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Intracerebroventricular or intrathecal injection of glycine produces analgesia in thermal nociception and chemical nociception via glycine receptors

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ABSTRACT

The present study was designed to investigate the role of glycine receptors in analgesia induced by injection of glycine in vivo. Glycine was injected intracerebroventricularly or intrathecally and strychnine, a glycine receptor antagonist, was injected intracerebroventricularly or intrathecally before glycine injection. The effects on the pain threshold index in hot-plate test and the writhing times in acetic acid-induced writhing test were observed. The locomotor activity and motor performance (rotarod test) were also observed. The dosages of glycine and strychnine we choose had no effect on locomotor activity or motor performance in conscious mice. Glycine increased the pain threshold index in hot-plate test and decreased the writhing times of the mice. Strychnine antagonized the effects induced by glycine above. These results demonstrated that intracerebroventricular or intrathecal injection of glycine can produce analgesia in thermal nociception and chemical nociception in vivo, which is mediated by glycine receptors.

1. Introduction

As the primary inhibitory neurotransmitter in the central nervous system, glycine is widely distributed throughout in the brain stem and spinal cord (Lynch, 2004). Since decades, general anesthetics are known to imitate or potentiate the effect of glycine on glycine receptors display anesthetic action (Belelli et al., 1999; Daniels and Roberts, 1998; Hadipour-jahromy and Daniels, 2003; Mihic et al., 1997; Mitchell et al., 2007; Wang et al., 2005; Zhang et al., 2001, 2003; Yamauchi et al., 2002). Analgesia is an important aspect of anesthesia (Franks and Lieb, 1994). It is traditionally accepted that general anesthetics have analgesic properties and their analgesic properties are mediated by the spinal cord (Borges and Antognini, 1994; Rampil et al., 1993; Rampil, 1994). Activation of glycine receptors typically yields an anti-nociceptive response (Chen et al., 2005; Harvey et al., 2004; Muller et al., 2003; Simpson et al., 1997). However, the roles of glycine and glycine receptors in the analgesic effects of general anesthetics are not fully understood.

In this regard, one consideration is whether glycine directly injected intracerebroventricularly and intrathecally can produce analgesic effects in hot-plate test and acetic acid-induced writhing test. Second is whether glycine receptors mediate the analgesia induced by glycine in vivo, application of strychnine, which antagonizes primarily glycine receptors, should decrease analgesic effects of glycine.

2. Materials and methods

2.1. Animals

This research was carried out according to the guidelines of the Jiangsu Council on Animal Care. Kunming mice (22 ± 3 g, certificate no. SCXK-SU-2002-0022) were obtained from the Experimental Animal Center of Xuzhou Medical College. Mice were housed in a 12-hr light:dark cycle at room temperature (22 ± 2 °C). Food and water were given ad libitum. All experiments were performed at the same time between 8:00 a.m. and 12:00 a.m. to avoid diurnal variation in behavioral tests. The Animal Ethics Committee of Xuzhou Medical College approved all procedures.

2.2. Drug

Glycine (Sigma) and strychnine (Fluka) were dissolved in artificial cerebrospinal fluid (aCSF, pH 7.35–7.45) and kept at 4 °C in a light-excluding vial. Fresh solutions were prepared every testing day. aCSF contained (in mmol/l) NaCl 117, KCl 4.5, $CaCl_2$ 2.5, $MgCl_2$ 1.2, NaH_2PO_4 1.2, $NaHCO_3$ 25 and dextrose 11.4.

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2.3. Intracerebroventricular injection and intrathecal injection

The intracerebroventricular administration was performed following the method described by Laursen and Belknap (1986). Briefly, the animal was injected at bregma with a 50-μl Hamilton syringe fitted with a 26-gauge needle of which the tip was adjusted to be inserted 2.4 mm deep. The intracerebroventricular injection volume was 5 μl and injection sites were verified by injecting the same volume of 1% methylene blue and then observing the distribution of the injected drugs or dye in the ventricular space. The dye injected intracerebroventricularly was found to be distributed in the ventricular spaces and ventral surface of the brain and in the upper cervical portion of the spinal cord.

Intrathecal injection was made in the conscious mice as described by Hylden and Wilcox (1980). We identified the fifth lumbar vertebra of the mouse and inserted a 26-gauge needle with a microsyringe into the spinal canal with a 30° angle at the fifth lumbar level. Transient tail extension was assessed as a successive injection sign. The solution was injected in a volume of 5 μl in 5 s. Lidocaine (2%) 5 μl was injected intrathecally into 10 mice which immediately exhibited hind limb paralysis that lasted for approximately 10 min in preliminary experiments.

2.4. Experimental design

The mice (male or female) were subdivided into four main experimental groups: the locomotor activity test groups, the rotarod test groups, the hot-plate test groups and the acetic acid-induced writhing test groups.

In the locomotor activity test groups or the rotarod test groups, one hundred and sixty mice (male or female) in each main experimental group were divided randomly into 16 subgroups ($n=10$): aCSF and glycine (25, 50 and 100 μg) intracerebroventricular groups; aCSF and glycine (25, 50 and 100 μg) intrathecally groups; aCSF and strychnine (1, 2, 4 μg) intracerebroventricularly groups; aCSF and strychnine (0.1, 0.2, 0.4 μg) intrathecally groups.

In the hot-plate test groups or the acetic acid-induced writhing test groups, two hundred and forty mice (male or female) in each main experimental group were divided randomly into 24 subgroups ($n=10$): aCSF and strychnine (1, 2, 4 μg) intracerebroventricularly groups; aCSF and strychnine (0.1, 0.2, 0.4 μg) intrathecally groups; aCSF and glycine (25, 50 and 100 μg) intracerebroventricularly groups; aCSF and glycine (25, 50 and 100 μg) intrathecally groups; glycine (100 μg) + aCSF or strychnine (1, 2, 4 μg) intracerebroventricularly groups; glycine (100 μg) + aCSF or strychnine (0.1, 0.2, 0.4 μg) intrathecally groups.

The glycine, aCSF and strychnine groups were injected intracerebroventricularly and intrathecally with aCSF or different doses of strychnine and glycine, respectively. The glycine + aCSF or strychnine group was injected intracerebroventricularly and intrathecally with aCSF or different doses of strychnine, and after 5 min, each mouse was injected intracerebroventricularly and intrathecally with analgesic doses of glycine. In our study, the glycine, aCSF and strychnine were used as the same volume (5 μl) injected. All data were collected in a blinded manner.

2.5. Locomotor activity test

Locomotor activity was recorded by an activity meter (Biological Equipment Co., Ltd., Zhenghua, Huaibei, China). The activity cage of this instrument is equipped with horizontal and vertical sets of infrared photocells, which send continuous unseen light beams. The number of light beam interruptions due to the animal's movement inside the cage was automatically recorded. Each mouse was placed in the activity cage and counts of motor activity were recorded automatically 10 min after 10 min following the glycine, aCSF and strychnine administration.

2.6. Rotarod test

In a rotarod test, the ability of mice to stay on an accelerating rotating rod was used to assess motor performance by a rotarod (Biological Equipment Co., Ltd., Zhenghua, Huaibei, China). The rotarod consisted of a rotating rod (2.8-cm diameter) and individual compartments for each mouse. Mice were trained for two consecutive days prior to glycine dosing in an acceleration mode (2–16 rpm) for over 2 min. The training was repeated at a fixed speed (16 rpm) until the mice were able to stay on the rod for at least 600 s.

Rotarod test was performed after 10 min following the glycine, aCSF and strychnine administration. The mice were assessed for their coordination capability on the rod at 16 rpm for a maximum recording time of 600 s, and the results were averaged to obtain a single value for each group.

2.7. Hot-plate test

A homeothermic water-box was heated to 55 ± 0.5 °C and then mice were placed onto the hot-plate (Eddy and Leimbach, 1953). The latency to licking the hind paw was recorded as the pain threshold in hot-plate test of mice. All mice were tested twice at 5-min intervals and the mean value was considered as the basal pain threshold (basal pain threshold in hot-plate test) before aCSF or strychnine and glycine administration. The latency between 5 and 30 s was qualified and the cutoff time was 60 s to avoid tissue damage. The basal pain threshold in hot-plate test and pain threshold in hot-plate test at 5, 15, 25, 35 and 45 min after injected aCSF or strychnine and glycine were observed. For each time point, pain threshold index in hot-plate test was calculated according to the formula: pain threshold index in hot-plate test = pain threshold in hot-plate test / basal pain threshold in hot-plate test. As determined in preliminary experiments, analgesic dose of glycine (100 μg) was injected intracerebroventricularly or intrathecally to establish the mouse model of analgesia.

2.8. Acetic acid-induced writhing tests

In acetic acid-induced writhing tests (Siegmund et al., 1957), 0.6% acetic acid is injected into the peritoneal cavity. It will produce the characteristic 'writhing' response that is observed in this assay. Each mouse was then placed individually in a Plexiglas observation cylinder (14 cm diameter; 30 cm in height). Five min after the administration of aCSF or strychnine and glycine, each group of mice was injected intraperitoneally with 0.6% acetic acid (10 ml/kg). The writhing times (arching of the back, development of tension in the abdominal muscles, elongation of the body and extension of the forelimbs) within 15 min were recorded after the last injection. As determined in preliminary experiments, analgesic dose of glycine (100 μg) was injected intrathecally or intracerebroventricularly to establish the mouse model of analgesia.

2.9. Statistical analysis

Results are expressed as the mean ± S.E.M. The results obtained were statistically treated by applying the SPSS 11.5 (SSPS Inc., Chicago, IL, USA). Multiple group comparisons were performed by one-way ANOVA followed by Dunnett's test. In hot-plate test, the difference between baseline and post-drug was analyzed using repeated-measures ANOVA. $P<0.05$ was considered statistically significant.

3. Results

3.1. Locomotor activity test

The locomotor activity of conscious mice was not affected by aCSF or glycine ($P>0.05$, Tables 1, 2). The strychnine groups exhibited no

Table 1
Effect of aCSF and glycine (intracerebroventricularly) on the locomotor activity and falling time in conscious mice.

Group	Intracerebroventricular injection	
	Locomotor activity (n)	Falling time (s)
aCSF	444 ± 24	566 ± 34
Glycine 25 μg	423 ± 29	535 ± 22
Glycine 50 μg	446 ± 34	577 ± 33
Glycine 100 μg	434 ± 25	543 ± 37

Data are expressed as the mean ± S.E.M. (n = 9–10 per group).

Table 2
Effect of aCSF and glycine (intrathecally) on the locomotor activity and falling time in conscious mice.

Group	Intrathecal injection	
	Locomotor activity (n)	Falling time (s)
aCSF	433 ± 26	555 ± 27
Glycine 25 μg	458 ± 21	534 ± 31
Glycine 50 μg	446 ± 33	573 ± 27
Glycine 100 μg	462 ± 36	542 ± 32

Data are expressed as the mean ± S.E.M. (n = 9–10 per group).

effects on the locomotor activity compared with aCSF groups (P>0.05, Tables 3, 4).

3.2. Rotarod test

The time fell from the drum of conscious mice was not affected by aCSF or glycine (P>0.05, Tables 1, 2). The strychnine groups exhibited no effects on the time fell from the drum compared with aCSF groups (P>0.05, Tables 3, 4).

3.3. Hot-plate test

The strychnine groups exhibited no effects on pain threshold index in hot-plate test compared with baseline values and aCSF groups (P>0.05, Figs. 1, 2). Glycine significantly increased the pain threshold index in hot-plate test (P<0.05, P<0.01, Figs. 3, 4) compared with baseline values and aCSF groups, and the increase of pain threshold index in hot-plate test induced by glycine can be antagonized by strychnine(P<0.05, P<0.01, Figs. 5, 6).

Table 3
Effect of aCSF and strychnine (intracerebroventricularly) on the locomotor activity and falling time in conscious mice.

Group	Intracerebroventricular injection	
	Locomotor activity (n)	Falling time (s)
aCSF	424 ± 35	540 ± 37
Strychnine 1 μg	439 ± 30	535 ± 32
Strychnine 2 μg	470 ± 43	565 ± 47
Strychnine 4 μg	440 ± 25	527 ± 29

Data are expressed as the mean ± S.E.M. (n = 10 per group).

Table 4
Effect of aCSF and strychnine (intrathecally) on the locomotor activity and falling time in conscious mice.

Group	Intrathecal injection	
	Locomotor activity (n)	Falling time (s)
aCSF	434 ± 32	565 ± 34
Strychnine 0.1 μg	452 ± 28	533 ± 27
Strychnine 0.2 μg	466 ± 36	568 ± 38
Strychnine 0.4 μg	428 ± 31	522 ± 31

Data are expressed as the mean ± S.E.M. (n = 8–10 per group).

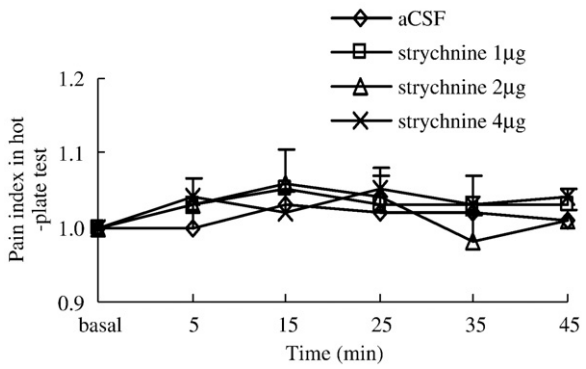

Fig. 1. Effect of strychnine on the pain threshold index in hot-plate test in conscious mice. Strychnine (1, 2 and 4 μg) and aCSF were injected intracerebroventricularly. Data are expressed as the mean ± S.E.M. (n = 9–10 per group).

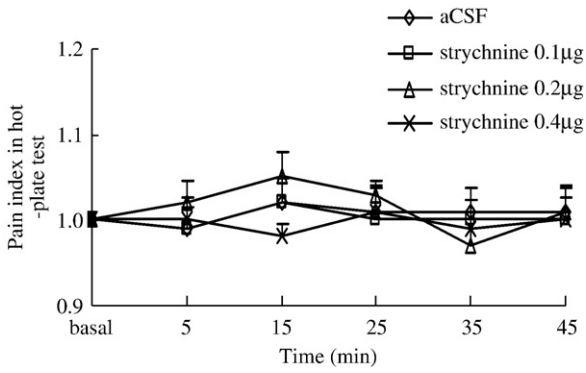

Fig. 2. Effect of strychnine on the pain threshold index in hot-plate test in conscious mice. Strychnine (0.1, 0.2 and 0.4 μg) and aCSF were injected intrathecally. Data are expressed as the mean ± S.E.M. (n = 8–9 per group).

At the 15-min time point after injecting drugs, glycine (25, 50 and 100 μg, intracerebroventricularly) increased the pain threshold index in hot-plate test by 25.0%, 46.2%, 83.7%, respectively (P<0.01, compared with aCSF group, Fig. 3); glycine (25, 50 and 100 μg, intrathecally) increased the pain threshold index in hot-plate test by 51.9%, 77.9%, 103.8%, respectively (P<0.01, compared with aCSF group, Fig. 4).

At the 15-min time point after injecting drugs, strychnine (0.1, 0.2, 0.4 μg, intracerebroventricularly) decreased the pain threshold index in hot-plate test by 10.2% (P<0.05), 24.7% (P<0.01), 35.5% (P<0.01),

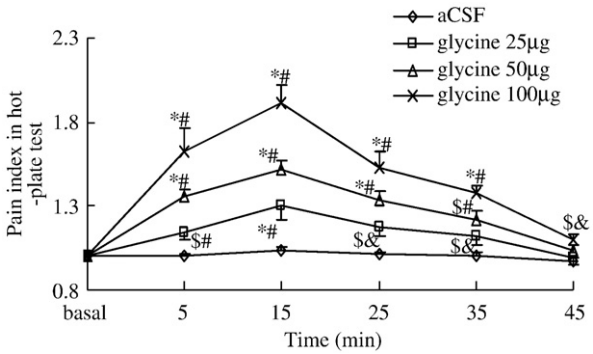

Fig. 3. Effect of glycine on the pain threshold index in hot-plate test in conscious mice. Glycine (25, 50 and 100 μg) and aCSF were injected intracerebroventricularly. Data are expressed as the mean ± S.E.M. Ten mice were used in each group. $ P<0.05 and * P<0.01 vs. basal pain threshold index in hot-plate test, using repeated-measures ANOVA; & P<0.05 and # P<0.01 vs. aCSF group, using one-way ANOVA, Dunnett's test. (n = 10 per group).

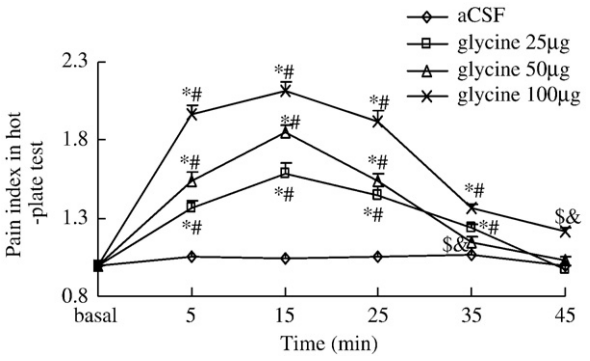

Fig. 4. Effect of glycine on the pain threshold index in hot-plate test in conscious mice. Glycine (25, 50 and 100 μg) and aCSF were injected intrathecally. Data are expressed as the mean ± S.E.M. Ten mice were used in each group. $ $P<0.05$ and * $P<0.01$ vs. basal pain threshold index in hot-plate test, using repeated-measures ANOVA; & $P<0.05$ and # $P<0.01$ vs. aCSF group, using one-way ANOVA, Dunnett's test. ($n=9$–10 per group).

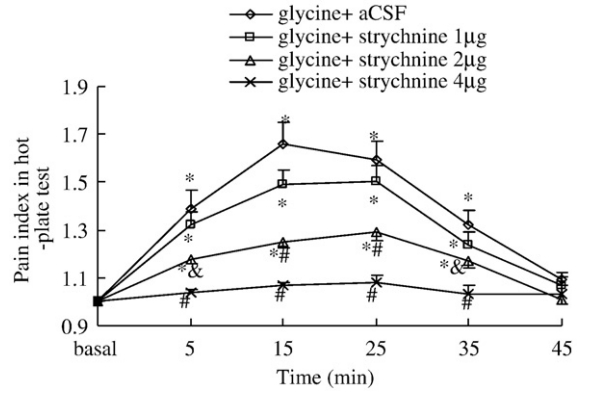

Fig. 5. Effect of strychnine on the pain threshold index in hot-plate test in glycine treated mice. Glycine 100 μg, aCSF and strychnine (1, 2 and 4 μg) were injected intracerebroventricularly. Data are expressed as the mean ± S.E.M. Ten mice were used in each group. $ $P<0.05$ and * $P<0.01$ vs. basal pain threshold index in hot-plate test, using repeated-measures ANOVA; & $P<0.05$ and # $P<0.01$ vs. glycine + aCSF group, using one-way ANOVA, Dunnett's test. ($n=9$–10 per group).

respectively, compared with glycine + aCSF group (Fig. 5); strychnine (0.05, 0.1, 0.2 μg, intrathecally) decreased the pain threshold index in hot-plate test by 20.4% ($P<0.01$), 35.0% ($P<0.01$), 47.6% ($P<0.01$), respectively, compared with glycine + aCSF group (Fig. 6).

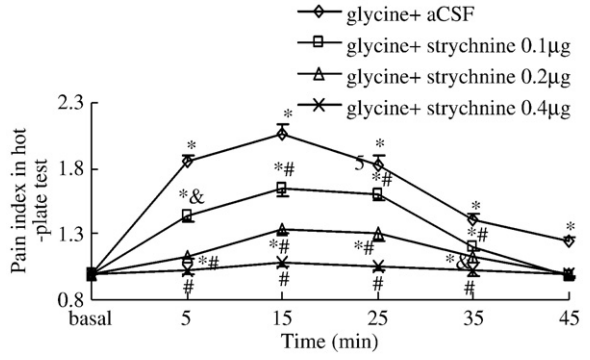

Fig. 6. Effect of strychnine on the pain threshold index in hot-plate test in glycine treated mice. Glycine 100 μg; aCSF and strychnine (0.1, 0.2 and 0.4 μg) were injected intrathecally. Data are expressed as the mean ± S.E.M. Ten mice were used in each group. $ $P<0.05$ and * $P<0.01$ vs. basal pain threshold index in hot-plate test, using repeated-measures ANOVA; & $P<0.05$ and # $P<0.01$ vs. glycine + aCSF group, using one-way ANOVA, Dunnett's test. ($n=9$ per group).

Table 5
Effects of aCSF and strychnine on writhing times in conscious mice.

Intracerebroventricular injection		Intrathecal injection	
Groups	Writhing times (n)	Groups	Writhing times (n)
aCSF	38.2 ± 2.5	aCSF	41.9 ± 2.7
Strychnine 1 μg	41.2 ± 3.3	Strychnine 0.1 μg	41.2 ± 3.3
Strychnine 2 μg	37.9 ± 2.9	Strychnine 0.2 μg	39.8 ± 2.5
Strychnine 4 μg	40.9 ± 2.6	Strychnine 0.4 μg	38.9 ± 2.3

Data are expressed as the mean ± S.E.M. ($n=9$ per group).

Table 6
Effects of aCSF and glycine on writhing times in conscious mice.

Intracerebroventricular injection		Intrathecal injection	
Groups	Writhing times (n)	Groups	Writhing times (n)
aCSF	40.2 ± 3.0	aCSF	44.1 ± 1.3
Glycine 25 μg	32.7 ± 2.1[a]	Glycine 25 μg	30.2 ± 2.5[b]
Glycine 50 μg	22.8 ± 1.6[b]	Glycine 50 μg	19.2 ± 1.0[b]
Glycine 100 μg	14.7 ± 0.6[b]	Glycine 100 μg	9.6 ± 0.5[b]

Data are expressed as the mean ± S.E.M. ($n=10$ per group).
[a] $P<0.05$ vs. aCSF groups, using one-way ANOVA, Dunnett's test.
[b] $P<0.01$ vs. aCSF groups, using one-way ANOVA, Dunnett's test.

3.4. Acetic acid-induced writhing test

The strychnine groups exhibited no effects on the writhing times compared with aCSF groups ($P>0.05$; Table 5). Glycine significantly decreases writhing times of the mice ($P<0.05$, $P<0.01$, Table 6) compared with aCSF groups. The decrease in writhing times induced by glycine was antagonized by pre-injection of strychnine ($P<0.05$, $P<0.01$, Table 7).

4. Discussion

An acute administration of aCSF or strychnine induced no change to pain threshold index in hot-plate test and writhing times in mice. However, in stimulated animals, intracerebroventricular or intrathecal injection of glycine significantly increased pain threshold index in hot-plate test and decreased writhing times. These effects induced by glycine were antagonized by strychnine.

As an important aspect of anesthesia, analgesia means the loss of pain sensation without loss of consciousness or immobility (Kendig, 2002). The mice in analgesic models not only show the obvious effects of analgesia, but also have an almost normal behavior and had no loss of the righting reflex (Chen et al., 2007). The righting reflex is a very insensitive measure of motoric capability, generally only being affected at extremely high doses of drug. As a widespread inhibitory neurotransmitter, glycine invariably affects an organism's ability to move; thus, it is crucial to quantify glycine's

Table 7
Effect of strychnine on the writhing times in glycine treated mice.

Intracerebroventricular injection		Intrathecal injection	
Groups	Writhing times (n)	Groups	Writhing times (n)
Glycine 100 μg + aCSF	12.9 ± 0.7	Glycine 100 μg + aCSF	9.2 ± 0.5
Glycine 100 μg + strychnine1 μg	19.7 ± 1.1[a]	Glycine 100 μg + strychnine0.1 μg	13.8 ± 0.6[a]
Glycine 100 μg + strychnine2 μg	25.4 ± 1.4[b]	Glycine 100 μg + strychnine0.2 μg	22.9 ± 1.4[b]
Glycine 100 μg + strychnine4 μg	34.6 ± 2.3[b]	Glycine 100 μg + strychnine0.4 μg	40.2 ± 2.7[b]

Data are expressed as the mean ± S.E.M. ($n=9$–10 per group).
[a] $P<0.05$ vs. glycine 100 μg + aCSF groups, using one-way ANOVA, Dunnett's test.
[b] $P<0.01$ vs. glycine 100 μg + aCSF groups, using one-way ANOVA, Dunnett's test.

potency in producing motoric effects using the same routes of administration used for the anti-nociceptive testing. We demonstrated that intracerebroventricular and intrathecal injection of aCSF or glycine (25, 50 and 100 μg) induced no change to locomotor activity and rotarod performance in conscious mice. The results of rotarod tests and locomotor activity experiments showed that the mice (glycine 25, 50 and 100 μg, intracerebroventricularly and intrathecally) were excluded to sedate effects, motor impairment and remained alert. So, we chose the doses of glycine (100 μg) intracerebroventricular and intrathecal injection to establish the analgesic model. Strychnine is a specific antagonist of glycine receptors, and can agitate or even convulse animals after administration. Chen reported that the dosage of strychnine (0.1, 0.2 and 0.4 μg intrathecally and 1, 2 and 4 μg intracerebroventricularly), did not exhibit its own behavioral effect (Chen et al., 2007). We also proved that the dosages of strychnine induced no change to locomotor activity and rotarod performance in conscious mice. So the dosages of strychnine in present study were selected. Intrathecal injection in mice is a good method to study the spinally mediated actions of drugs. According to Hylden and Wilcox (1980) report, 5 μl of methylene blue dye never extended rostral of thoracic segments from injection site and intrathecal [^{3}H] morphine was not found in significant quantities in either the midbrain or forebrain. Hence, it gives us the desired ability to observe behavioral effects of drugs at the spinal level.

Studies in vivo indicated that the effects of intravenous anesthetics on pain thresholds are mediated by enhancement of the inhibitory action of glycine receptor in selected regions of the central nervous system (Mitchell et al., 2007; Wang et al., 2005.). In mice during inhalational anesthesia, former studies found that administration of glycine receptor antagonists can increase the minimum alveolar anesthetic concentration (MAC) of volatile anesthetics which implicated glycine and glycine receptors may in part be responsible for the immobilizing action of inhalational anesthetics (Zhang et al., 2001, 2003; Yamauchi et al., 2002.). Simpson provided that glycine administered intrathecally reduces the pain response evoked by the hot-plate analgesia meter method (Simpson et al., 1997). Huang proved that intrathecal glycine administration increased the normal mechano-nociceptive responses and prevented the development of mechano-nociceptive hyperalgesia of neuropathic pain evoked by unilateral loose ligation of the sciatic nerve in the rat (Huang and Simpson, 2000). The studies above suggest that glycine and glycine receptors play an important role in analgesic effects, which are in accord with our observations.

While, Dumka found that intracerebroventricular administration of glycine did not alter the pain threshold significantly in comparison to control in formalin-induced acute peripheral inflammation (Dumka et al., 1998). Dumka's results are not in accord with our observation. The explanations of the probable mechanisms may include the following points: Pain is the net effect of multidimensional mechanisms that involve most parts of the central nervous system (Le et al., 2001). According to this view, a variety of drugs have been tested in different preclinical models of pain with variable results, depending on the models and tests used. Writhing test shows more analogy to visceral pain, while hot-plate test is pain reaction involving higher brain centers and is affected by both motor and emotional components. In formalin-induced pain, the first phase may result from a direct activation of myelinated and unmyelinated fibers, both low-threshold mechanoreceptive and nociceptive types, and the second phase may be caused by the activation of central sensitized neurons due to peripheral inflammation stimuli as well as ongoing activity of primary afferents (Puig and Sorkin, 1996). The possible mechanism of neuropathic pain may include inhibition of glycine release from the terminals of glycinergic interneurons, reduction in the transmembrane chloride gradient rendering the inhibitory tone of GABAergic and glycinergic synaptic input less efficient, and loss of inhibitory innervation due to selective death of GABAergic or glycinergic interneurons (Zeilhofer, 2005). Different nociception causes different pain and the analgesic mechanisms of glycine are also different.

In conclusion, intracerebroventricular or intrathecal injection of glycine can produce anti-nociceptive effects in vivo. Glycine receptor mediated the analgesic effects of glycine at the spinal and supra-spinal level.

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