

Effects of tiletamine-xylazine-tramadol combination and its specific antagonist on AMPK in the brain of rats

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Abstract

Introduction: Tiletamine-xylazine-tramadol (XFM) has few side effects and can provide good sedation and analgesia. Adenosine 5'-monophosphate-activated protein kinase (AMPK) can attenuate trigeminal neuralgia. The study aimed to investigate the effects of XFM and its specific antagonist on AMPK in different regions of the brain. **Material and Methods:** A model of XFM in the rat was established. A total of 72 Sprague Dawley (SD) rats were randomly divided into three equally sized groups: XFM anaesthesia (M group), antagonist (W group), and XFM with antagonist interactive groups (MW group). Eighteen SD rats were in the control group and were injected intraperitoneally with saline (C group). The rats were sacrificed and the cerebral cortex, cerebellum, hippocampus, thalamus, and brain stem were immediately separated, in order to detect AMPK α mRNA expression by quantitative PCR. **Results:** XFM was able to increase the mRNA expression of AMPK α 1 and AMPK α 2 in all brain regions, and the antagonist caused the opposite effect, although the effects of XFM could not be completely reversed in some areas. **Conclusion:** XFM can influence the expression of AMPK in the central nervous system of the rat, which can provide a reference for the future development of anaesthetics for animals.

Keywords: brain, AMPK, xylazine, tramadol, tiletamine.

Introduction

Tiletamine is a dissociative anaesthetic and can act as a narcotic analgesic for small animals such as mice, when used as part of a combination of anaesthetics. It is compatible with the antagonistic action of N-methyl-D-aspartate (1), and inhibits the nitric oxide-cyclic guanosine monophosphate (NO-cGMP) signal transduction system by decreasing the content of cGMP (2), which plays a role in inhibiting the conduction of the nerve excitability process, thereby changing the state of consciousness, and resulting in sedative and hypnotic effects (3, 4). Using xylazine only reduces the quantity of isoflurane required during anaesthesia of dogs but also decreases the dosage of pentobarbital (5). Xylazine is a commonly used clinical α 2-adrenergic receptor agonist and has good analgesic and sedative effects, often being combined with other preparations for general anaesthesia of animals (6). Tramadol can

replace opioids as a pain inhibitor and can effectively complement and is synergistic with analgesic drugs by improving their pain-relieving effects and enhancing the body's tolerance for them (7). It is recommended as an adjuvant when comparing the effects of other sustained-release opioid analgesics. The most common adverse reactions to tramadol are nausea and vomiting, but by combining with antiemetics these phenomena can be avoided (9).

Through pre-experiments, scientific formulation tests, verification and orthogonal prescription screening experiments (10), the tiletamine-xylazine-tramadol (XFM) combination was found to be a balanced anaesthetic based on theoretical considerations (11). Induction of anaesthesia using XFM is rapid, time for maintenance of anaesthesia is appropriate and recovery is stable. Therefore, XFM can meet the needs of clinical diagnosis, treatment, and research work. Fan et al. (7) in their research on the mechanism of XFM and relevant

studies of Na⁺/K⁺-ATPase and Ca²⁺/Mg²⁺-ATPase, found that XFM was able to inhibit Na⁺/K⁺-ATPase activity in the cerebral cortex, brain stem, and thalamus, and could also inhibit the remaining two areas of the brain for Ca²⁺/Mg²⁺-ATP enzyme activity (10).

Flumazenil (FLU) has a strong affinity for receptors of benzodiazepines in the brain and can reverse their pharmacological effects on the central nervous system (12). This could activate the GABA_A receptor and inhibit its combining with GABA (13), interfering with the opening of the calcium channel and increasing the influx of Ca²⁺, thereby promoting the release of Glu from the presynaptic membrane and indirectly affecting the activation of the NMDA receptor in the postsynaptic membrane. The NO-cGMP signal transduction system was involved in the regulation of molecular mechanisms that were produced by the α₂-adrenergic receptor agonist (14). Atipamezole (ATI) can inhibit the conduction of K⁺ and the production of synaptic hyperpolarisation, increase the conductivity of calcium channels and the flow of Ca²⁺, activate NOS, and increase the content of NO and cGMP. Atipamezole can also completely antagonise the anaesthetic effect of α₂-adrenergic receptor agonist produced by xylazine in XFM (15), and the patients who receive it revive quickly with no adverse cardiovascular reactions. Naloxone (NAL), a specific antagonist of opioid receptors, can block and reverse the toxic effects of endogenous opioid peptides (16). Due to the interaction of NO-cGMP signal transduction system and the GABA receptor pathway (18), ATI-FLU-NAL, an XFM antagonist, can activate NMDA receptors in synapses and lead to the activation of NO-cGMP signal transduction system in different brain areas, which can inhibit the activity of GABA_A receptor so that it activates NO-cGMP signal transduction system in turn. The research of Lu et al. (19) suggests that the revival mechanism of ATI-FLU-NAL can enhance Na⁺/K⁺-ATPase and Ca²⁺-ATPase activity by inhibiting the phosphorylation of the protein kinase closely linked to cyclic adenosine monophosphate (cAMP), doing so by playing a catalytic role and ultimately promoting the formation of NO which increases the expression of cGMP. This is contrary to the role of XFM.

Adenosine 5'-monophosphate-activated protein kinase (AMPK) can regulate pain, glucose, protein, and the metabolism of other energy sources in vivo, and the changes in the energy state of the body can also play a role in the regulation of AMPK (20). The catalytic subunit of AMPK has two isoforms, α₁ and α₂ (21). There are reports that the α₂ subunit is also distributed in brain neurons and the hippocampus (22). Researchers have found that AMPK is also associated with the mammalian target of rapamycin (mTOR) signal transduction pathway (8) in such a way that when AMPK activity is elevated, mTOR is inhibited by negative feedback (23). Sheng (26) and other researchers (24) found that XFM can affect the protein

and mRNA expression of Akt and mTOR. Being a negative regulator of mTOR, it is reasonable to speculate that AMPK may also be negatively regulated by XFM (25).

To the best of our knowledge, no data are available on the gene expression patterns of AMPK in different brain regions of XFM-treated rats. We measured the mRNA levels of AMPKα₁ and AMPKα₂ in the cerebellum, cerebral cortex, thalamus, hippocampus, and brain stem of rats following injection of XFM. The goal of this study was to determine the anaesthetic effect of XFM on AMPK, which would provide a reference for the future development of animal anaesthetics.

Material and Methods

A total of 90 healthy Sprague Dawley (SD) rats (220–240 g) were selected for experimentation. Of those 90, 72 rats were randomly divided into three equal groups: XFM (M group), ATI-FLU-NAL (W group), and XFM with ATI-FLU-NAL interaction (MW group) groups. The rats in each group were intraperitoneally injected with anaesthetics (0.15 mL/kg) and antagonist (0.08 mL/kg). Eighteen rats, comprising the control group (C group), were injected intraperitoneally with saline (0.55 mL of 0.9% NaCl) and were sacrificed 15 min later. The XFM consisted of 1.32 mg/kg xylazine (Rompun; Bayer, Germany), 3.5 mg/kg of tiletamine (Zoletil 100; Virbac Corporation, France), and 1.8 mg/kg of tramadol (Tramal 100; Grunenthal GmbH, Germany). ATI-FLU-NAL was composed of 4 mg/kg of atipamezole (Sigma-Aldrich, USA), 4 mg/kg of naloxone (Pfizer Animal Health Group, USA), and 2 mg/kg of flumazenil (Nhwa Pharma, China). After anaesthetising with XFM, the rats were sacrificed after 10 min (M1 group, n = 6), 20 min (M2 group, n = 6), 40 min (M3 group, n = 6), or 60 min (M4 group, n = 6). After treatment with ATI-FLU-NAL, the rats were sacrificed after 10 min (W1 group, n = 6), 20 min (W2 group, n = 6), 40 min (W3 group, n = 6), or 60 min (W4 group, n = 6). In group MW, the rats received a peritoneal injection of XFM and then an injection of ATI-FLU-NAL after 10 min to initiate revival. Rats were sacrificed after a further 10 min (MW1 group, n = 6), 20 min (MW2 group, n = 6), 40 min (MW3 group, n = 6), or 60 min (MW4 group, n = 6). A saline control group (C group, 0.55 mL 0.9% NaCl, n = 18) was injected intraperitoneally, then sacrificed 15 min later.

The rats in each group were euthanised by cervical dislocation and all efforts were made to minimise suffering. The brains were removed and immediately washed with cold saline, quickly isolating the various cerebral areas. Samples were then placed in a frozen storage tube without RNA enzyme, then placed in liquid nitrogen to induce rapid freezing and transferred to –80°C until analysis.

Table 1. Sequences of primers used for the quantitative real-time PCR

Gene	GenBank number	Primer sequences (5' to 3')	Product size (bp)
β -actin	NM_031144.3	Forward: AGGGAAATCGTGCGTGACAT Reverse: CCTCGGGGCATCGGAA	163
AMPK α 1	NM_019142.2	Forward: GAAGTCAAAGCCGACCCAAT Reverse: AGGGTCTTCTCCTCGCACAC	116
AMPK α 2	NM_023991.1	Forward: ATGATGAGGTGGTGGAGCAG Reverse: GTGAATGGTTCTCGGCTGTG	117

AMPK α 1 – adenosine 5'-monophosphate-activated protein kinase α 1; AMPK α 2 – adenosine 5'-monophosphate-activated protein kinase α 2

Total RNA was extracted with Trizol Reagent (TransGen Biotech, China) according to the manufacturer's instructions. Briefly, lysis of the cells in Trizol was followed by centrifugation at $12,000 \times g$ for 15 min at 4°C in the presence of chloroform. The upper aqueous phase was collected and RNA precipitated by the addition of isopropyl alcohol, which was left to stand for 10 min at -20°C before centrifugation at $12,000 \times g$ for 15 min at 4°C . The resultant RNA pellet was washed twice with 75% ethanol, dried, resuspended in sterile water, and quantified by spectrometry then stored at -80°C .

Reverse transcription was performed using a Rever Tra Ace qPCR RT Master Mix with gDNA Remover (Toyobo, USA), according to the manufacturer's instructions. The cDNA thus obtained was used to amplify AMPK α 1 and AMPK α 2 transcriptions. The primer sequences used in this study (Sangon Biotech, China) are shown in Table 1. RT-PCR amplification and quantification were carried out using SYBR green master mix (Toyobo) in a LightCycler 2.0 (Toyobo) using the following conditions: initial denaturation at 95°C for 30 s; 45 cycles of amplification with denaturation at 95°C for 5 s, annealing at 57°C for 20 s, and extension at 72°C for 20 s; 1 cycle of melting curves at 95°C for 0 s, 65°C for 15 s, and 95°C continuously; and a final cooling step at 40°C for 30 s. All samples were performed in triplicate. Quantitative PCR results were analysed using the comparative cycle threshold (CT) method.

Statistical analysis. The relative expression levels of mRNA were analysed by the $2^{-\Delta\Delta\text{Ct}}$ method (17). All data were statistically analysed with GraphPad Prism v5.0 software (GraphPad Software Inc, USA). Data are expressed as mean \pm standard deviation. Analysis of significance was calculated using one-way analysis of variance (ANOVA). The analysis was achieved using a single factor analysis of variance, where $P < 0.01$ indicated a highly significant difference, but results were considered statistically significant in all analyses at $P < 0.05$.

Results

The depth of anaesthesia was different for the various times of anaesthesia. When complete anaesthesia was achieved, no response could be evoked

from any rat. XFM rapidly induced sedation and anaesthesia without vomiting during its induction. During anaesthesia, the rats had a uniform heartbeat with smooth breathing, the muscles were relaxed, touching the eyelid did not cause a response and the pedal reflex had disappeared.

Effect of XFM on AMPK α 1 and AMPK α 2 in different brain regions. After administration of XFM, the PCR-determined transcription of AMPK α 1 mRNA and AMPK α 2 mRNA in different brain regions increased significantly. AMPK α 1 was significantly increased in the cerebral cortex in the M2 and M4 groups. In the hippocampus, the increase in AMPK α 1 was significant. In the thalamus, AMPK α 1 was significantly increased in the M1 and M4 groups. In the cerebellum, AMPK α 1 was significantly increased in all groups except M2. In the brain stem, AMPK α 1 was significantly increased in the M2, M3, and M4 groups (Fig. 1). AMPK α 2 expression increased in the cerebral cortex and hippocampus in the M4 group. In the cerebellum, it increased significantly in the M1 and M4 groups. In the brain stem, the trend of expression in the M4 and M3 groups was increasing compared with the control group. In the thalamus, the M4 was significantly elevated compared with the control; in M3 group a significant decrease was observed (Fig. 2).

Effects of the ATI-FLU-NAL on AMPK α 1 and AMPK α 2 in different brain regions. The relative expression of AMPK α 1 mRNA in the cerebral cortex of groups W1 and W3 decreased significantly, which was the opposite to that of the W2 group. The relative expression of AMPK α 1 mRNA in the cerebellum of group W4 decreased significantly, while it increased significantly in W3 group. In the hippocampus, there was a significant decrease in W1 and W3 groups and significant increase in W2 and W4 groups. In the brain stem, the expression also reduced significantly in the W3 and W4 groups compared with the control, and increased in group W1, while the expression was higher in the thalamus for groups W1 and W2 compared with the control group (Fig. 3). The expression of AMPK α 2 decreased significantly in all W groups compared with the control in the cerebral cortex, hippocampus, thalamus, and brain stem, although there was a small, but not significant, rebound. In the cerebellum, its expression only decreased significantly in the W1 and W3 groups (Fig. 4).

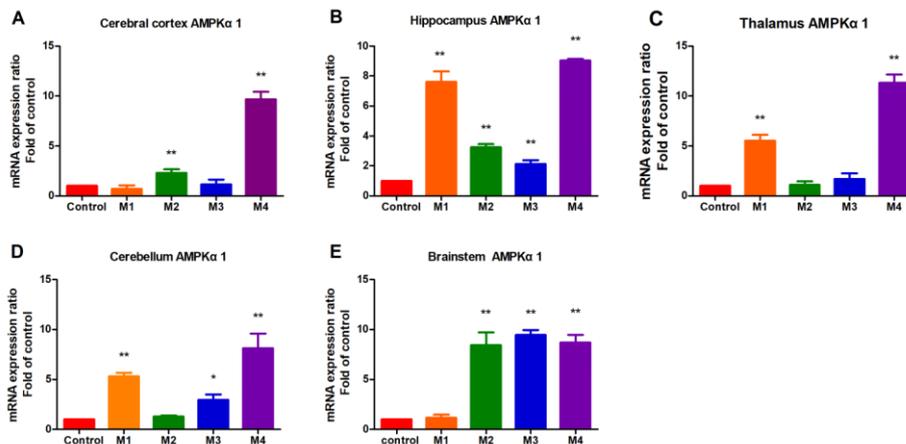


Fig. 1. Effect of tiletamine-xylazine-tramadol (XFM) on the mRNA levels of adenosine 5'-monophosphate-activated protein kinase $\alpha 1$ (AMPK $\alpha 1$) in rats. Rats received saline (0.55 mL) or XFM intraperitoneally and then were sacrificed after 10 (M1), 20 (M2), 40 (M3), or 60 min (M4). RNA from each brain region was extracted and subjected to qPCR analysis. β -actin was used as a control. Data are presented as the mean \pm standard deviation, $n = 6$. * $P < 0.05$ compared with control group, ** $P < 0.01$ compared with control group

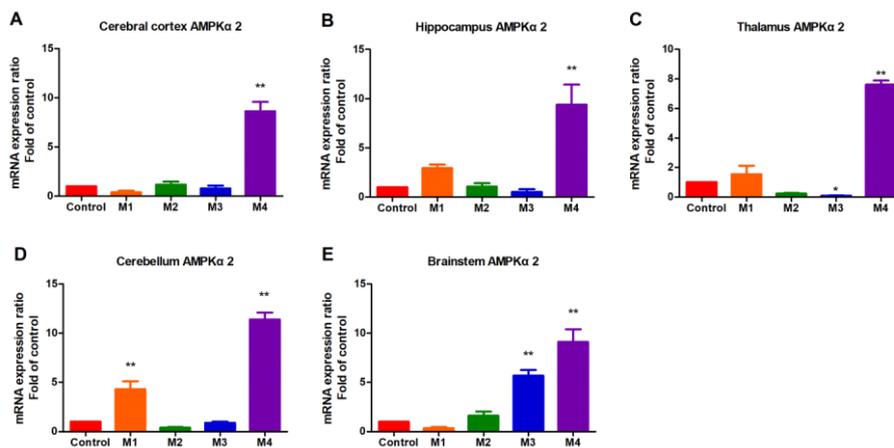


Fig. 2. Effect of tiletamine-xylazine-tramadol (XFM) on the mRNA levels of adenosine 5'-monophosphate-activated protein kinase $\alpha 2$ (AMPK $\alpha 2$) in rats. Rats received saline (0.55 mL) or XFM intraperitoneally and then were sacrificed after 10 (M1), 20 (M2), 40 (M3), or 60 min (M4). RNA from each brain region was extracted and subjected to qPCR analysis. β -actin was used as a control. Data are presented as the means \pm standard deviation, $n = 6$. * $P < 0.05$ compared with control group, ** $P < 0.01$ compared with control group

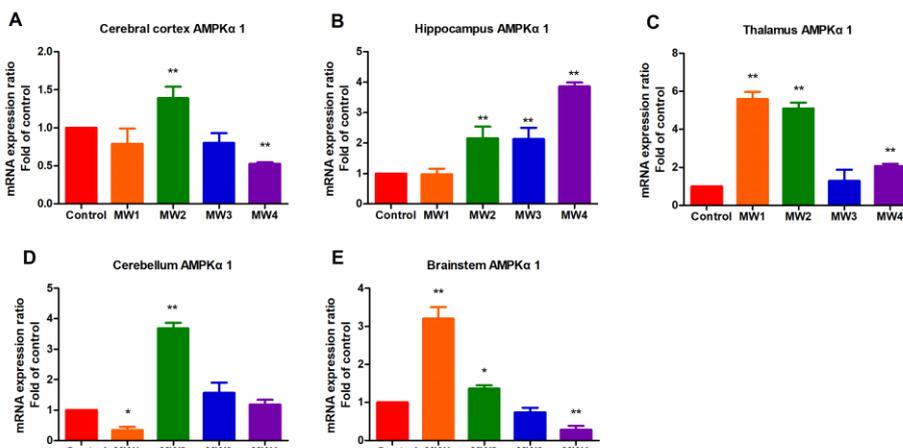


Fig. 3. Effects of atipamezole-flumazenil-naloxone (ATI-FLU-NAL) on levels of adenosine 5'-monophosphate-activated protein kinase $\alpha 1$ (AMPK $\alpha 1$) in rats. Rats received saline (0.55 mL) or ATI-FLU-NAL intraperitoneally and then were sacrificed after 10 (W1), 20 (W2), 40 (W3), or 60 min (W4). RNA from each brain region was extracted and subjected to qPCR analysis. β -actin was used as a control. Data are presented as the means \pm standard deviation, $n = 6$. * $P < 0.05$ compared with control group, ** $P < 0.01$ compared with control group

Effects of the specific antagonist for XFM for arousal from anaesthesia on AMPK α 1 and AMPK α 2 in different brain regions. Using the specific antagonist for XFM, ATI-FLU-NAL to arouse rats anaesthetised with XFM caused a significant increase in the transcription of AMPK α 1 mRNA in the thalamus in the MW1 and MW2 groups and a decrease in MW4. In the cerebral cortex, the expression of AMPK α 1 decreased significantly in the MW4 group compared with the control, and increased in the MW2 group. In the hippocampus, it increased significantly in all groups except MW1. In the cerebellum, the

expression of AMPK α 1 increased significantly in the MW2 group and decreased significantly in the MW1 group. In the brain stem, the MW1 and MW2 groups disclosed an increase while the MW4 group showed a significant decrease (Fig. 5). The transcription of AMPK α 2 mRNA declined significantly in the cerebellum, thalamus (MW2, MW4), and brain stem, while its transcription increased significantly in the cerebral cortex in the MW3 and MW4 groups. In the hippocampus, the transcription of AMPK α 2 mRNA increased in the MW4 group and decreased significantly in the MW1 group (Fig. 6).

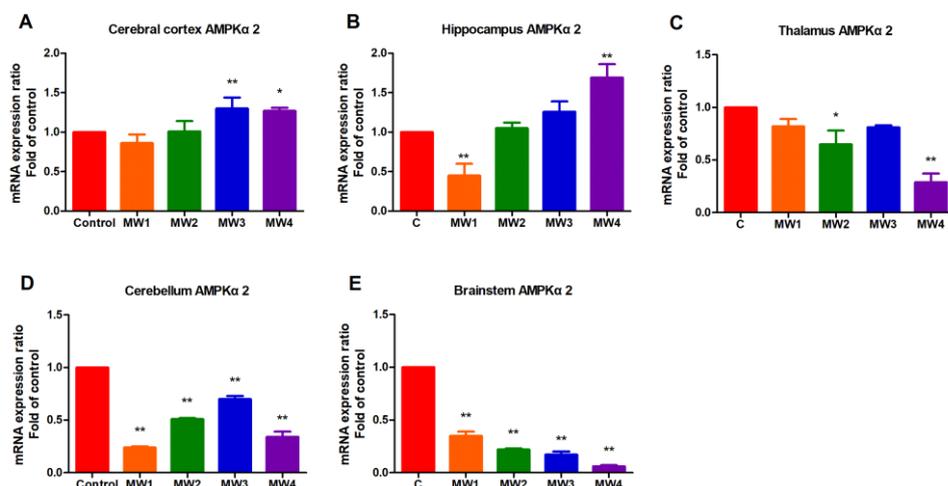


Fig. 4. Effects of atipamezole-flumazenil-naloxone (ATI-FLU-NAL) on the levels of adenosine 5'-monophosphate-activated protein kinase α 2 (AMPK α 2) in rats. Rats received saline (0.55 mL) or ATI-FLU-NAL intraperitoneally and then were sacrificed after 10 (W1), 20 (W2), 40 (W3), or 60 min (W4). RNA from each brain region was extracted and subjected to qPCR analysis. β -actin was used as a control. Data are presented as the means \pm standard deviation, N = 6. * P < 0.05 compared with control group, ** P < 0.01 compared with control group

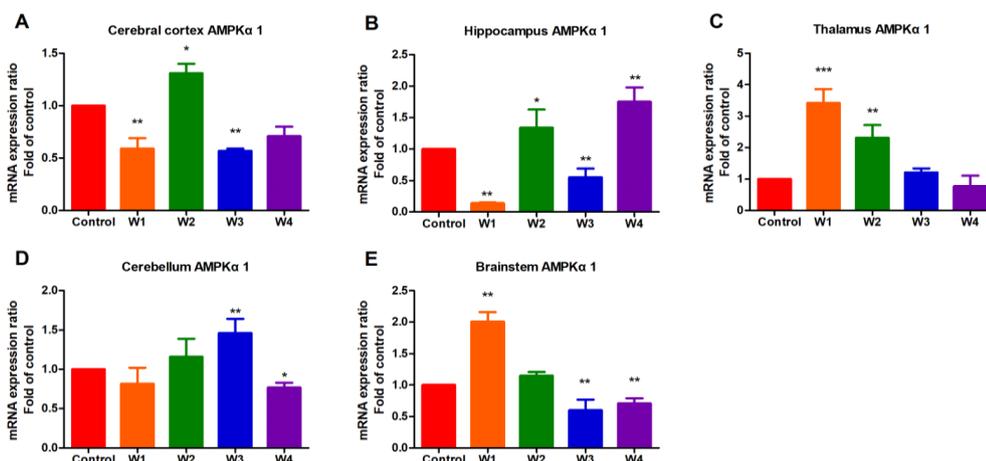


Fig. 5. Effects of the specific antagonist for tiletamine-xylazine-tramadol (XFM) in anaesthetic arousal on adenosine 5'-monophosphate-activated protein kinase α 1 (AMPK α 1) in different brain regions of rats. Rats in group MW received an intraperitoneal injection of XFM then atipamezole-flumazenil-naloxone and were sacrificed after 10 min (MW1), 20 min (MW2), 40 min (MW3), or 60 min (MW4). Saline control rats received intraperitoneal injection (0.55 mL of 0.9% NaCl, n = 6) and were then sacrificed 15 min later. RNA from each brain region was extracted and subjected to qPCR analysis. β -actin was used as a control. Data are presented as the means \pm standard deviation, n = 6. * P < 0.05 compared with control group, ** P < 0.01 compared with control group

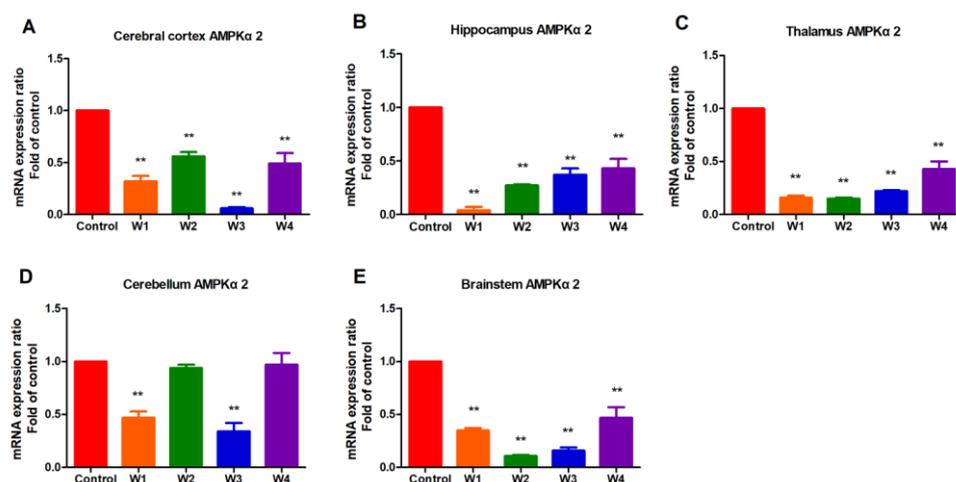


Fig. 6. Effects of the specific antagonist for tiletamine-xylazine-tramadol (XFM) in anaesthetic arousal on adenosine 5'-monophosphate-activated protein kinase $\alpha 2$ (AMPK $\alpha 2$) in different brain regions of rats. Rats in group MW received an intraperitoneal injection of XFM then 20 min later atipamezole-flumazenil-naloxone and were sacrificed after 10 min (MW1), 20 min (MW2), 40 min (MW3), or 60 min (MW4). Saline control rats received intraperitoneal injection (0.55 mL 0.9% NaCl, n = 6) and were then sacrificed 15 min later. RNA from each brain region was extracted and subjected to qPCR analysis. β -actin was used as a control. Data are presented as the means \pm standard deviation, n = 6. * P < 0.05 compared with control group, ** P < 0.01 compared with control group

Discussion

In this study, rats were sacrificed at different time points after injection of anaesthetic or antagonist, and the relative mRNA expression of AMPK $\alpha 1$ and AMPK $\alpha 2$ in the cerebral cortex, cerebellum, thalamus, hippocampus, and brain stem were detected using qPCR. The results demonstrated that the expression of both subunits increased significantly in each brain region. Adenosine 5'-monophosphate-activated protein kinase (AMPK) can be expressed in all regions of the brain, and only inhibited in the brain stem (27). It is hypothesised that the effects of XFM in the brain stem on elevation of AMPK expression is due to the combined interaction of all three anaesthetic compounds.

The results of antagonist treatment showed that the expression of AMPK $\alpha 2$ mRNA in each brain region decreased significantly. AMPK $\alpha 1$ mRNA expression only decreased in the cerebral cortex (W1 and W3) and brain stem (W3 and W4), mirroring the change in AMPK $\alpha 2$ mRNA expression, while not changing significantly in the cerebellum or hippocampus. The difference in the mRNA expression of AMPK $\alpha 1$ and AMPK $\alpha 2$ in the cerebellum and hippocampus indicated that the antagonists had no effect on AMPK $\alpha 1$ in these regions. One cause may be that the content in the brain of AMPK $\alpha 2$ is higher than that of the $\alpha 1$ subunit (28). Another reason may be that the nuclear localisation complex may also regulate gene expression when a stress response to ATP depletion occurs in the organism (29). Lastly, a stress reaction to ATP loss can result in the nuclear localisation of the complex, which may also regulate the expression of genes.

Using the specific antagonist for XFM to arouse the rats anaesthetised with that combination caused

a significant decrease in the expression of AMPK $\alpha 1$ mRNA in the cerebral cortex (MW4) and brain stem (MW4) and a significant increase in the cerebellum (MW2), thalamus (MW1, MW2 and MW4), and hippocampus (MW2, MW3 and MW4). The results showed inhibition of AMPK $\alpha 1$ mRNA in the cerebral cortex and brain stem that was stronger than that of the combined anaesthetic agent, while its expression in the cerebellum, thalamus, and hippocampus was the opposite. This suggests that the antagonists can reverse the effect of XFM on AMPK $\alpha 1$ mRNA expression in the cerebral cortex and brain stem, but not completely in the cerebellum, thalamus, and hippocampus. AMPK $\alpha 2$ mRNA expression in the cerebral cortex (MW3 and MW4) and hippocampus (MW4) increased significantly, but in the cerebellum, thalamus and brain stem the opposite effect was observed, indicating that the interaction of anaesthetic and antagonist is manifested at the molecular level. The results suggest that the combined antagonists can reverse the promoting effects of XFM on AMPK $\alpha 2$ mRNA expression in the cerebellum, thalamus, and brain stem, and that there are other signal transduction networks in the central nervous system which can influence the mechanisms of anaesthesia and arousal (29).

The anaesthetic effects of XFM were validated clinically and the results showed that heart rate, body temperature, respiratory rate, oxygen saturation, and blood pressure were all within the normal physiological range. After intramuscular injection of XFM in a cat, analgesia and sedation was complete, muscle relaxation was good, and the biological reflex was slightly affected during anaesthesia (30). It was observed that XFM had no significant effect on the heart function of mini swine and provided sufficient depth of anaesthesia. During surgery, reflexes of the cornea,

eyelid, and anus were still present, but the swine had no response to external sounds and the sedative effect was good (18). For various operations, having no muscle tension or other phenomena can meet clinical requirements. XFM can provide 60–75 min of anaesthesia and meet the relevant requirement for research, clinical diagnosis, and treatment, and furthermore does not result in any significant damage to heart or brain function (30). To summarise, XFM can be used as a special anaesthetic for clinical diagnosis or scientific research.

To the best of our knowledge, this is the first study on the effects of a combination of tramadol, tiletamine, and xylazine. This combination provides better sedation and analgesia than any used alone, with relatively few side effects (18), but the mechanism has been unclear. In this study, we choose only one point of many signal pathways to study the mechanisms of XFM and their antagonists and demonstrated that XFM may play an important role in gene expression. Meeting the target of general anaesthesia requires diverse solutions given that the site of action is diverse, and the mechanisms of action require a diversification of hypotheses. Thus, more efforts are required at the molecular level for a deeper understanding.

In summary, our study demonstrated that XFM could alter mRNA expression, suggesting that the effect of anaesthesia can be realised through AMPK. However, the antagonist cannot completely reverse the activation of XFM on AMPK molecules in some brain regions, suggesting that the mechanism of general anaesthesia is complex and has multiple loci (29). Thus, more work is required to clarify the relationship between XFM and anaesthesia.

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Animal Rights Statement: The authors declare that the experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals. All procedures were approved by the Local Ethical Commission (Northeast Agricultural University, Harbin, China).

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