

Xylazine regulates the release of glycine and aspartic acid in rat brain

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Abstract

Introduction: Xylazine, a type of α_2 -adrenoceptors, is a commonly used drug in veterinary medicine. Xylazine-induced changes in the content of amino acid neurotransmitters – glycine (Gly) and aspartic acid (Asp), in different brain regions and neurons were studied. **Material and Methods:** Wistar rats were administered 50 mg/kg or 70 mg/kg of xylazine by intraperitoneal injection. In addition, *in vitro* experiments were conducted, in which neurons were treated with 15 $\mu\text{g/mL}$, 25 $\mu\text{g/mL}$, 35 $\mu\text{g/mL}$, and 45 $\mu\text{g/mL}$ of xylazine. Test methods were based on the enzyme-linked immunosorbent assays (ELISA). **Results:** During anaesthesia, Asp levels in each brain area were significantly lower compared to the control group. Except for the cerebrum, levels of Gly in other brain areas were significantly increased during the anaesthesia period. *In vitro*, xylazine-related neuron secretion of Gly increased significantly compared to the control group at 60 min and 90 min. Moreover, xylazine caused a significant decrease in the levels of Asp secreted by neurons at 20 min, but gradually returned to the level of the control group. **Conclusion:** The data showed that during anaesthesia the overall levels of Asp decreased and overall levels of Gly increased. In addition, the inhibitory effect of xylazine on Asp and the promotion of Gly were dose-dependent. Our data showed that different effects of xylazine on excitatory and inhibitory neurotransmitters provided a theoretical basis for the mechanism of xylazine activity in clinical anaesthesia.

Keywords: rats, brain, xylazine, glycine, aspartic acid.

Introduction

Xylazine, which is commonly used as an anaesthetic in animals, has an impact on general sedation, analgesia, and muscle relaxation, and can directly inhibit the central nervous system, thereby leading to a central nerve conduction block (5). Xylazine in combination with other drugs has been widely used during clinical examination and surgery of animals, including horses, cattle, sheep, dogs, cats, and rabbits. Moreover, xylazine has been widely used as the main component of anaesthetics in clinical practice (10, 20). Recently, a growing number of studies have focused on the molecular and cellular mechanisms of its anaesthetic action.

General anaesthetics, including xylazine, mainly act directly on synaptic electro-chemical delivery processes, in which inhibition and promotion of neurotransmitters are important. The synapse is a basic structure of

extensive contact between neurons and plays an important role in the regulation of the central nervous system activity (9, 29). General anaesthesia mainly affects synaptic transmission, and it is believed that commonly used anaesthetics reduce the presynaptic membrane release of excitatory neurotransmitters and increase presynaptic membrane release of inhibitory neurotransmitters by the excitatory postsynaptic potential (EPSP) amplitude decrease and inhibitory postsynaptic potential (IPSP) amplitude increase (6, 25). Glycine (Gly) is an inhibitory neurotransmitter, which is released from the brain stem and spinal postsynaptic neurons (23). Gly is a necessary agonist of the N-methyl-D-aspartic acid receptor. Although the metabolic pathway is not entirely clear, it can inhibit the central nervous system (24). Aspartic acid (Asp) is a neurotransmitter in the central nervous system; it is unevenly distributed and has a strong excitatory effect on cerebral cortex cells (28). Studies on Asp and Gly during anaesthesia are

limited, therefore the present study focused on xylazine-induced changes in Asp and Gly release during anaesthesia.

Material and Methods

Animals and experimental groups. Fifty-five three-month-old Wistar rats, weighing 200 ± 20 g, and 10 pregnant rats, weighing 260 ± 20 g, were provided by the animal experimental centre of the Affiliated Hospital of Harbin Medical University (Harbin, China). Control group was given an intraperitoneal injection of saline (0.005 mL/g) (group C, $n = 5$). Rats in other groups were either given an intraperitoneal injection of 50 mg/kg of xylazine (low dose group, $n = 25$) or 70 mg/kg of xylazine (high dose group, $n = 25$). Rats were examined at five different periods (five rats per time period): the induction period (I group, low dose group – 8 min after the xylazine injection, and high dose group – 5 min after the xylazine injection), the anaesthesia period (group A, righting reflex disappeared), the recovery period (group R1, righting reflex recovery), the recovery period (group R2, 3 h after the xylazine injection), and the recovery period (group R3, 5 h after xylazine injection). At termination of the experiment, rats were euthanised by cervical dislocation.

Judgment criteria of the righting reflex assumed that 15–20 min after xylazine injection the righting reflex disappeared for more than 1 min, and neither needle nor clamp claw and tail could reactivate the righting reflex, in which case the result was defined as positive.

Sample collection. The control group was injected intraperitoneally with saline solution and after 8 min the cerebrum, cerebellum, thalamus, hippocampus, and brainstem were quickly removed and stored on ice. The organs were immediately frozen in vials using liquid nitrogen. For all other experimental groups, rats were euthanised at each time point. The tissues in different encephalic regions of the brain were removed from the liquid nitrogen tank and stored at -80°C until further investigation.

Culture of neurons. Ten pregnant rats were killed at days 16–18 of pregnancy by cervical dislocation and the foetuses were removed from each rat. Afterwards, the head of the foetus was cut off and a small incision was made on the midline from the basis of the skull dividing the skull in half to expose the whole brain. The entire brain was smoothly removed from the skull and immediately placed in the dissection medium. The cerebral cortex tissues of the foetus were peeled and collected for the culture of neurons. Neurons cultured for 24 h were identified. After removal of the supernatant, cells were fixed with a 1 mL paraformaldehyde/sugar mixture for 10 min and placed in 1 mL of 0.1% (v/v) Triton X-100 for 10 min at room temperature. After two washes with phosphate-buffered saline (PBS), the cells were incubated in 10% foetal bovine serum (Invitrogen,

USA) for 1 h. Then the cells were incubated overnight at 4°C with a mouse anti-MAP2 antibody (Invitrogen, USA), followed by goat anti-mouse IgG (1:100; Santa Cruz Biotechnology, USA) for 1 h and washed three times with PBS.

Treatment management. Neurons harvested from foetal rats were treated with one of the following xylazine concentrations: 15 $\mu\text{g}/\text{mL}$, 25 $\mu\text{g}/\text{mL}$, 35 $\mu\text{g}/\text{mL}$, or 45 $\mu\text{g}/\text{mL}$. The control group received an equal amount of PBS. The neurons were divided into the control group, 5 min dose group, 10 min dose group, 15 min dose group, 20 min dose group, 25 min dose group, 30 min dose group, 45 min dose group, 60 min dose group, 90 min dose group, and 120 min dose group. The experiment was performed in triplicate per each group ($n = 3$).

Determination of Asp and Gly. Levels of Asp and Gly were determined by ELISA using the Asp and Gly kits (Mlbio, China). The samples were diluted 1:1 with the sample diluent, and 50 μL was added to the reaction micropore. A total of 50 μL of biotin labelled antibody solution was added to the standard micropore and sample micropore and incubated at $36 \pm 2^{\circ}\text{C}$ for 60 min. The mixture was washed five times, each time left to settle for 10–20 s. Then, 60 μL affinity streptavidin-HRP was added to all micropores and mixed well before incubation at $36 \pm 2^{\circ}\text{C}$ for 30 min. The mixture was again washed five times and left to settle for 10 s. Next, 50 μL of A solution and 50 μL of B solution were added to each pore and incubated at $36 \pm 2^{\circ}\text{C}$ without light for 15 min. Then, 50 μL termination solution was added to each micropore to terminate.

Statistical analysis. SPSS software (SPSS version 18.0 for Windows, SPSS incorporated, USA) was used for all statistical analyses. Data are expressed as the mean \pm standard error of the mean (SD). Statistical comparisons of *in vivo* and *in vitro* studies were analysed using the Dunnett's test after the test of normality and the assumption for homogeneity of variances. $P < 0.05$ was considered statistically significant and $P < 0.01$ was considered very significant.

Results

Effects of low-dose xylazine anaesthesia on amino acid neurotransmitters in different regions of rat brains. After anaesthetising the rats with a low dose of xylazine, Gly levels in the cerebellum, thalamus, brain stem, and hippocampus initially increased, and then decreased. However, in the cerebrum Gly levels first decreased and then increased. The highest Gly levels in the cerebellum and hippocampus were observed in the first stage of recovery ($P < 0.01$) (Figs 1A and E). In the thalamus and brainstem, Gly reached the highest levels during anaesthesia period ($P < 0.01$) (Figs 1C and D). However, in the cerebrum, the levels of Gly decreased to the lowest value in the anaesthesia period ($P < 0.01$) (Fig. 1B). The level of Asp in different brain regions

initially decreased and then increased. Asp levels in the cerebellum, cerebrum, and hippocampus were the lowest in anaesthesia period ($P < 0.01$) (Figs 2A, B, and E). In addition, in the thalamus, Asp levels were the lowest in the first stage of recovery ($P < 0.01$) (Fig. 2C), whereas in the brainstem, Asp levels in the control group were the highest, and the lowest levels were found in the first stage of recovery ($P < 0.01$) (Fig. 2D).

Effects of high-dose xylazine anaesthesia on amino acid neurotransmitters in different regions of rat brains. When rats were anaesthetised with a high dose of xylazine, the Gly levels were similar to those when rats were administered a low dose of xylazine. In the cerebellum and hippocampus, the highest levels of

Gly were found in the first stage of recovery ($P < 0.01$) (Figs 3A and E). Gly levels showed irregular fluctuations in the cerebrum. The lowest levels were observed in the anaesthetic period, whereas in the control group the highest levels were found ($P < 0.01$) (Fig. 3B). In the thalamus and brainstem, the highest levels of Gly were found in the anaesthetic period ($P < 0.01$) (Figs 3C and 3D). When administered a high dose of xylazine, the levels of Asp were similar to those of rats administered a low dose of xylazine. The levels of Asp in the cerebellum, cerebrum, thalamus, and hippocampus were the lowest in anaesthesia period ($P < 0.01$) (Figs 4A, B, C, and E). In the brainstem, the lowest Asp levels were found in the first stage of recovery ($P < 0.01$) (Fig. 4D).

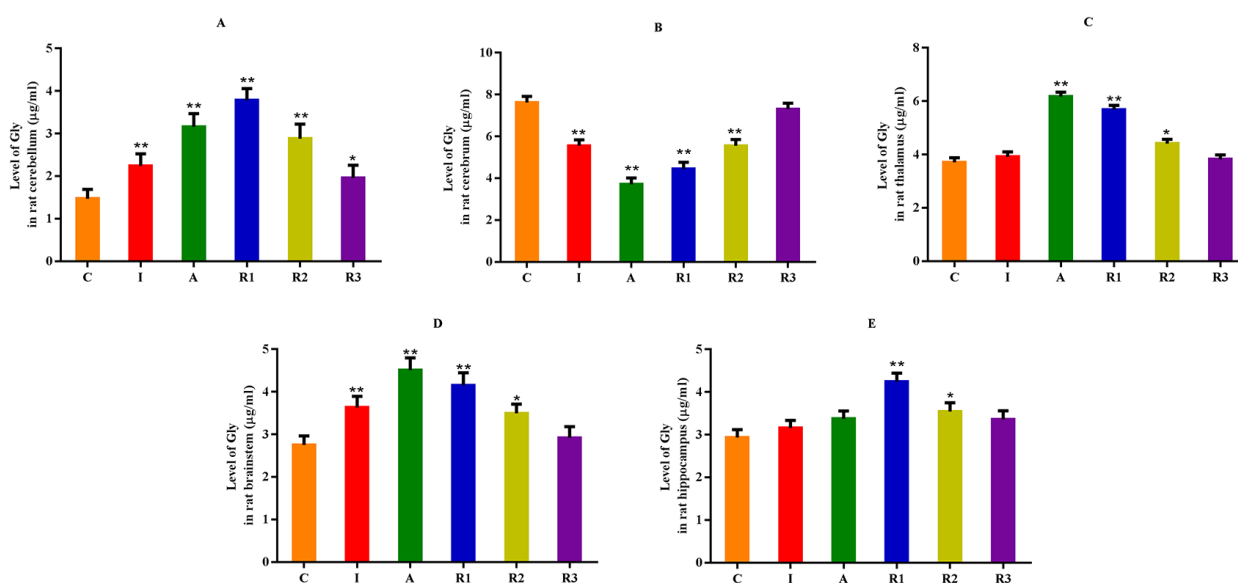


Fig. 1. The effect of low-dose xylazine anaesthesia on Gly neurotransmitters in rat brain. C – control group, I – induction period, A – anaesthesia period, R1 – recovery period (group R1), R2 – recovery period (group R2), R3 – recovery period (group R3). * $P < 0.05$; ** $P < 0.01$

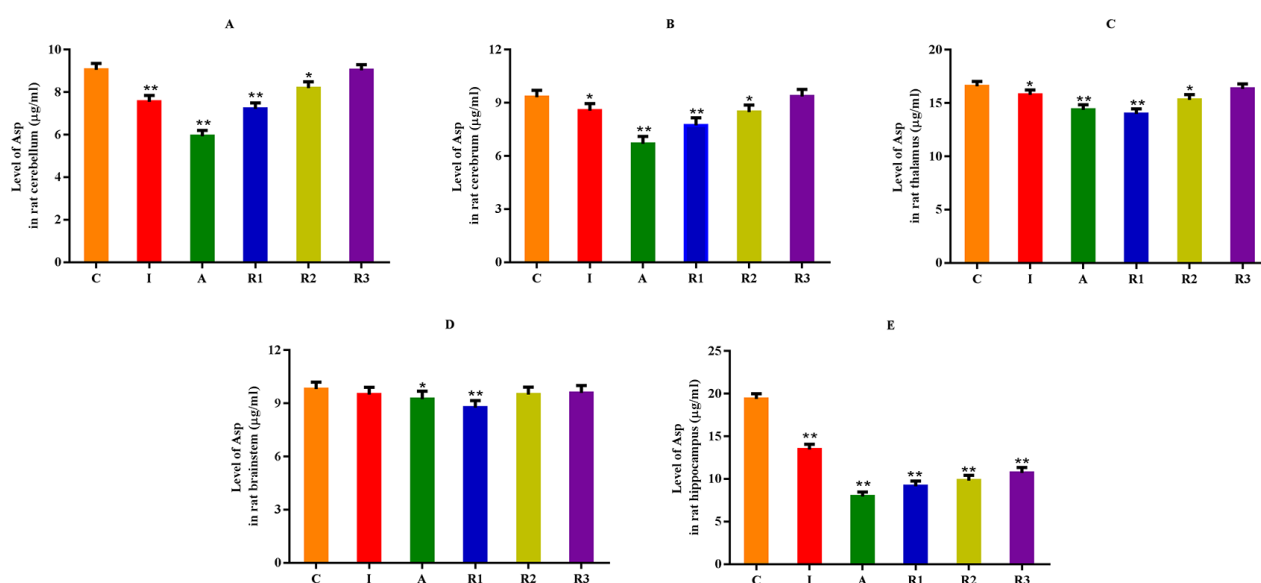


Fig. 2. The effect of low-dose xylazine anaesthesia on Asp neurotransmitters in rat brain. C – control group, I – induction period, A – anaesthesia period, R1 – recovery period (group R1), R2 – recovery period (group R2), R3 – recovery period (group R3). * $P < 0.05$; ** $P < 0.01$

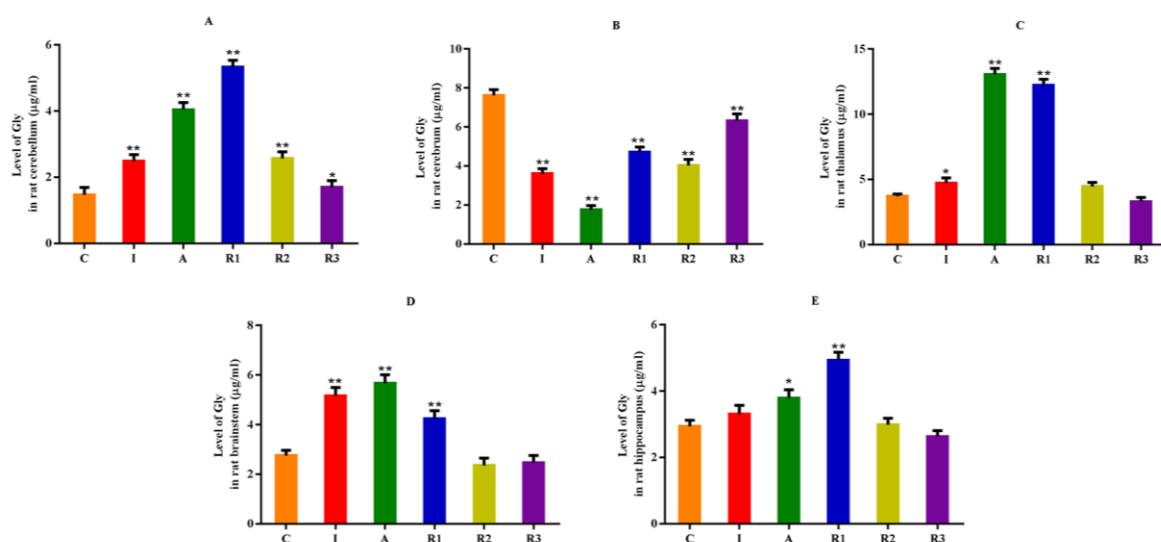


Fig. 3. The effect of high-dose xylazine anaesthesia on Gly neurotransmitters in rat brain. C – control group, I – induction period, A – anaesthesia period, R1 – recovery period (group R1), R2 – recovery period (group R2), R3 – recovery period (group R3). * $P < 0.05$; ** $P < 0.01$

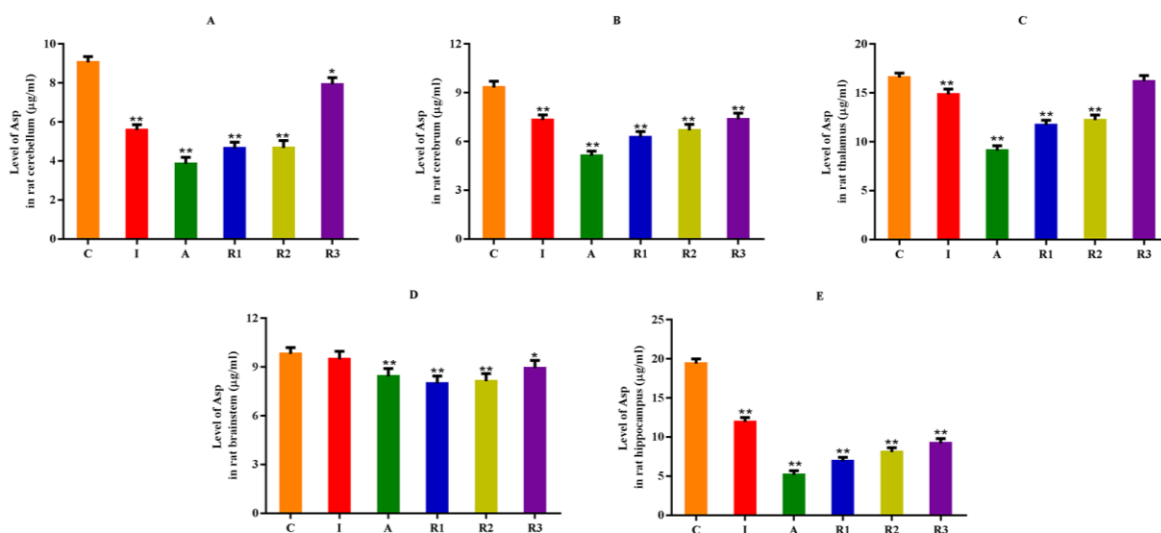


Fig. 4. The effect of high-dose xylazine anaesthesia on Asp neurotransmitters in rat brain. C – control group, I – induction period, A – anaesthesia period, R1 – recovery period (group R1), R2 – recovery period (group R2), R3 – recovery period (group R3). * $P < 0.05$; ** $P < 0.01$

Effects of different doses of xylazine on neuronal amino acid neurotransmitters *in vitro*.

Figure 5 shows that at a final xylazine concentration of 25 µg/mL, 35 µg/mL, 45 µg/mL, the Gly levels gradually decreased prior to 20 min, and that the lowest value was observed at 20 min ($P < 0.01$) (Figs 5B, C, and D). However, Gly levels in 15 µg/mL group did not significantly change during this period (Fig. 5A). Then the levels of Gly began to increase, and the Gly levels of the 15 µg/mL, 25 µg/mL, 35 µg/mL, and 45 µg/mL groups at 60 min and 90 min were significantly different from those at 20 min ($P < 0.01$) (Figs 5A, B, C, and D). Moreover, when compared with the control group, the content of Gly was significantly higher at 60 min and 90 min ($P < 0.01$) (Figs 5A, B, C, and D).

Figure 6 shows that Asp levels in 15 µg/mL, 25 µg/mL, 35 µg/mL, and 45 µg/mL groups were the lowest at 20 min, and decreased significantly when compared with the control group ($P < 0.05$) (Figs 6A, B, C, and D). After 20 min, Asp levels gradually increased. Asp levels in 25 µg/mL group increased to a maximum at 60 min, which was significantly different from those at 20 min ($P < 0.01$) (Fig. 6B). Asp levels in 15 µg/mL and 35 µg/mL groups increased to a maximum at 45 min, which was significantly different from those at 20 min ($P < 0.01$) (Figs 6A and C). Asp levels in 45 µg/mL group increased to a maximum at 90 min, which was significantly different from those at 20 min ($P < 0.01$) (Fig. 6D). The levels in each group returned to the level of the control group at 120 min.

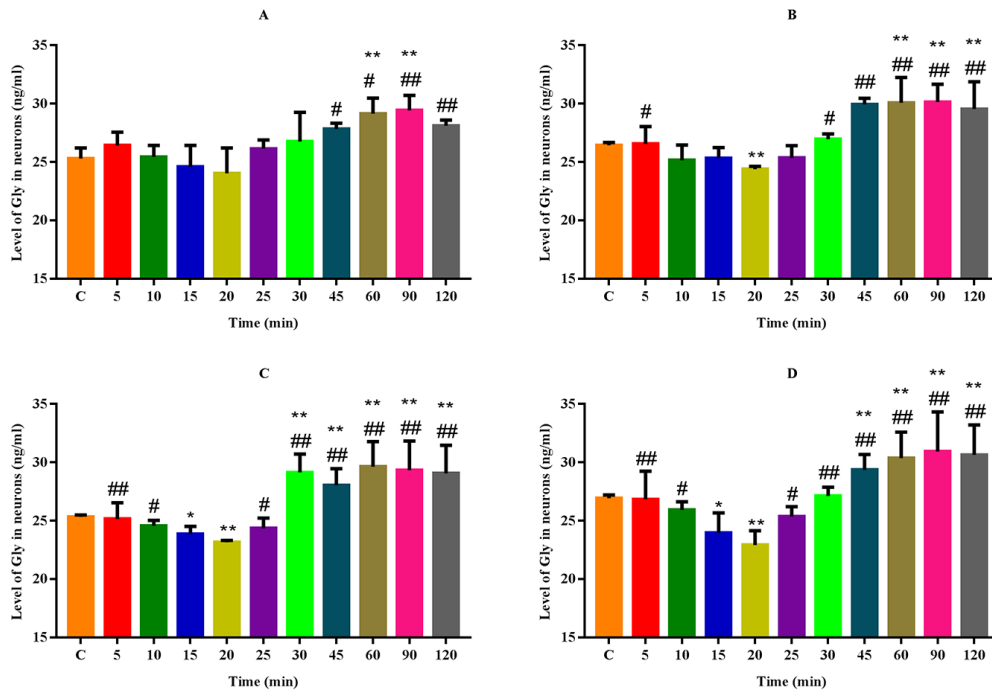


Fig. 5. Effects of different concentrations of xylazine on neurons secreting the neurotransmitter Gly. At 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min xylazine was allowed to act on the neurons. (A) 15 µg/mL xylazine, (B) 25 µg/mL xylazine, (C) 35 µg/mL xylazine, (D) 45 µg/mL xylazine. *P < 0.05; **P < 0.01 versus control, #P < 0.05; ##P < 0.01 versus the minimum value

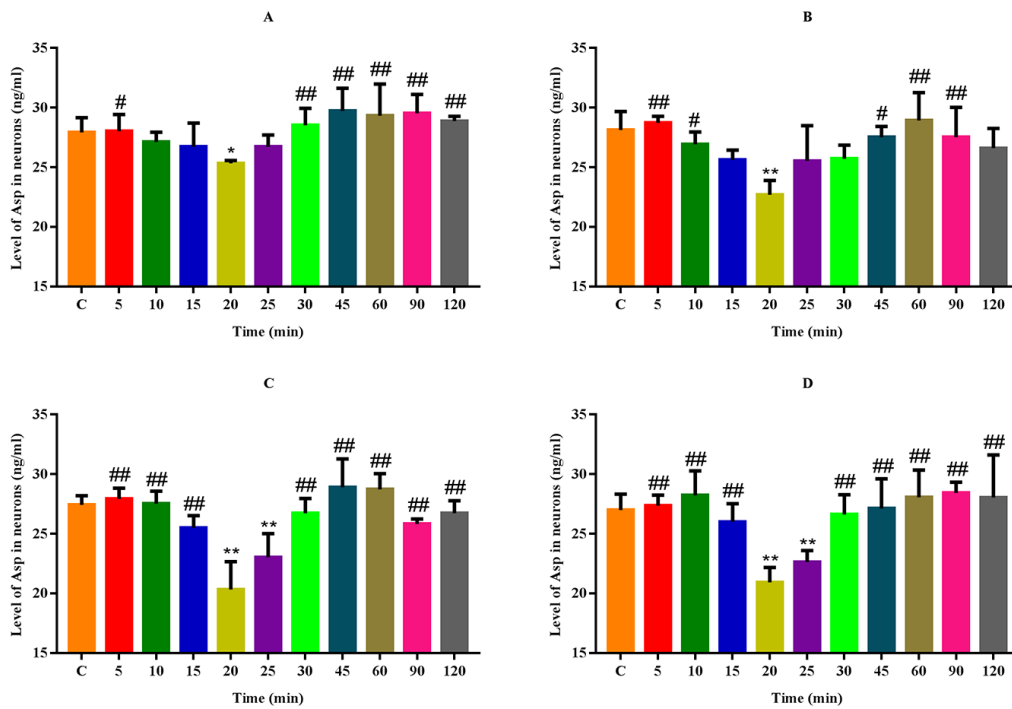


Fig. 6. Effects of different concentrations of xylazine on neurons secreting the neurotransmitter Asp. At 5, 10, 15, 20, 25, 30, 45, 60, 90, and 120 min xylazine was allowed to act on the neurons. (A) 15 µg/mL xylazine, (B) 25 µg/mL xylazine, (C) 35 µg/mL xylazine, (D) 45 µg/mL xylazine. *P < 0.05; **P < 0.01 versus control, #P < 0.05; ##P < 0.01 versus the minimum value

Discussion

The present study revealed that Asp and Gly levels were altered in different regions of the brain of rats anaesthetised with xylazine. Moreover, by analysing

the different effects of xylazine on excitatory and inhibitory neurotransmitters, we speculated that xylazine may alter the efficiency of neurotransmission by affecting the release of different neurotransmitters. However, several factors could influence the rats, such

as anaesthetic stress, oxygen saturation, oppression of blood vessels, and other elements (4, 12). To exclude these effects, we cultured neurons *in vitro* and explored the effect of xylazine on Asp and Gly levels.

Asp is an excitatory neurotransmitter in the central nervous system, and is commonly found in different regions in the brain. In physiology, it is important to maintain excitability of the brain (1). However, studies on the effects of general anaesthesia on amino acid neurotransmitters, especially on Asp, are limited. At present, only few theories exist about the mechanism of general anaesthesia, and it is generally believed that anaesthetics act at the protein level. Their sites of action may involve a synaptic receptor, an ion channel, or a corresponding regulatory system in the synapse, and general anaesthetics may influence the synapse directly during the process of electro-chemical transfer, thereby inhibiting excitatory neurotransmitters and promoting inhibitory neurotransmitters (9, 21). In the central nervous system, the action of Asp on the postsynaptic membrane can regulate the internal state of neuronal cells, which is similar to the role of Glu. Nerve impulses reach nerve endings, resulting in presynaptic membrane depolarisation (3). The synaptic vesicles release the excitatory neurotransmitter Asp, leading to Asp and postsynaptic membrane receptor binding, resulting in the production of excitatory action potentials by postsynaptic neurons (11). Previous studies have shown that clinical doses of barbiturates and hydroxy progesterone can inhibit the release of Asp, thereby inhibiting the central nervous system neurotransmission, eventually leading to narcotic effects (13, 30). In *in vivo* studies, the changes of Asp levels in the five brain regions of rats during the process of anaesthesia showed a trend of a decrease followed by an increase, suggesting that xylazine may inhibit Asp synthesis and release, reducing excitatory neurotransmitter and receptor binding, ultimately reducing the excitability of neurons and producing anaesthetic effects. When rabbits were anaesthetised with dexmedetomidine, it was found that Asp levels during the anaesthesia were significantly lower compared to the levels in the control group, indicating that the anaesthesia mechanism of dexmedetomidine may be related to the decreased levels of Asp, which is consistent with the findings of the current study (27). Related studies have confirmed that Asp increased the influx of Ca^{2+} in neuronal cells. The release of neurotransmitters in the vesicles of central nerve endings cannot be separated from Ca^{2+} , therefore Asp may also play an important role in the transmission of information between neurons (15). In *in vivo* studies, the inhibitory effect of xylazine on neuronal secretion of Asp levels was the strongest at 20 min. During the entire duration of ketamine action on neurons, Asp levels in the experimental group did not exceed the levels of Asp in the control group, which may be related to the inhibitory effect of xylazine. In addition, the inhibitory effect of xylazine at high concentration

was more obvious. In agreement with the findings of *in vivo* studies, the inhibitory effect of xylazine on Asp was increased in a dose-dependent manner. The above studies confirmed that Asp levels are in close relationship with the central inhibition of anaesthesia.

In brain tissue, Gly is an important inhibitory neurotransmitter. Gly release by binding of the presynaptic membrane with postsynaptic membrane receptors increases the permeability of the postsynaptic membrane to K^+ and Cl^- , which hyperpolarises the postsynaptic membrane and produces an inhibitory postsynaptic potential, leading to the production of inhibitory action potentials by postsynaptic neurons (17, 18). Excess Asp levels can activate its receptors, which can lead to excitatory neurotoxicity in the central nervous system. Gly plays a key role as an inhibitory neurotransmitter in the inhibition of excitatory neurotoxicity, and excitatory amino acids and inhibitory amino acids are in a state of dynamic equilibrium, having a pivotal effect in maintaining the function of the nervous system. Changes in the content of these amino acids are closely related to nervous system disorders (19, 26). In a recent study, it was suggested that propofol could enhance Gly-induced currents, and that general anaesthetics increased the affinity of Gly and its receptor, thereby inducing inhibitory postsynaptic currents (2, 14). During anaesthesia total Gly levels in the rat brain increased, and gradually decreased when the rats woke up. In the cerebrum, Gly levels were changed irregularly, suggesting that the cerebrum may not be the main site of anaesthesia when using xylazine, and that the action site of Gly may vary for different anaesthetics. This statement needs to be verified in the future. Elevation of the total Gly levels may be due to the role of xylazine in these brain regions, and may promote the synthesis and release of Gly, producing an inhibitory potential, reducing the excitability of neurons, thus resulting in anaesthesia. In several studies, it was demonstrated that isoflurane inhibited the transmission of Asp in the cerebral cortex and hippocampus, and enhanced the role of Gly in hippocampus and spinal cord. This inhibited the excitatory amino acid synaptic transmission and enhanced the inhibitory amino acid synaptic transmission in the central nervous system, thereby producing anaesthetic effects (8, 22). Combined with the findings in previous studies, we speculated that the anaesthetic effects of xylazine on rats may be due to the combined inhibition of excitatory neurotransmitters and promotion of the release of inhibitory neurotransmitters. However, Gly can freely diffuse in the brain extracellular fluid and cerebrospinal fluid. Gly receptors and GABAA receptors function similarly and can mediate the increase in chloride concentration in neurons, resulting in a hyperpolarisation effect. In a study of rat neurons, general anaesthetics enhanced the inhibitory Cl^- current that was induced by low concentration of Gly (7, 16). *In vivo*, the first 20 min may represent the time that

neurons needed to interact and to adapt to xylazine. Moreover, the promoting effect of xylazine on Gly began to emerge; Gly levels at 60 and 90 min were higher when compared to the control group, indicating that the anaesthetic effects of xylazine may be related to the increased secretion of Gly in neurons.

Due to the action of xylazine, the homeostatic state of excitatory and inhibitory neurotransmitters is destroyed, resulting in a decrease in excitatory neurotransmitters and an increase in inhibitory neurotransmitters. This blocks the signal transmission between neurons, and reduces the effect of the transmission of excitement, leading to the anaesthetic effect of xylazine.

In conclusion, xylazine (50 and 70 mg/kg) significantly inhibited Asp levels in the hippocampus and thalamus of rats, indicating that the hippocampus and thalamus may be the main sites in which xylazine changes Asp. Moreover, xylazine significantly increased Gly levels in the cerebellum, thalamus, and brainstem, thereby indicating that these parts of the brain may be the main sites in which xylazine increased levels of Gly. *In vitro*, xylazine has significant inhibitory effects on Asp, and promotes the effects of Gly. The results of our study indicated that xylazine has different effects on amino acid neurotransmitters. Through the intervention of synthesis and release of neurotransmitters, the Asp levels were decreased and Gly levels were increased. Thus, the inhibitory effects of xylazine on Asp levels, and the promotion of the effects of Gly are dose-dependent, which may be one of the underlying mechanisms of xylazine anaesthesia.

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Animal Rights Statement: The authors declare that all animal experiments were performed in accordance with a protocol that was approved by the Animal Experimentation Committee of the Northeast Agricultural University, Harbin, China. Rats were euthanised by cervical dislocation, and all efforts were made to minimise their suffering.

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