was measured three times at each time point, and the average of three blood pressure values was recorded as the measurement result. Blood oxygen saturation was monitored using a portable blood oxygen saturation meter. During the experiment, the photoelectric sensor (ear tongue clip) was clamped onto the ear of the tested goat, and the value was measured three times at each time point.

2.7. Sample Collection

Three goats were euthanized with 2,4-xylene thiazole. The goat brain and spinal cord L4-L6 were immediately removed from the skull and lumbar spinal canal, respectively.

The nuclei and brain area were collected with 4–10 cm diameters in DEPC-treated plastic tubes based on protocols described in our previous work [20]. The following nuclei and regions related to pain signal transmission and modulation were selected in this study (Figure 1): forebrain: caudate putamen nucleus (Cpu), nucleus accumbens (Acb), paraventricular thalamus (PVT), hypothalamic paraventricular nucleus (PVN), arcuate nucleus (Arc), amygdala (Amy), ventromedial hypothalamic nucleus (VMH), hippocampus (Hip), thalamic nucleus submedius (Sm), habenular nucleus (Hb) and parafascicular nucleus (Pf); midbrain: substantia nigra (SN) and periaqueductal gray (PAG); hindbrain: parabrachial nucleus (PBN), locus coeruleus (LC), nucleus raphe magnus (NRM), gigantocellular reticular nucleus (GRN) and nucleus of the solitary tract (NTS); and spinal cord L4-L6 dorsal horn (SCDH).

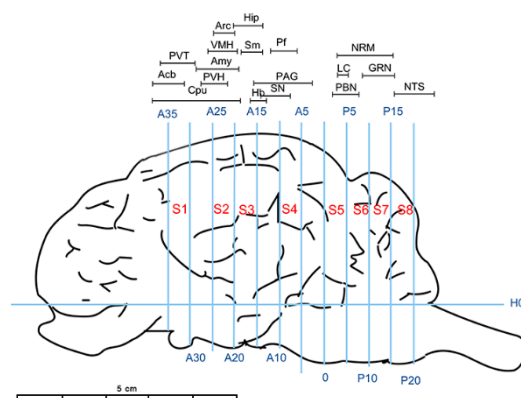


Figure 1. Brain section: H0: the horizontal zero plane. The 0 plane represents the interaural line. A35, A25, A20, A15, A10 and A5 show transverse planes located 35, 30, 25, 20, 15, 10 and 5 mm rostral to the interaural line, and P5, P10, P15 and P20 show transverse planes located 5, 10, 15 and 20 mm caudal to the interaural line, respectively. S1–8, take the base edge of the olfactory bulb as the starting line, and make a sagittal plane, each 5mm. The locations of nuclei or areas in the brain blocks are presented at the top of the figure. The nuclei and areas identified include the caudate putamen nucleus (Cpu), nucleus accumbens (Acb), paraventricular thalamus (PVT), hypothalamic paraventricular nucleus (PVN), arcuate nucleus (Arc), amygdala (Amy), ventromedial hypothalamic nucleus (VMH), hippocampus (Hip), thalamic nucleus submedius (Sm), habenular nucleus (Hb), parafascicular nucleus (Pf), substantia nigra (SN), periaqueductal gray (PAG), parabrachial nucleus (PBN), locus coeruleus (LC), nucleus raphe magnus (NRM), gigantocellular reticular nucleus (GRN) and nucleus of the solitary tract (NTS).

Peripheral tissues, including the myocardium (Myo), liver, spleen, lung, kidney, aorta, superior vena cava (SVC), inferior vena cava (IVC), arterioles, venules and blood mononuclear macrophages (M ϕ s), were collected to investigate the distribution and density of α 2DR, α 2BR and α 2CR.

Blood mononuclear macrophages were isolated from heparinized blood samples using the instructions provided in the goat peripheral blood mononuclear cell separation kit (P5290, Solarbio, Beijing, China). The cells obtained through density gradient centrifugation were resuspended in monocyte media at a density of 106 cells/mL, plated on cell culture plates and placed in a cell incubator for adherent culture. After 3 h, all nonadherent cells were removed, and the adherent cells were blood mononuclear macrophages.

2.8. RNA Isolation and Real-Time Quantitative PCR

After the tissue was sampled, it was ground in liquid nitrogen and treated with 1 mL of TRIzol (ThermoFisher Scientific, Waltham, MA, USA) reagent. Then, 200 μ L of chloroform was added and incubated at 25 $^{\circ}$ C for 15 min. The sample mixture was centrifuged at 12,000 \times g for 10 min at 4 $^{\circ}$ C. The supernatant was mixed with an equal amount of isopropanol. After gentle inversion, the sample was frozen at -20 $^{\circ}$ C for 30 min

and then centrifuged at $12,000 \times g$ for 10 min at 4°C . Subsequently, the sample was washed twice with precooled 75% ethanol, and then DEPC water was added to dissolve the total RNA to $1000 \text{ ng}/\mu\text{L}$. Then, reverse transcription PCR was performed. Real-time qPCR was performed using SYBR[®] Green I according to the manufacturer's protocol (ABclonal Technology, RK21203, Woburn, MA, USA). The relative expression of the target gene relative to the housekeeping gene (GAPDH) was determined using the Cq method. The average value of the Cq values of different groups was used as $\Delta\Delta\text{Ct}$, and the relative quantification was calculated using the equation $\text{RQ} = 2^{-\Delta\Delta\text{Ct}}$. All samples were measured in triplicate.

2.9. Protein Separation and Western Blot Analysis

The collected tissues were ground in liquid nitrogen, and the protein was extracted with RIPA buffer containing PMSF and phosphatase inhibitors for 30 min according to the manufacturer's instructions (Beyotime, P0013B, Shanghai, China). The protein concentration was then analyzed using a BCA protein detection kit (Beyotime, P0010, Shanghai, China). Thirty micrograms of each protein sample was separated on a 10% SDS-PAGE gel and then transferred to a polyvinylidene fluoride membrane (Life Technologies, ThermoFisher Scientific, Waltham, MA, USA). After an incubation with 5% skim milk for 2 h at 25°C , the membrane was immunolabeled with a primary antibody at 4°C overnight. The membrane was washed with TBST buffer and then incubated with $\alpha 2\text{B}$ -, $\alpha 2\text{C}$ - and $\alpha 2\text{D}$ -specific multiclonal antibodies for 2 h at 25°C . After washes with TBST buffer, the membrane was treated with horseradish peroxidase substrate, and then the signal was detected with a chemiluminescence imaging system.

2.10. Statistical Analysis

All data were statistically analyzed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA). The data are presented as the means \pm SD. The pain threshold and physiological indicator data were analyzed using two-way ANOVA followed by Bonferroni's post hoc test. The decreases in body temperature were analyzed using one-way ANOVA followed by Bonferroni's post hoc test. The least significant difference (LSD) test was used for multiple comparisons. * Indicates a significant difference between the indicated groups: * $p < 0.05$ and ** $p < 0.013$.

3. Results

3.1. Real-Time Quantitative PCR Analyses of $\alpha 2\text{DR}$, $\alpha 2\text{BR}$ and $\alpha 2\text{CR}$ mRNA Abundance in Selected CNS Nuclei and Regions, as well as Peripheral Tissues

The levels of the $\alpha 2\text{DR}$, $\alpha 2\text{BR}$ and $\alpha 2\text{CR}$ mRNAs were detected in different nuclei and regions in the CNS and peripheral tissues of goats to assess the distribution of $\alpha 2\text{DR}$, $\alpha 2\text{BR}$ and $\alpha 2\text{CR}$ in the CNS and peripheral tissues in goats (Figure 2). The relative fold change in the expression of each $\alpha 2\text{R}$ mRNA within a specific CNS nucleus or peripheral tissue was calculated as the ratio of their expression to that of a housekeeping gene (GAPDH). The mRNAs encoding the receptor subtypes were widely but unevenly distributed in different tissues.

In pain-related nuclei, the $\alpha 2\text{BR}$ mRNA (Figure 2A) was distributed at relatively high levels in the Cpu, Acb, PVT, PVN, Arc and SCDH. The $\alpha 2\text{BR}$ mRNA was abundant in the following goat peripheral tissues: arteriole, spleen, aorta and venule ($p < 0.05$).

The mRNA encoding $\alpha 2\text{C}$ (Figure 2B) in the Cpu and PVT was among the pain-related nuclei and regions. The Acb, Arc and Amy also exhibited high levels of $\alpha 2\text{C}$ mRNA but lower levels than the Cpu and PVT. Among the peripheral tissues, a higher $\alpha 2\text{CR}$ mRNA abundance was detected in the spleen, lung, SVC, arterioles and venule than Myo, hepatic, kidney, aorta and M ϕ tissues ($p < 0.5$).

Figure 2C shows a more equal distribution of the $\alpha 2\text{DR}$ mRNA between various nuclei in the CNS, with relatively lower levels in the Cpu, Acb and Pvt. The $\alpha 2\text{DR}$ mRNA was most abundant in the goat arteriole among the peripheral tissues, and significant levels were also detected in the goat kidney, aorta, SVC, IVC, venule and M ϕ ($p < 0.5$).

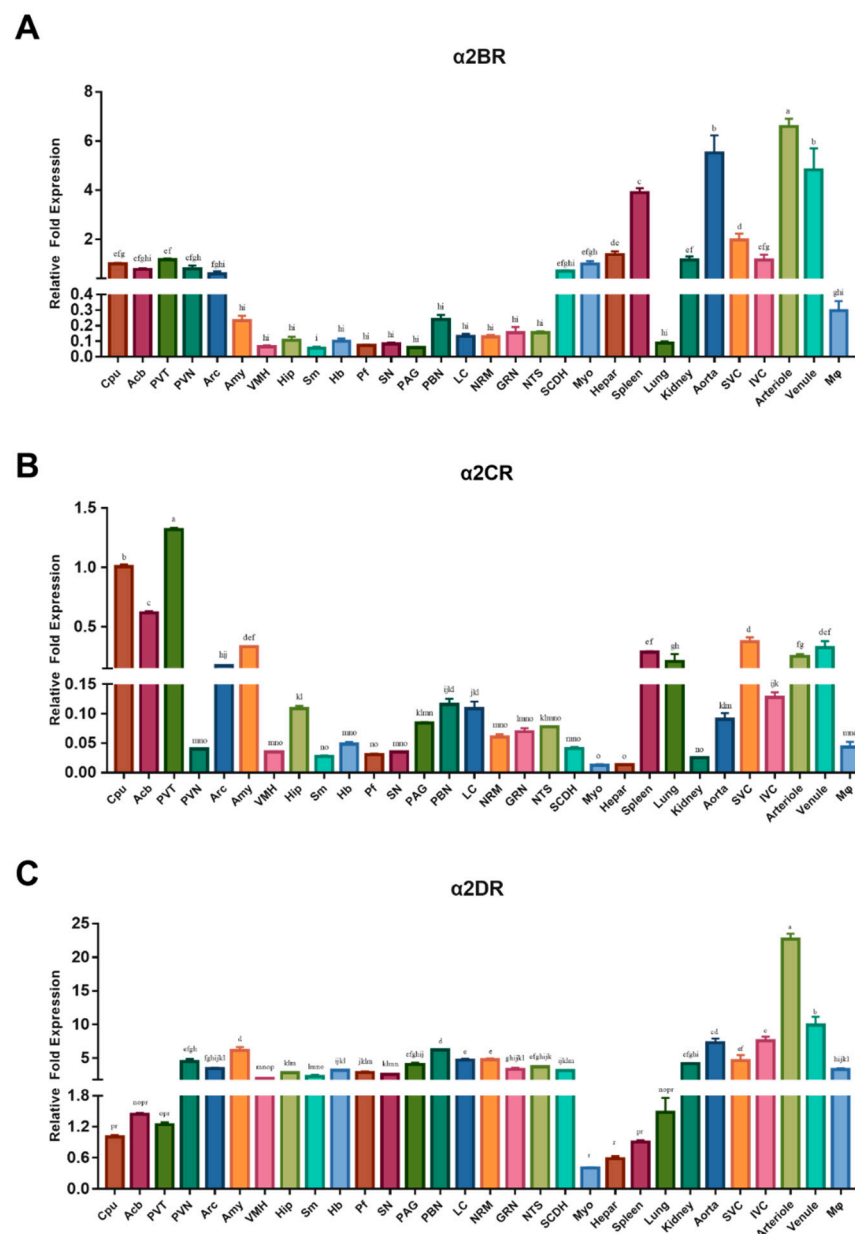


Figure 2. Real-time qPCR analyses of $\alpha 2BR$ (A), $\alpha 2CR$ (B) and $\alpha 2DR$ (C) mRNA abundances in the Cpu, Acb, PVT, PVN, Arc, Amy, VMH, Hip, Sm, Hb, Pf, SN, PAG, PBN, LC, NRM, GRN, NTS, SCDH, Myo, liver, spleen, lung, kidney, aorta, SVC, IVC, arterioles, venules and M ϕ of goats. The results were normalized to GAPDH as a housekeeping gene and are presented as the means \pm SD ($n = 3$). Bars with different letters differ ($p < 0.05$).

Simultaneously, we also calculated and compared the relative levels of $\alpha 2DR$, $\alpha 2BR$ and $\alpha 2CR$ mRNAs within each selected CNS nucleus and peripheral tissue in goats (Figure S2). The $\alpha 2DR$ mRNA was expressed at significantly higher levels in the PVN, Sm, Pf, PAG, LC, NRM, SCDH, kidney, aorta, IVC, arterioles and M ϕ than in the $\alpha 2BR$ and $\alpha 2CR$ ($p < 0.05$). The $\alpha 2CR$ mRNA was more abundant in the Cpu, Acb, PVT, Arc, Amy, Hip, spleen, lung, SVC and venule than $\alpha 2BR$ and $\alpha 2DR$ mRNAs ($p < 0.05$). Lower levels of the $\alpha 2BR$ mRNA were detected in most central nervous tissues than levels of the $\alpha 2CR$ and $\alpha 2DR$ mRNAs, except for the GRN and SCDH. In peripheral tissues, the $\alpha 2BR$ mRNA was expressed at higher levels than the $\alpha 2DR$ mRNA in the spleen and lungs and at higher levels than both the $\alpha 2CR$ and $\alpha 2DR$ mRNAs in macrophages and the liver.

3.2. Western Blot Analyses of α 2DR, α 2BR and α 2CR mRNA Abundances in Selected CNS Nuclei and Regions, as well as Peripheral Tissues

Western blot analyses were performed in this study to further confirm the relative levels of α 2DR and α 2BR and α 2CR in goat CNS nuclei, regions and peripheral tissues. Figure 3A–C demonstrates the Western blotting results of The relative expression level was defined as the ratio of the protein level of each α 2R subtype to the housekeeping protein. The pain-related nuclei that we selected were distributed in the forebrain, midbrain and hindbrain. Similar to the mRNA results, proteins for all three α 2R subtypes were detected in the selected nuclei, spinal cord and peripheral tissues.

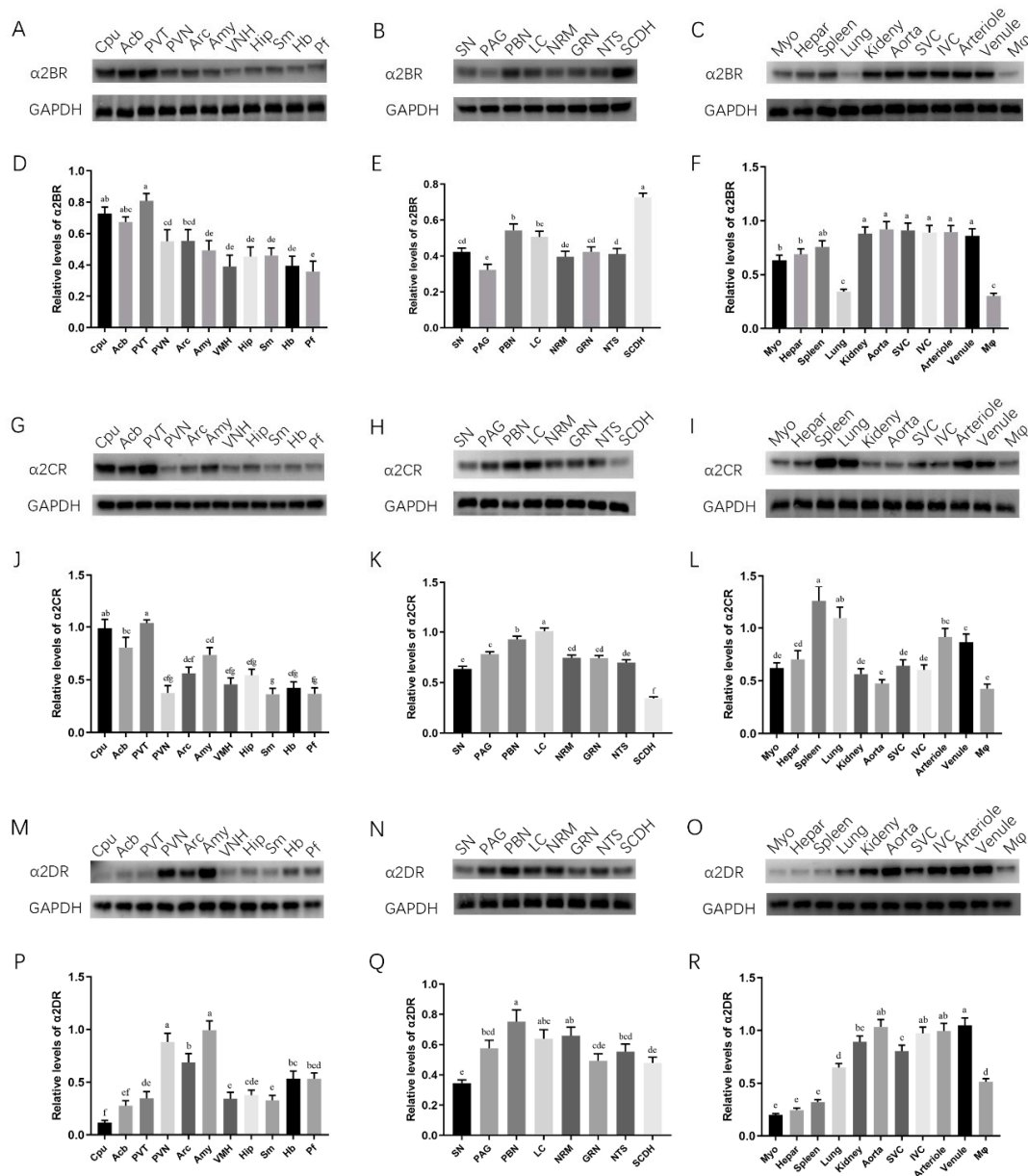


Figure 3. Western blot analyses of α 2DR, α 2BR and α 2CR protein abundances in the Cpu, Acb, PVT, PVN, Arc, Amy, VMH, Hip, Sm, Hb, Pf, SN, PAG, PBN, LC, NRM, GRN, NTS, SCDH, Myo, liver, spleen, lung, kidney, aorta, SVC, IVC, arterioles, venules and M ϕ of goats. (A–C) Representative bands for α 2BR and GAPDH. (G–I) Representative bands for α 2CR and GAPDH. (M–O) Representative bands for α 2DR and GAPDH. (D–F, J–L, P–R) Densitometry analyses of protein bands. Data were normalized to GAPDH as the housekeeping protein and are presented as the means \pm SD ($n = 3$). Bars with different letters differ ($p < 0.05$) Original Western blot figures are in Figure S5.

In the forebrain (Figure 3A,D,G,J,M,P), the protein levels of α 2BR and α 2CR were predominantly detected in the Cpu, Acb and PVN, whereas α 2DR was mainly distributed in the PVT, Arc and Amy. α 2CR and α 2DR had relatively equal distributions in the midbrain, hindbrain and SCDH; nevertheless, α 2BR was expressed at higher levels in the SCDH than in the midbrain and hindbrain. Regarding the peripheral tissues, α 2CR was predominantly expressed in the spleen, lung, arteriole and venule, and a high abundance of α 2DR was detected in the aorta, IVC arteriole and venule, whereas α 2BR showed a relatively equal distribution. These results were generally consistent with the mRNA data and indicated that α 2R subtypes are unevenly expressed in different CNS nuclei and tissues.

3.3. α 2R-Mediated Analgesia in the Goat Spinal Cord

The characteristics of α 2R-mediated analgesia in the goat spinal cord were explored by intrathecally injecting the neutralizing antibodies (Frdbio, Wuhan, China) against the α 2R subtypes (30 μ g/kg), nonspecific immunoglobulin (30 μ g/kg) and yohimbine (3 μ g/kg) in each goat according to the group (IgG, Yoh, α 2BR Ab, α 2CR Ab and α 2DR Ab groups), followed by an intramuscular injection of xylazine (0.05 mg/kg) 30 min after the antibody injection. The potassium iontophoresis threshold and HWT threshold were recorded every 10 min, as shown in Figure 4. Yoh was used as an α 2R antagonist in the control group, and neither the potassium iontophoresis threshold nor the HWT threshold changed significantly with time, as shown in Figure 4, indicating that the effect of xylazine was blocked by yohimbine.

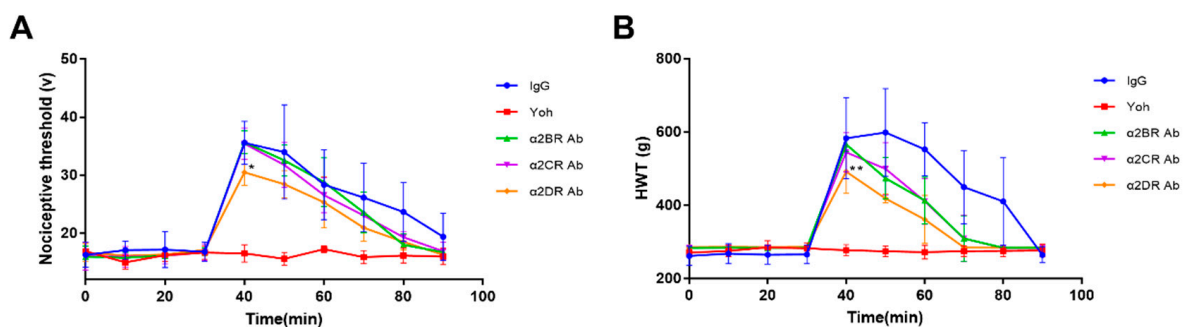


Figure 4. The effect of different α 2R subtypes expressed in the spinal cord on the pain threshold of goats. Potassium iontophoresis (A) and HWT (B) thresholds of the tested goats represent the effect of the intrathecal injection of α 2R subtype antibody on the potassium iontophoresis and HWT thresholds of goats, respectively. Data are presented as the means \pm SD ($n = 6$ in each group). * $p < 0.05$ compared with the IgG group and ** $p < 0.01$ compared with the IgG group.

The peak effect of xylazine occurred at 40 min in every group except for the Yoh group. The α 2DR Ab group showed the lowest value for the peak effect in both Figure 4A,B, and a significant difference was not observed between the IgG group and the α 2BR Ab or the α 2CR Ab group ($p > 0.05$). Based on these results, α 2DR expressed in the spinal cord may play a major role in α 2R-mediated analgesia in goats.

3.4. Effect of α 2Rs Expressed in the Spinal Cord on Physiological Indices of Goats

The effects of α 2Rs expressed in the spinal cord on the physiological indices of goats were assessed by monitoring the physiological indicators in goats from each group before and after the intrathecal injection of antibodies.

The HR decreased slightly in the IgG group and α 2CR antibody group after the xylazine injection, but still fluctuated in a normal range. SPO2 generally did not show obvious fluctuations, with relatively lower values observed in the IgG and Yoh groups. NIBP-SYS, -MEAN and DIA started to decrease in all groups within 20–30 min after the xylazine injection (time 30) by no more than 20 mmHg and then began to slowly increase (Figure 5A–G). Furthermore, the temperature showed a slowly decreasing trend in all

groups after the xylazine injection. The value for the decrease in temperature in each group was also calculated (Figure 5H), showing no distinct differences between different groups.

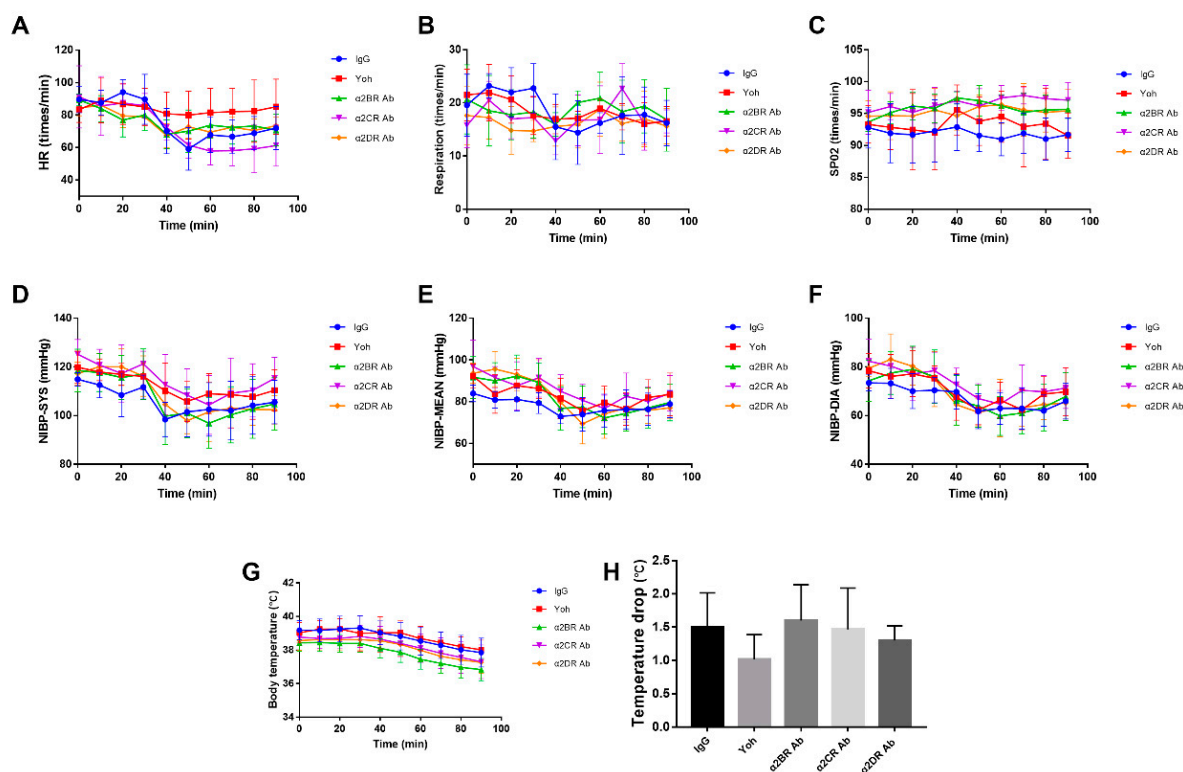


Figure 5. Monitoring of physiological indices in the goats from each group. (A–G) Effects of different α 2R subtypes expressed in the goat spinal cord on HR, RESP, SPO2, NIBP-SYS, NIBP-MEAN, NIBP-DIA and body temperature. (H) Decreased body temperature in the goats of each group. Data are presented as the means \pm SD ($n = 6$ in each group).

3.5. α 2R-Mediated Analgesia in the Goat Brain

The characteristics of α 2R-mediated analgesia in the goat brain were also explored with a similar method, i.e., intracerebroventricularly injecting the neutralizing antibodies against the α 2R subtypes (30 μ g/kg), nonspecific immunoglobulin (30 μ g/kg) and yohimbine (3 μ g/kg) within each goat according to the group (IgG, Yoh, α 2BR Ab, α 2CR Ab and α 2DR Ab groups) followed by an intramuscular injection of xylazine (0.05 mg/kg) 30 min after the antibody injection. Similar results were obtained as in the spinal cord experiment. As showed in Figure 6, the peak effect of xylazine at 40 min was significantly lower in the α 2DR Ab group than in the IgG, α 2BR and α 2CR Ab groups ($p < 0.01$), whereas no significant difference was observed between the IgG group and the α 2BR or α 2CR Ab group ($p > 0.05$). Thus, among the three α 2R subtypes of goats, α 2DR expressed in the goat brain plays a major role in the analgesic effects.

We also monitored the physiological indicators in goats from each group after the intracerebroventricular injection of antibodies and xylazine to investigate the effects of α 2Rs expressed in the brain on the physiological indices of the goats. The physiological indicators also included HR, RESP, SPO2, NIBP-SYS, NIBP-MEAN, NIBP-DIA and body temperature. All physiological indices monitored throughout the experiment were within the normal range and exhibited small fluctuations (Figure 7A–G).

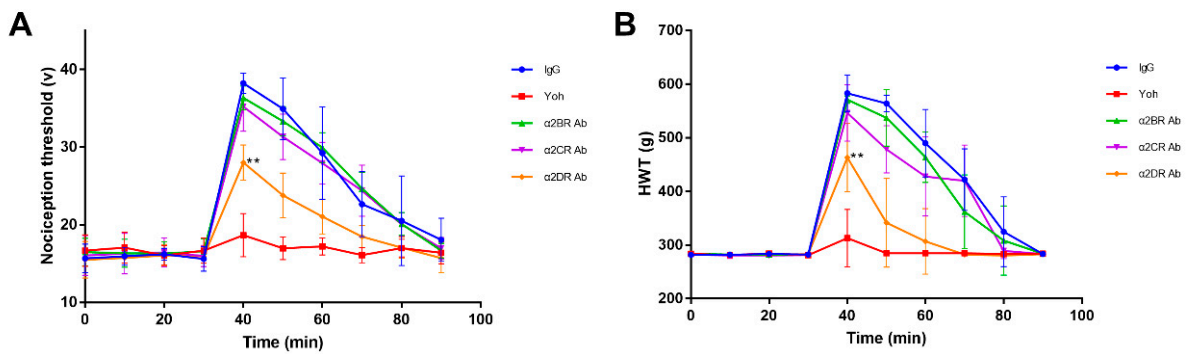


Figure 6. The effects of different $\alpha 2R$ subtypes expressed in the brain on the pain threshold of goats. (A,B) Effects of the intrathecal injection of $\alpha 2R$ subtype-specific antibodies on potassium iontophoresis and the HWT threshold of goats, respectively. Data are presented as the means \pm SD ($n = 6$ in each group). ** $p < 0.01$ compared with the IgG group. 3.6 Effects of $\alpha 2Rs$ expressed in the brain on the physiological indices of goats.

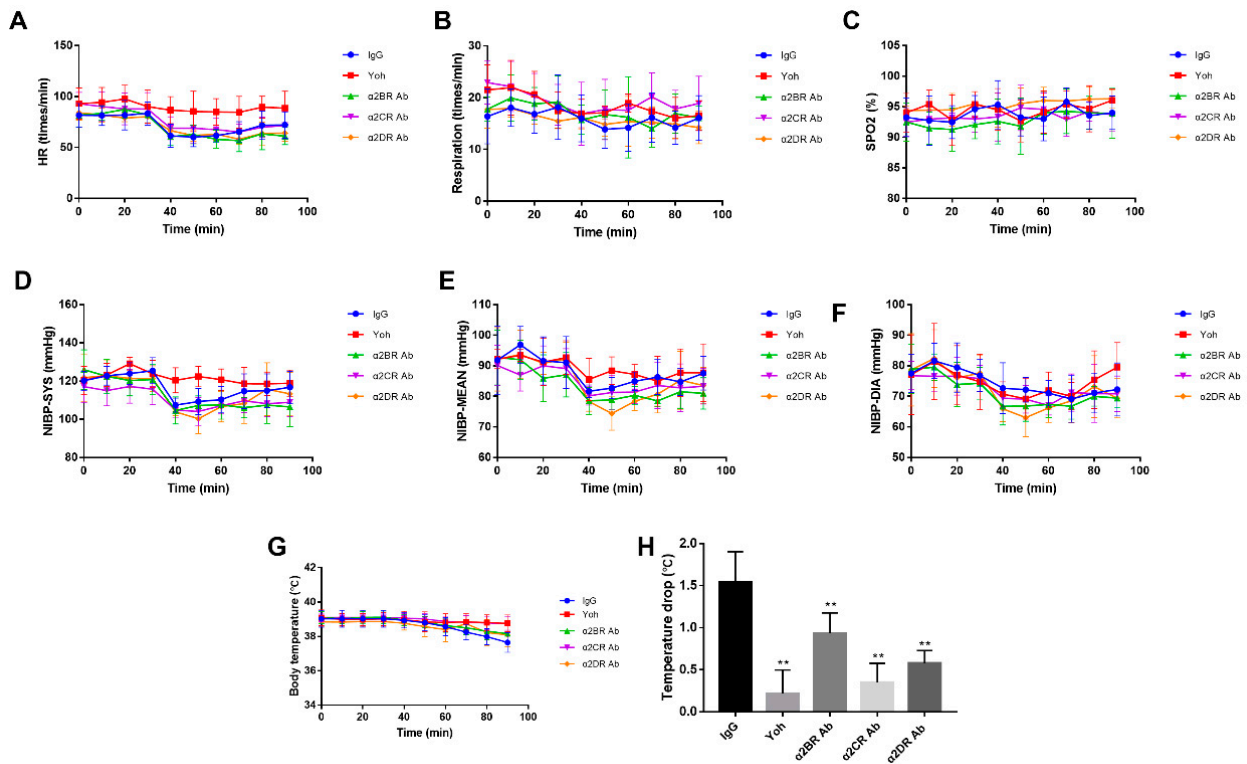


Figure 7. Effects of different $\alpha 2R$ subtypes expressed in the brain on the physiological indices of goats. (A–G) Effects of different $\alpha 2$ -AR subtypes expressed in the goat brain on HR, RESP, SPO2, NIBP-SYS, NIBP-MEAN, NIBP-DIA and body temperature. (H) The range of decrease in the body temperature of goats from each group. Data are presented as the means \pm SD ($n = 6$ in each group). ** $p < 0.01$ compared with the IgG group.

HR started to decrease slightly after the xylazine injection in all groups except the Yoh group and remained at a lower level until the end of the experiment. No distinct changes in RESP and SPO2 were noticed in any group. IgG, $\alpha 2BR$, $\alpha 2CR$ and $\alpha 2DR$ Ab groups exhibited slight decreases in NIBP-SYS, MEAN and DIA 10 to 20 min after xylazine was injected, and then the values gradually increased after the nadir was reached. Similar to the results from the intrathecal injection experiment, all groups except the Yoh group also showed decreasing trends in these parameters. After the intraventricular injection of xylazine, the body temperature gradually and continuously decreased in all groups except

the Yoh group, starting at 30 to 40 min till the end of the experiment, but the difference was insignificant ($p < 0.01$). The $\alpha 2CR$ antibody group presented the greatest decrease, as shown in Figure 7H. Therefore, blocking $\alpha 2Rs$ in the goat brain ameliorates the decrease in body temperature induced by xylazine, and $\alpha 2BR$ plays a more important role in modulating body temperature.

4. Discussion

$\alpha 2R$ agonists are some of the most commonly used sedative and analgesic drugs in ruminants [23]. Interestingly, the dose of $\alpha 2R$ agonists is less than one-tenth of that in other animals, while side effects in ruminants are still very prominent [19]. The present study is the first to report the distribution and function of $\alpha 2DR$, $\alpha 2BR$ and $\alpha 2CR$ in goats. These results not only contribute to explaining the characteristics of the effects of $\alpha 2R$ agonists on ruminants but also lay the foundation for developing $\alpha 2R$ agonists with high $\alpha 2R$ subtype specificity, which may increase sedative and analgesic effects and decrease side effects in ruminants.

In our study, the mRNA results were generally consistent with the Western blot results. All three $\alpha 2R$ subtypes were detected in the goat brain, and the $\alpha 2DR$ and $\alpha 2CR$ subtypes were the most abundant. These results are similar to those of a previous report examining humans and rats. Harrison et al. reported that $\alpha 2AR$, which is the orthologue of $\alpha 2DR$ [24], and $\alpha 2CR$ are the dominant subtypes expressed in the brains of rats and humans [25,26]. $\alpha 2AR$ and $\alpha 2CR$ are both adrenoreceptors expressed in noradrenergic neurons and usually coexist in the same area in the central nervous system [27]. Interestingly, the $\alpha 2DR$ and $\alpha 2BR$ mRNAs were expressed at high levels in the spinal cord, in contrast to a previous report that the human spinal cord expresses both $\alpha 2CR$ and $\alpha 2BR$, while the rat spinal cord predominantly expresses $\alpha 2CR$ subtypes.

Regarding the differences in $\alpha 2R$ subtypes at the nuclear level in the brain, the mRNA and Western blot results revealed different ratios of $\alpha 2R$ subtypes in the different nuclei. $\alpha 2AR$ was the dominant subtype in most nuclei tested in this study, except for the CPu, Arc, Amy, Acb, hip and PVT, where $\alpha 2CR$ was the main subtype expressed. The Amy, AcB and Hip belong to the limbic system, and the Cpu and Arc are regions of the basal ganglia. Both the limbic system and basal ganglia are related to pain signal modulation in a chronic pain status [28]. The high expression level of $\alpha 2CR$ in these areas makes it a potential therapeutic target for treating chronic pain in the future. In addition, the level of the $\alpha 2BR$ mRNA was much lower than that of the $\alpha 2DR$ and $\alpha 2CR$ subtypes in our study. A previous report showed that $\alpha 2BR$ expression increases in response to neuropathic pain [29]. Thus, $\alpha 2BR$ is an inducible receptor that may play a role in pathology. Recent studies also confirmed that $\alpha 2R$ agonists are effective treatments for neuropathic pain [30,31]; however, the role of $\alpha 2R$ subtypes in this chronic pain disease is unclear, and related studies are still ongoing.

The distribution of $\alpha 2DR$, $\alpha 2BR$ and $\alpha 2CR$ was also investigated in peripheral tissues, such as the kidney, liver, aorta, vena cava and macrophages. Our results showed a distinct expression pattern of $\alpha 2R$ subtypes in different areas of the vascular system. $\alpha 2BR$ is the major subtype expressed in myocardium. $\alpha 2DR$ is predominantly expressed in aortas and arterioles, whereas $\alpha 2CR$ is mainly expressed in the vena cava and venules of goats. In humans, $\alpha 2CR$ is mainly responsible for venous constriction, and $\alpha 2AR$ is responsible for arterial constriction [32], consistent with our findings in goats. However, in dogs, $\alpha 2AR$ is mainly detected in veins [33]. The myocardium of goats expressed mainly $\alpha 2BR$ and $\alpha 2CR$ in our study; however, Regitz-Zagrosek reported that the human myocardium mainly expresses $\alpha 2CR$ [34] and that mice mainly express $\alpha 2AR$ [35]. These results indicated species variation in the $\alpha 2R$ distribution pattern. Notably, although the expression level of $\alpha 2BR$ is relatively low in the nervous system, it is predominant in the liver. However, the biological function of $\alpha 2BR$ in the liver is still unknown. A large dose of $\alpha 2R$ agonist was reported to activate pulmonary intravascular macrophages (PIMs) and cause severe pulmonary edema in ruminants [14]. PIMs are not able to be isolated in large amounts, because they originate from peripheral mononuclear cells. We isolated mononuclear cells

from the blood as a surrogate of PIMs. Mononuclear cells mainly expressed $\alpha 2DR$ and $\alpha 2CR$, but did not express $\alpha 2BR$. Therefore, $\alpha 2R$ agonists that are more selective for $\alpha 2BR$ may be less likely to induce pulmonary edema in the ruminant. In addition, a balanced anesthesia could decrease the dosage of alpha2 agonist and reduce pulmonary edema in clinical practice.

The injection of alpha 2 adrenergic receptor agonists such as xylazine will exert certain side effects on goats, such as a lower body temperature, excessive salivation and an initial increase in blood pressure followed by a decrease. In the $\alpha 2CR$ antibody intracerebroventricular injection group, the body temperature of goats did not decrease significantly 90 min after the $\alpha 2CR$ antibody injection, while no difference in body temperature was observed between the $\alpha 2CR$ antibody intrathecal injection group and other five groups. Based on this result, $\alpha 2CR$ activation exerts a greater effect on body temperature than $\alpha 2BR$ and $\alpha 2DR$ activation. The thermoregulation center is located in the hypothalamus, and the insignificant decrease in temperature after the injection of $\alpha 2CR$ antibody may be due to the low expression of $\alpha 2CR$ in the hypothalamus. In our experiment, the hypothalamic nuclei PVN, Arc and VMH expressed $\alpha 2CR$ at lower levels than the other two receptors, which also supports this result to a certain extent. However, the blood pressure of the goats did not first increase and then decrease in the experiment. Since the effect of $\alpha 2R$ agonists on blood pressure is related to its dose [36] and the dose of xylazine used in the experiment was lower than the clinical dose, biphasic blood pressure changes in goats could not be detected.

After the xylazine injection, the pain threshold of potassium iontophoresis and HWT increased and peaked after 10 min. By comparing the pain thresholds of each group at this time point, we found that blocking $\alpha 2DR$ in both the intrathecal injection experiment and the intracerebroventricular injection experiment antagonized the effect of xylazine on the pain threshold, while no significant difference in the effects of the antagonists of the other two subtypes were observed compared with the IgG group. Based on our previous research, we selected 11 pain-related nuclei in the forebrain, 2 in the midbrain and 5 in the hindbrain, and measured the distribution and expression of different subtypes of $\alpha 2R$. Among them, the PAG–Cpu–Amy–Hb–PAG constitutes the limbic analgesia circuit. The PAG–rostral ventromedial medulla (RVM)–dorsal horn of the spinal cord circuit is the main part of the brainstem descending inhibitory pathway [37]. The Sm–ventral lateral orbitofrontal cortex (VLO)–PAG pathway has also been identified. These three pathways play important roles in regulating pain or nociception. Combined with the results of the antibody injection test, the expression of $\alpha 2DR$ in the PAG, NRM, GRN, NTS and SCDH, which play important roles in the descending inhibitory pathway of pain, was higher than that of the other two $\alpha 2R$ subtypes. Similarly, the PAG, Amy and Hb, which play important roles in a mesolimbic loop of analgesia, expressed $\alpha 2DR$ at higher levels than the other two $\alpha 2R$ subtypes. The Sm, which plays an important role in the Sm–VLO–PAG pathway, displayed a significantly higher expression of $\alpha 2DR$ than the other two $\alpha 2R$ subtypes. We inferred that $\alpha 2DR$ plays a major role in $\alpha 2R$ -mediated analgesia in goats. Therefore, $\alpha 2DR$ -selective agonists may exert better analgesic effects on ruminants.

5. Conclusions

In summary, we demonstrate the presence and heterogeneity of $\alpha 2R$ subtype mRNA and protein in different nuclei of the goat brain, selected regions of the spinal cord (L4–L6) and various peripheral tissues. We also demonstrate the functional characteristics of $\alpha 2BR$, $\alpha 2CR$ and $\alpha 2DR$ in goats. Our study revealed that $\alpha 2DR$ is more essential for $\alpha 2R$ -mediated analgesia, whereas $\alpha 2CR$ activation exerts a greater effect on body temperature. Such information enables the development of $\alpha 2R$ agonists with high $\alpha 2R$ subtype specificity, which may increase sedative and analgesic effects and decrease side effects in ruminants. In addition, the elucidation of the relationship of $\alpha 2R$ subtypes to CNS pathways will be important in understanding the mechanisms of nociception, autonomic function and muscle tone at the CNS level.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/ani12050664/s1>, Figure S1: Amino acid sequence alignment of goat α 2-adrenergic receptor subtypes. α 2B, α 2C and α 2D subtypes; Figure S2: Amino acid sequence alignment of human (NP_000672.3), mouse (NP_031443.4), cattle (NP_776924.1), and goat (XP_017897206.1) α 2A/D-adrenergic receptor subtypes; Figure S3: Amino acid sequence alignment of human, mouse, cattle, and goat α 2B-adrenergic receptor subtypes; Figure S4: Amino acid sequence alignment of human, mouse, cattle, and goat α 2C-adrenergic receptor subtypes; Figure S5: Original western blot figure in Figure 3.

Author Contributions: Conceptualization: Y.D.; Project Administration: Y.D. and M.D.; Visualization: Q.W. and D.P.; Writing: M.X. and Y.D.; Experiments: M.X., Q.Z. and Q.W.; Supervision: Y.D. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: The study was conducted under the ethical guidelines approved by the Institutional Animal Care and Use Committee of Huazhong Agricultural University (HZAUGO-2019-03) and adhered to the guidelines of the Committee for Research and Ethical Issues of the International Association for the Study of Pain.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Abbreviations

α 2BR Ab	α 2BR antibody;
α 2CR Ab	α 2CR antibody;
α 2DR Ab	α 2DR antibody;
Acb	nucleus accumbens;
Amy	amygdala;
Arc	arcuate nucleus;
Cpu	caudate putamen nucleus;
GRN	gigantocellular reticular nucleus;
Hb	habenular nucleus;
Hip	hippocampus;
HWT	hindlimb withdrawal threshold;
IgG	nonspecific immunoglobulin;
IVC	inferior vena cava;
LC	locus coeruleus;
M ϕ	blood mononuclear macrophages;
Myo	myocardium;
NRM	nucleus raphe magnus;
NTS	nucleus of the solitary tract;
PBN	parabrachial nucleus;
Pf	parafascicular nucleus;
PAG	periaqueductal gray;
PVN	hypothalamic paraventricular nucleus;
PVT	paraventricular thalamus;
SCDH	spinal cord L4-L6 dorsal horn;
Sm	thalamic nucleus submedius;
SN	substantia nigra;
SVC	superior vena cava;
VMH	ventromedial hypothalamic nucleus;
Yoh	yohimbine

References

1. Bantel, C.; Maze, M.; Stone, L.; Wilcox, G. Alpha(α) 2-Adrenergic Agonists in Pain Treatment. In *Encyclopedia of Pain*; Springer: Berlin/Heidelberg, Germany, 2007; pp. 58–61.
2. Chan, A.K.; Cheung, C.W.; Chong, Y.K. Alpha-2 agonists in acute pain management. *Expert Opin. Pharmacother.* **2010**, *11*, 2849–2868. [[CrossRef](#)]
3. McKelvey, D.; Hollingshead, K.W. *Veterinary Anesthesia and Analgesia*, 3rd ed.; Mosby: St Louis, MO, USA, 2003.
4. Shah, Z.; Ding, M.-X.; Hu, M.-L. A review on the current use of alpha2 agonists in small ruminants. *Kafkas Univ. Vet. Fak. Derg.* **2014**, *20*, 633–639.
5. Carroll, G.L.; Hartsfield, S.M. General anesthetic techniques in ruminants. *Vet. Clin. N. Am. Food Anim. Pract.* **1996**, *12*, 627–661. [[CrossRef](#)]
6. Sinclair, M.D. A review of the physiological effects of alpha2-agonists related to the clinical use of medetomidine in small animal practice. *Can. Vet. J. Rev. Vet. Can.* **2003**, *44*, 885–897.
7. McCallum, J.B.; Boban, N.; Hogan, Q.; Schmelting, W.T.; Kampine, J.P.; Bosnjak, Z.J. The Mechanism of [alpha] 2-Adrenergic Inhibition of Sympathetic Ganglionic Transmission. *Anesth. Analg.* **1998**, *87*, 503–510. [[CrossRef](#)] [[PubMed](#)]
8. Rajkowski, J.; Kubiak, P.; Aston-Jones, G. Locus coeruleus activity in monkey: Phasic and tonic changes are associated with altered vigilance. *Brain Res. Bull.* **1994**, *35*, 607–616. [[CrossRef](#)]
9. Atzori, M.; Cuevas-Olguin, R.; Esquivel-Rendon, E.; Garcia-Oscos, F.; Salgado-Delgado, R.C.; Sadari, N.; Miranda-Morales, M.; Treviño, M.; Pineda, J.C.; Salgado, H. Locus coeruleus norepinephrine release: A central regulator of CNS spatio-temporal activation? *Front. Synaptic Neurosci.* **2016**, *8*, 25. [[CrossRef](#)]
10. Yaksh, T.L.; Pogrel, J.W.; Lee, Y.W.; Chaplan, S.R. Reversal of nerve ligation-induced allodynia by spinal alpha-2 adrenoceptor agonists. *J. Pharmacol. Exp. Ther.* **1995**, *272*, 207–214.
11. Peng, Y.B.; Lin, Q.; Willis, W.D. Involvement of alpha-2 adrenoceptors in the periaqueductal gray-induced inhibition of dorsal horn cell activity in rats. *J. Pharmacol. Exp. Ther.* **1996**, *278*, 125–135.
12. Herbert, H.; Flügge, G. Distribution of alpha 2-adrenergic binding sites in the parabrachial complex of the rat. *Anat. Embryol.* **1995**, *192*, 507–516. [[CrossRef](#)]
13. Wijesundera, D.N.; Bender, J.S.; Beattie, W.S. Alpha-2 adrenergic agonists for the prevention of cardiac complications among patients undergoing surgery. *Cochrane Database Syst. Rev.* **2009**, *7*, CD004126. [[CrossRef](#)]
14. Bylund, D.B. Subtypes of alpha 2-adrenoceptors: Pharmacological and molecular biological evidence converge. *Trends Pharmacol. Sci.* **1988**, *9*, 356–361. [[CrossRef](#)]
15. Simonneaux, V.; Ebadi, M.; Bylund, D.B. Identification and characterization of alpha 2D-adrenergic receptors in bovine pineal gland. *Mol. Pharmacol.* **1991**, *40*, 235–241. [[PubMed](#)]
16. Celly, C.; McDonnell, W.; Young, S.; Black, W. The comparative hypoxaemic effect of four α_2 adrenoceptor agonists (xylazine, romifidine, detomidine and medetomidine) in sheep. *J. Vet. Pharmacol. Ther.* **1997**, *20*, 464–471. [[CrossRef](#)] [[PubMed](#)]
17. Ongioco, R.R.; Richardson, C.D.; Rudner, X.L.; Stafford-Smith, M.; Schwinn, D.A. Alpha2-adrenergic receptors in human dorsal root ganglia: Predominance of alpha2b and alpha2c subtype mRNAs. *Anesthesiology* **2000**, *92*, 968–976. [[CrossRef](#)]
18. Giron, L.T., Jr.; McCann, S.A.; Crist-Orlando, S.G. Pharmacological characterization and regional distribution of alpha-noradrenergic binding sites of rat spinal cord. *Eur. J. Pharmacol.* **1985**, *115*, 285–290. [[CrossRef](#)]
19. Kästner, S.B. A2-agonists in sheep: A review. *Vet. Anaesth. Analg.* **2006**, *33*, 79–96. [[CrossRef](#)]
20. Qiu, Z.Y.; Ding, Y.; Cui, L.Y.; Hu, M.L.; Ding, M.X. The Expression Patterns of c-Fos and c-Jun Induced by Different Frequencies of Electroacupuncture in the Brain. *Evid. Based Complementary Altern. Med. Ecam* **2015**, *2015*, 343682. [[CrossRef](#)]
21. Humphries, S.A.; Long, N.R.; Johnson, M.H. Iontophoretically applied potassium ions as an experimental pain stimulus for investigating pain mechanisms. *Percept. Psychophys.* **1994**, *56*, 637–648. [[CrossRef](#)]
22. Spadavecchia, C.; Arendt-Nielsen, L.; Andersen, O.K.; Spadavecchia, L.; Doherr, M.; Schatzmann, U. Comparison of nociceptive withdrawal reflexes and recruitment curves between the forelimbs and hind limbs in conscious horses. *Am. J. Vet. Res.* **2003**, *64*, 700–707. [[CrossRef](#)]
23. Gómez, F.G. Advances in the Knowledge of the α 2-Adrenergic Agonists Action Mechanisms in Domestic Animals: Clinical Implications and Future Perspectives. In Proceedings of the World Small Animal Veterinary Association World Congress Proceedings, Mexico City, Mexico, 11–14 May 2005.
24. Harrison, J.K.; Pearson, W.R.; Lynch, K.R. Molecular characterization of α_1 - and α_2 -adrenoceptors. *Trends Pharmacol. Sci.* **1991**, *12*, 62–67. [[CrossRef](#)]
25. Rosin, D.L.; Talley, E.M.; Lee, A.; Stornetta, R.L.; Gaylinn, B.D.; Guyenet, P.G.; Lynch, K.R. Distribution of α_{2C} -adrenergic receptor-like immunoreactivity in the rat central nervous system. *J. Comp. Neurol.* **1996**, *372*, 135–165. [[CrossRef](#)]
26. Ruffolo, R.R., Jr.; Nichols, A.; Stadel, J.; Hieble, J. Pharmacologic and therapeutic applications of alpha2-adrenoceptor subtypes. *Annu. Rev. Pharmacol. Toxicol.* **1993**, *33*, 243–279. [[CrossRef](#)]
27. Hein, L.; Altman, J.D.; Kobilka, B.K. Two functionally distinct α 2-adrenergic receptors regulate sympathetic neurotransmission. *Nature* **1999**, *402*, 181–184. [[CrossRef](#)]
28. Borsook, D.; Upadhyay, J.; Chudler, E.H.; Becerra, L. A key role of the basal ganglia in pain and analgesia—insights gained through human functional imaging. *Mol. Pain* **2010**, *6*, 27. [[CrossRef](#)] [[PubMed](#)]

29. Ogon, I.; Takebayashi, T.; Iwase, T.; Emori, M.; Tanimoto, K.; Miyakawa, T.; Terashima, Y.; Kobayashi, T.; Tohse, N.; Yamashita, T. Sympathectomy and sympathetic blockade reduce pain behavior via alpha-2 adrenoceptor of the dorsal root ganglion neurons in a lumbar radiculopathy model. *Spine* **2015**, *40*, E1269–E1275. [[CrossRef](#)]
30. Di Cesare Mannelli, L.; Micheli, L.; Crocetti, L.; Paola Giovannoni, M.; Vergelli, C.; Ghelardini, C. α_2 adrenoceptor: A target for neuropathic pain treatment. *Mini Rev. Med. Chem.* **2017**, *17*, 95–107. [[CrossRef](#)]
31. Engelman, E.; Marsala, C. Efficacy of adding clonidine to intrathecal morphine in acute postoperative pain: Meta-analysis. *Br. J. Anaesth.* **2013**, *110*, 21–27. [[CrossRef](#)]
32. Gavin, K.; Colgan, M.-P.; Moore, D.; Shanik, G.; Docherty, J. α_2 C-adrenoceptors mediate contractile responses to noradrenaline in the human saphenous vein. *Naunyn Schmiedeberg's Arch. Pharmacol.* **1997**, *355*, 406–411. [[CrossRef](#)]
33. Lee, J.; Warner, R.; Brune, M.; DeBernardis, J. Calcium-induced vasoconstrictions after alpha-2 adrenergic receptor activation in the dog saphenous vein: Comparison to calcium-induced contractions after potassium-depolarization. *Gen. Pharmacol. Vasc. Syst.* **1990**, *21*, 647–654. [[CrossRef](#)]
34. Regitz-Zagrosek, V.; Hocher, B.; Bettmann, M.; Brede, M.; Hadamek, K.; Gerstner, C.; Lehmkuhl, H.B.; Hetzer, R.; Hein, L. Alpha_{2C}-adrenoceptor polymorphism is associated with improved event-free survival in patients with dilated cardiomyopathy. *Eur. Heart* **2006**, *27*, 454–459. [[CrossRef](#)] [[PubMed](#)]
35. Wang, Y.; Yu, X.; Wang, F.; Wang, Y.; Wang, Y.; Li, H.; Lv, X.; Lu, D.; Wang, H. Yohimbine Promotes Cardiac NE Release and Prevents LPS-Induced Cardiac Dysfunction via Blockade of Presynaptic α_2A -Adrenergic Receptor. *PLoS ONE* **2013**, *8*, e63622.
36. Pypendop, B.H.; Verstegen, J.P. Hemodynamic effects of medetomidine in the dog: A dose titration study. *Vet. Surg.* **1998**, *27*, 612–622. [[CrossRef](#)] [[PubMed](#)]
37. Pertovaara, A.; Almeida, A. Descending inhibitory systems. *Handb. Clin. Neurol.* **2006**, *81*, 179–192.