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Glycine Receptors Contribute to Hypnosis Induced by Ethanol

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Abstract

Background—Glycine is a major inhibitory neurotransmitter in the adult central nervous system (CNS), and its receptors (GlyRs) are well known for their effects in the spinal cord and the lower brainstem. Accumulating evidence indicates that GlyRs are more widely distributed in the CNS, including many supraspinal regions. Previous in vitro studies have demonstrated that ethanol potentiates the function of these brain GlyRs, yet the behavioral role of the brain GlyRs has not been well explored.

Methods—Experiments were conducted in rats. The loss of righting reflex (LORR) was used as a marker of the hypnotic state. We compared the LORR induced by systematic administration of ethanol and of ketamine in the absence and presence of the selective glycine receptor antagonist strychnine. Ketamine is a general anesthetic that does not affect GlyRs.

Results—Systemically administered (by intraperitoneal injection) ethanol and ketamine dosedependently induced LORR in rats. Furthermore, systemically administered (by subcutaneous injection) strychnine dose-dependently reduced the percentage of rats exhibiting LORR induced by ethanol, increased the onset time, and decreased the duration of LORR. Strychnine had no effect, however, on the LORR induced by ketamine.

Conclusions—Given that hypnosis is caused by neuronal depression in upper brain areas, we therefore conclude that brain GlyRs contribute at least in part to the hypnosis induced by ethanol.

Keywords

Alcohol; Strychnine; Loss of Righting Reflex; Ketamine; Sedation; Hypnosis; Glycine Receptor

Glycine is one of the major neurotransmitters that mediate inhibitory synaptic transmission in the central nervous system (CNS) (Betz, 1992). When bound to glycine receptors (GlyRs), glycine increases the permeability of neuronal membranes to chloride ions, thus hyperpolarizing adult mammalian neurons and producing inhibitory effects (Ye, 2008). GlyRs are highly concentrated in the lower brain stem and spinal cord (Legendre, 2001). Much evidence has been presented demonstrating the role of spinal GlyRs in the mediation of motor and sensory responses. They have been shown to control motor rhythm generation (Baldissera et al., 1981; Grillner, 1981; Grillner et al., 1998), and mediate reciprocal inhibition in stretch reflex circuits (Fyffe, 1991; Kandel et al., 2000; Lodge et al., 1977).

Glycine released by the spinal Renshaw interneurons regulates motoneuron excitability and firing (Curtis et al., 1976; Fyffe, 1991; Kandel et al., 2000). GlyRs are major mediators of sensory inputs, including pain (Huang and Simpson, 2000). They are also major targets of general anesthetics, such as propofol and isoflurane (Dong and Xu, 2002; Downie et al., 1996). Current evidence clearly implicates spinal GlyRs mediate part of the immobilization produced by inhaled anesthetics (Sonner et al., 2003; Zhang et al., 2003).

Accumulating evidence strongly indicates that GlyRs exist throughout the CNS (Rampon et al., 1996; Ye, 2008), including many areas of the brain, such as the prefrontal cortex, hippocampus, amygdala, hypothalamus, cerebellum, nucleus accumbens, ventral tegmental area, and substantia nigra (Chattipakorn and McMahon, 2002; Flint et al., 1998; Gaiarsa et al., 2002; Laube et al., 2002; Mangin et al., 2003; McCool and Botting, 2000; McCool and Farroni, 2001; Mori et al., 2002; Tapia et al., 2000; Ye et al., 1998, 1999; Zhou, 2001). In vitro studies have demonstrated that ethanol potentiates the function of GlyRs, including the GlyRs in the spinal cord and in the cerebral cortex (Aguayo and Pancetti, 1994; van Zundert et al., 2000), as well as in the midbrain (Jiang and Ye, 2003; Ye et al., 2001; Zhu and Ye, 2005). Furthermore, a structural–function relationship study has shown a binding site in GlyRs for ethanol (Mihic et al., 1997).

However, the behavioral roles of brain GlyRs are not well explored. A previous in vivo study in rats has indicted the hypothalamic GABA_A receptor as a contributor to the hypnosis, or loss of consciousness, induced by propofol and several other general anesthetics (Nelson et al., 2002). The GABA_A receptor is the receptor for the other major inhibitory neurotransmitter in the brain. The hypothalamus is a crucial brain area in the sleep pathways. We reasoned that the GlyRs in the brain may also contribute to the hypnotic effects of CNS depressants. In support of this possibility, recent studies in our laboratory have demonstrated that the specific GlyR antagonist strychnine decreased the number of rats exhibiting loss of righting reflex (LORR) induced by propofol. Furthermore, the glycine current of hypothalamic neurons was potentiated by propofol (Nguyen et al., 2009). The object of the current study was to investigate the role of brain GlyRs in the hypnotic response to ethanol. This is accomplished via behavioral analyses on rats.

MATERIALS AND METHODS

Protocol

The experimental protocol conforms to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and was approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey (UMDNJ). The UMDNJ Animal Facility is AAALAC approved.

Subjects

Animals used were female adult Sprague-Dawley rats weighing 350 to 450 g. All were individually housed under controlled conditions (20 to 22°C), with plentiful access to water and food ad libitum.

Assessment of Hypnosis

The primary endpoint for evaluation of the hypnotic state was the LORR. LORR was defined as the inability of rats to right themselves when positioned in a supine position. After sequential injections of ethanol or ketamine, then either saline or strychnine, the animals were positioned on their backs at 1-minute intervals. If they did not regain their posture within 10 seconds, then the rats were recorded as having lost their righting reflex, and the righting reflex was considered restored when the animals were able to regain an upright position. The time lapsed in between the injections and the presence of LORR was recorded in minutes and referred to as the onset of LORR, and the time between the initial LORR and the return of the righting reflex was recorded in minutes and referred to as the duration of LORR. The total percentage of animals that demonstrated the LORR was also recorded.

Chemicals

Ethanol was purchased from Pharmco Products, Inc. (Brookfield, CT), and ketamine and strychnine were each obtained from Sigma Chemical Company (St Louis, MO). Propofol was purchased from AstraZeneca Pharmaceuticals LP (Wilmington, DE).

Statistical Analysis

Data were statistically compared using Student's *t* test using Sigma plot (Systat Software Inc., San Jose, CA), or ANOVA using SPSS (SPSS, Chicago, IL) at a significance level of $p < 0.05$. A two-way ANOVA and post hoc comparison was used for the data in Fig. 1B, Fig. 1C, Fig. 2B, and Fig. 2C. For all experiments, average values are expressed as mean $\pm$ SEM. Dose-response data (in Fig. 1A and Fig. 2A) were fitted as previously described (Waud, 1972) to a logistic equation of the following form:

$$P = 100 D^n / (D^n + (ED_{50})^n)$$

where P is the percent of the population anesthetized, D is the drug dose, n is the slope parameter, and ED_{50} is the drug dose for a half-maximal effect.

RESULTS

Ethanol Induces LORR in a Dose-Dependent Manner

We first tested whether or not ethanol would induce LORR in a dose-dependent manner. Animals ($n = 7$) were given intraperitoneal (i.p.) injections of a range of concentrations of ethanol (2.50, 2.75, 2.90, 3.00, 3.25, 3.50, and 3.75 g/kg) (20% w/v), followed immediately by a subcutaneous (s.c.) injection of saline. We used the LORR score as our primary measure for hypnosis because the concentrations of anesthetics that are necessary to produce the hypnotic state in humans are similar to those needed to induce LORR in rodents (Bonin and Orser, 2008; Franks, 2008; Nelson et al., 2002; Rudolph and Antkowiak, 2004). As exemplified by Fig. 1, the percentage of rats exhibiting LORR increased (Fig. 1A), the onset time of LORR decreased (Fig. 1B, two-way ANOVA, $p < 0.001$), and the duration of LORR increased (Fig. 1C, two-way ANOVA, $p = 0.01$) with increasing doses of ethanol.

Strychnine Dose-Dependently Reduces Ethanol-Induced Hypnosis

We then tested whether strychnine (s.c. administration) could attenuate the hypnosis induced by ethanol. Animals ($n = 7$) were given an i.p. injection of 3.0 g/kg ethanol, which was the minimum dose of ethanol required to produce LORR in 100% of the subjects, followed by s.c. injections of strychnine (0.00 to 1.25 mg/kg). Figure 1D shows that as the percentage of animals displaying LORR in response to ethanol (3.0 g/kg, i.p.) was decreased, the onset of LORR was increased (Fig. 1E, one-way ANOVA, $p < 0.001$), and the duration of LORR was decreased (Fig. 1F, one-way ANOVA, $p < 0.05$) with increasing doses of strychnine (0.00, 0.75, 1.00, and 1.25 mg/kg, s.c.).

Strychnine Shifted the Dose–Response Curve of Ethanol to the Right

We next determined the effect of strychnine (1.0 mg/kg, s.c.) on the LORR induced by a range of concentrations of ethanol. As per the first experiment, animals were given i.p. injections of ethanol (2.50 to 3.75 g/kg, 20% w/v), but this time the ethanol injections were followed by s.c. injection of 1.0 mg/kg strychnine. This concentration of strychnine was estimated to be the ED_{50} (Fig. 1D). Figure 1 shows that strychnine (s.c.) reduced the percentage of animals displaying LORR, increased the onset time of LORR, and decreased the duration of LORR at each dose of ethanol (i.p.). Strychnine (1.0 mg/kg, s.c.) induced a statistically significant rightward shift of the LORR in response to ethanol (i.p.) and significantly increased the median effective dose (ED_{50}) of ethanol from 2.67 ± 0.02 g/kg (mean $\pm$ SEM) to 3.00 ± 0.06 (paired t -test, $n = 7$, $p = 0.011$) (Fig. 1A). In the rats that exhibited LORR, an s.c. injection of 1.0 mg/kg strychnine after the i.p. administration of 2.50, 2.75, 2.90, 3.00, 3.25, 3.50, and 3.75 g/kg ethanol significantly prolonged the onset time (Fig. 1B, two-way ANOVA, $p < 0.001$), and significantly reduced the mean duration time (Fig. 1C, two-way ANOVA, $p < 0.01$). In the above experiments, the animals were considered to regain their righting reflex if the animals right themselves one time. To determine whether this contributed to the shorter duration observed in the presence of strychnine, we repeated the experiments in which animals were considered to regain their righting reflex only when they were able to regain an upright position 3 times within 30 to 60 seconds. In four rats, the duration of LORR induced by 3.0 g/kg ethanol (i.p.) was 32 ± 5 minutes, which was significantly longer than 10 ± 3 minutes in the presence of 1.0 mg/kg strychnine ($p < 0.01$, not illustrated). These results indicate that strychnine reduces the LORR induced by ethanol.

Strychnine is Unable to Attenuate Hypnosis Induced by Ketamine

Ketamine has been shown to reduce excitatory neurotransmission by inhibiting the *N*-methyl-D-aspartic acid (NMDA) subtype of glutamate receptors, thus inducing hypnosis (Anis et al., 1983). Figure 2 demonstrates that the percentage of animals exhibiting LORR in response to 20, 35, 50, 100, and 150 mg/kg (i.p.) of ketamine was not altered in the presence and the absence of strychnine (1.0 mg/kg, s.c.). The ED_{50} of ketamine was the same in the absence (33.3 ± 0.5 mg/kg) and the presence (33.3 ± 0.5 mg/kg) of strychnine ($p = 1.0$). The addition of strychnine did not significantly change the onset time and the duration of LORR induced by ketamine (Fig. 2B and Fig. 2C, $p > 0.2$). This finding, consistent with data collected in a recent study also conducted in our laboratory (Nguyen et al., 2009), shows that

systemic strychnine does in fact act specifically on GlyRs to attenuate responses to CNS depressants, as opposed to causing an indiscriminate escalation in neuronal excitability.

DISCUSSION

Our major finding is that brain GlyRs contribute, at least in part, to ethanol-induced hypnosis. Our data demonstrate that strychnine dose-dependently reduced the percentage of animals exhibiting LORR induced by ethanol, extended the onset time of LORR, and reduced the LORR duration. In contrast, strychnine had no effect on the ketamine-induced LORR. Ketamine reduces excitatory neurotransmission by inhibiting the NMDA subtype of glutamate receptors (Anis et al., 1983). These results indicate that strychnine attenuates the LORR caused by ethanol via a blockage of CNS GlyRs, as opposed to a nonspecific excitatory effect.

In this study, ethanol and strychnine were administered systemically, and thus were capable of interacting with the GlyRs throughout the CNS, including those GlyRs in the spinal cord. We believe that strychnine attenuated the LORR induced by ethanol through its effect on the GlyRs in the supraspinal regions of the CNS based on the following reasoning: as mentioned, we used the LORR as an indicator for hypnosis because the concentrations of anesthetics that are necessary to produce the hypnotic state in humans are similar to those needed to induce LORR in rodents (Bonin and Orser, 2008; Franks, 2008; Nelson et al., 2002; Rudolph and Antkowiak, 2004). Furthermore, past studies have shown that while anesthetic-induced ablation of movement in response to pain is mediated primarily by the spinal cord (Antognini and Carstens, 2002), hypnosis and amnesia are supraspinal effects (Eger et al., 1997). This suggests that spinal GlyRs are the main players in immobility, and brain GlyRs have a considerable role in hypnosis. Nevertheless, we cannot exclude the involvement of GlyRs in the other parts of the CNS, as the righting reflex requires a complex coordination of perception and response. The perception of position by the inner ear must communicate with cerebral centers responsible for signaling motor responses, the last of these mediated by the spinal cord.

In addition, in the present study, we found that the GlyR antagonist strychnine failed to attenuate the hypnotic effects of ketamine, while it did attenuate the hypnotic effects of ethanol. These results further support the idea that ethanol exerts its hypnotic effects by acting on the strychnine-sensitive GlyRs.

Furthermore, in support of this notion, a recent study in our laboratory demonstrated that strychnine, when injected into the intracerebroventricular (ICV) space of rats, could attenuate the LORR induced by propofol. Additionally, propofol was shown to potentiate the glycine current of hypothalamic neurons. As mentioned, the hypothalamus is a critical area in the sleep pathway. These in vivo and in vitro data provided solid indications of brain GlyRs' role in mediating hypnosis (Nguyen et al., 2009).

Our conclusion is also supported by a previous study in male mice. Williams and colleagues (1995) demonstrated the ability of exogenous glycine applied to the ICV space to augment the central depressant effects of systemically administered (i.p.) ethanol. In that experiment,

glycine and either saline or strychnine were administered simultaneously into the ICV space of mice after the animals had awakened from an ethanol-induced LORR. In the current study on female rats, we demonstrated that systemically administered strychnine attenuated the LORR induced by systemically administered ethanol. Taken together, the results of our study on female rats further confirm the results from the Williams' study on male mice, indicating that brain GlyRs play a considerable role in ethanol-induced hypnosis.

In the current study, we showed that the systemic injection of 1.0 mg/kg of strychnine increases the ED₅₀ of LORR induced by ethanol by 12.4%. However, this 12.4% increase in ED₅₀ by 1.0 mg/kg strychnine is not the upper limit of the effects of blocking GlyRs. The dose–response curve of strychnine (Fig. 1D) indicates that strychnine (1.25 mg/kg) has about 140% of the effect of 1.0 mg/kg strychnine on the LORR induced by 3.0 g/kg of ethanol. Assuming that this is also true for the other concentrations of ethanol, we can estimate that the increase of ED₅₀ of ethanol by 1.25 mg/kg strychnine is 140% of the original, or a 17.4% increase. Thus, the GlyR is one of the targets for ethanol-induced hypnosis. It is known that ethanol works on many other receptors/channels, such as GABA_AR (Allan et al., 1987) and NMDA receptors (Grant and Lovinger, 1995; Vengeliene et al., 2008; Weight et al., 1991, 1992). These receptor/channels, the GABA_ARs in particular, are expected to play an even greater role in the hypnosis induced by ethanol.

SUMMARY AND CONCLUSIONS

Though the roles of the GlyRs in the lower brainstem and spinal cord have been well studied, much less is currently known about the role played by the GlyRs in the supraspinal regions of the CNS. In this investigation, we demonstrated that ethanol and ketamine dose-dependently increased the percentage of animals displaying LORR. Strychnine dosedependently attenuated the responses induced by ethanol, but not by ketamine. Given that the depression of the activity of neurons in upper brain areas is responsible for hypnosis, the current study provides substantial evidence that GlyRs in the upper brain areas play a considerable role in ethanol-induced hypnosis.

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Fig. 1.

Strychnine (STR, 1.0 mg/kg, s.c.) shifts the dose–response curve of ethanol (2.50 to 3.75 g/kg, i.p.) to the right in the percentage of rats exhibiting LORR, increasing the ED_{50} of ethanol from 2.67 ± 0.02 to 3.00 ± 0.06 g/kg (paired *t*-test, $n = 7$, $p = 0.011$) (A), prolongs the mean ($\pm$ SEM) onset time of LORR, and decreases the mean ($\pm$ SEM) duration time of LORR. Ethanol dose-dependently increases the percentage of rats exhibiting LORR (A), reduces the onset time of LORR (B, two-way ANOVA, $p < 0.001$), and prolongs the duration of LORR (C, two-way ANOVA, $p < 0.01$). Strychnine (STR, 0.00 to 1.25 mg/kg, s.c.) dose-dependently decreases the percentage of rats exhibiting LORR induced by ethanol (3.0 g/kg, i.p.) with an estimated ED_{50} of 1.00 mg/kg (s.c.) (D), increases the onset time of LORR (E, one-way ANOVA, $p < 0.001$), and decreases the LORR duration (F, one-way ANOVA, $p < 0.05$).

Fig. 2.

Strychnine (STR, 0.75 mg/kg, s.c.) has no effect on the percentage of rats exhibiting LORR induced by ketamine (20 to 150 mg/kg, i.p.). The presence of strychnine had no impact on the ED_{50} of ketamine, which was 33.3 ± 0.5 mg/kg ($n = 7$) both with and without strychnine. The ketamine and ketamine + strychnine curves are superimposed (**A**). The presence of strychnine had no significant impact on the onset time (**B**) and duration (**C**) of LORR induced by ketamine.