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Research Article

Molecular investigation of ergot alkaloid ergotamine's modulatory effects on glycine receptors expressed in *Xenopus* oocytes

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ABSTRACT

The relationship between oxidative stress and glycine receptors is complex, involving multiple mechanisms through which reactive oxygen species can modify glycine receptor function. Understanding these interactions is essential for developing therapeutic strategies to mitigate the effects of oxidative stress on inhibitory neurotransmission in various neurological disorders. Inhibitory glycine receptors play a critical role in regulating the final grand postsynaptic potential by attenuating excitatory postsynaptic potentials through inhibitory postsynaptic potentials in postsynaptic neurons. This is particularly important in rapid signal transmission systems, where it determines whether the grand postsynaptic potential exceeds the activation threshold. Glycine receptors are known to be expressed not only in the spinal cord and brainstem but also in the hippocampus, as evidenced by studies conducted over the past decade. Interestingly, these regions share a common cellular architecture, predominantly composed of pyramidal neurons. In hippocampal pyramidal neurons, glycine receptors contribute to the regulation of synapse formation and plasticity, and they are crucial in motor neuron control within the pyramidal tract. However, there is limited research on glycine receptor antagonism, which is necessary to fully understand their biological functions in these regions. We conducted a comprehensive molecular-level analysis of the pharmacological properties of glycine receptors, examined their interaction mechanisms through electrophysiological studies, and identified binding sites using structural modeling and site-directed mutagenesis. Our findings suggest that ergotamine may serve as a promising antioxidant candidate to address issues associated with excessive or prolonged inhibitory postsynaptic potentials, offering a potential new therapeutic pathway.

1. Introduction

The primary function of the glycine receptor is to allow chloride ions into the cell to generate inhibitory postsynaptic potentials (IPSPs) in response to glycine binding. The relationship between oxidative stress and glycine receptors is complex and involves multiple mechanisms through which reactive oxygen species (ROS) can alter glycine receptor function. These IPSPs regulate grand postsynaptic potentials (GPSPs), which integrate excitatory postsynaptic potentials (EPSPs) and IPSPs from multiple synapses by diminishing EPSPs via membrane hyperpolarization. If a GPSP reaches the threshold for firing an action potential, then voltage-gated calcium channels (VGCCs) are opened to transiently increase intracellular calcium levels. Downstream signaling ensues (e.g., cell proliferation, neurogenesis, or transmission informational action potentials) [1–3]. In situations where intracellular calcium concentrations are sustained or excessively high due to massive EPSPs, apoptosis ensues. In situations where IPSPs become massive, problems in cell-specific functions ensue (e.g., new synapse/dendritic spine generation, dendritic shooting, and voluntary motor control) [4,5]. Antagonists of glycine receptors can ameliorate these neurobiological

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dysfunctions. Glycine receptors are pentameric ligand-gated ion channels that are found throughout the central nervous system, the brainstem, and the spinal cord [6,7]. Notably, the locations where glycine receptors are expressed are not only the pyramidal tract (related to body movement control) but also the hippocampus (related to cognitive abilities) [8–10]. Glycine is an inhibitory neurotransmitter. When glycine binds to its receptor, the ion channel allows anions to flow into the intracellular space and the membrane potential hyperpolarizes [11]. Massive hyperpolarization results in a communication failure with other cells because it is more difficult for the cell to generate an action potential. These glycinergic transmissions modulate motor coordination, respiration rhythms, and sensory reflex activity [5,12,13]. Glycine receptor dysfunctions lead to motor neuron impairment that leads to hyperekplexia, convulsions, epilepsy, seizures, pain signaling, and cognitive disorders [14–18]. The cells that inhabit these regions and express glycine receptors are pyramidal neurons. Pyramidal neurons have a single axon and numerous dendrites, resembling a pyramid, hence their name. Thanks to this structural feature, they play a key role in integrating vast amounts of informational input from other neurons and are located in the cerebral cortex, hippocampus, and amygdala [19]. They form pyramidal tracks in the brainstem and spinal cord, controlling voluntary motor functions [20,21]. Each neuron possesses dendritic spines that receive excitatory postsynaptic potentials from presynaptic neurons [22]. Pyramidal neurons are responsible for integrating numerous input signals necessary for memory and learning, especially in the hippocampus, and affect whether memories are consolidated [23]. The expression of glycine receptors in hippocampal pyramidal neurons was confirmed [9], which used electrophysiology techniques to observe glycine-induced currents and the effects of strychnine, a glycine receptor-specific antagonist. This study not only revealed the presence of glycine receptors in hippocampal pyramidal cells but also confirmed their existence in hippocampal interneurons. Additionally, other studies have shown that non-neuronal cells such as astrocytes and glial cells also express glycine receptors [24–26]. However, our understanding of molecular-level electrophysiological mechanisms of how these receptors operate remains insufficient. The relationship between oxidative stress and glycine receptors involves the impact of ROS on the function and integrity of these receptors. Glycine receptors are crucial for inhibitory neurotransmission in the central nervous system, playing a significant role in modulating neuronal excitability and maintaining synaptic balance. Oxidative stress is a common feature of neurodegenerative diseases like Alzheimer's, Parkinson's, and Amyotrophic Lateral Sclerosis. Dysfunctional glycine receptors due to oxidative stress can contribute to the pathophysiology of these diseases by disrupting inhibitory neurotransmission and promoting neuronal damage [27–29]. Inhibitory glycine receptors have subtypes: the α1 - α4 subunits can form functional homopentamers, whereas a β-subunit forms functional heteropentamers consisting of three α- and two β-subunits [30,31]. In this study, we focused on the electrophysiological and structural function of the α1 homopentamer. Pentameric glycine receptors exhibit a dynamic interplay between distinct functional states, known as resting, open, and desensitized [32–34]. Following glycine binding, the channel undergoes transitions from its resting state to the open and desensitized conformations [35]. The structure of the glycine receptor consists of an intracellular, a transmembrane, and an extracellular domain [17,36,37]. The intracellular domain interacts with endogenous and exogenous modulators to regulate receptor trafficking, synaptic clustering, and being a hub for posttranslational modifications [38,39]. The transmembrane domain mediates selective ion permeation. The extracellular domain contains binding pockets for specific ligands, making them attractive targets for drug development [40]. Binding sites of new agents are evaluated using techniques such as electrophysiological research, mutation analysis, electron microscopy, and computational modeling. Two-electrode voltage clamps (TEVC) and modeling are advantageous for understanding electrophysiological and structural functions [41]. Therefore, we employed these methods to identify substances capable of modulating electrical signals and electrophysiological functions. Through computational modeling, we derived candidate compounds, performed point mutations, investigated binding sites using TEVC, and analyzed the underlying mechanisms. Ergotamine is prescribed to treat acute migraines a member of a family of alkaloids extracted from the ergot fungus, used for therapeutic purposes since the 16th century. A portion of ergotamine's structure is similar to a neurotransmitter, so it interacts with nerve receptors at the synapse. It acts as an agonist at some serotonin receptors, contributing to vasoconstriction and migraine relief. Additionally, it partially activates dopamine D2 receptors, influencing mood and motor control, while also modulating α-adrenergic receptors, affecting vascular tone. Through these interactions, ergotamine regulates neurotransmitter release and neuronal excitability. Despite its known pharmacological actions, its precise mechanisms in neural circuits remain incompletely understood, highlighting the need for further research into its neurological effects. Ergotamine functions biologically as a vasoconstrictor. It binds to the 5-HT1 and 5-HT2 receptors [42] and induces signaling pathways that constrict blood vessels and suppress nerve inflammation in the dura mater [43]. Ergotamine is known to have relatively poor penetration across the blood-brain barrier [44], albeit to varying extents. However, there is no research on how ergotamine interacts with glycine receptors, which are related to neuroplasticity, cognition, pain, and motor coordination. Therefore, we investigated the interactions between ergotamine and other neuroreceptors. We identified an N-methyl-d-aspartate neuroreceptor in a previous study that interacts with ergotamine [45]. We studied the structural and electrophysiological functions of glycine receptors that bind ergotamine at the molecular and cellular levels. The left-central nervous system diagram in Fig. 1 illustrates the expression sites of glycine receptors in the hippocampus's pyramidal neurons and pyramidal tract [3,46,47]. While glycine receptors are conventionally known to be expressed in the spinal cord and brainstem, this image highlights their expression in the hippocampus and the pyramidal tract (encompassing the corticospinal tract and corticobulbar tract). These regions share a commonality in the presence of pyramidal neurons, and the diagram on the right elucidates the mechanism of glycine receptors expressed in pyramidal neurons, modulating signal transmission. Ultimately, the failure to form action potentials may lead to functional impairment. In situations characterized by persistent or excessive IPSP, ergotamine demonstrates the potential to improve functional deficits.

2. Materials and methods

2.1. Materials

Fig. 2A shows the chemical structure of ergotamine. Stock solutions (100 mM) of ergotamine were prepared with dimethyl sulfoxide (DMSO). Stock solutions (100 mM) were diluted with bath solution to appropriate concentration. In all experiments the final concentration of DMSO was less than 0.01 %. A human neuronal glycine receptor α1 cDNA was supplied by OriGene Technologies, Inc. (Rockville, MD 20850, USA). Ergotamine and all other compounds were purchased from Merck KGaA (Darmstadt, Germany).

2.2. Preparation of microinjection of Xenopus oocytes

According to the Chonnam National University Animal Care Guidelines (CNU IACUC-YB-2016–07), *Xenopus laevis* were cared for and handled following the manual from the Korean Xenopus Resource Center for Research (KXRCR000001). Surgery on *Xenopus laevis* were conducted to manually collect oocytes for experiments. Oocytes were incubated with shaking in Ringer solution (96 mM NaCl, 1 mM KCl, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM HEPES, pH 7.5) supplemented with 0.5 μg/μL collagenase (Type II, Merck, Germany) for 2 h and then maintained in ND96 solution (96 mM NaCl, 1 mM KCl, 0.33 mM Ca2 $(NO)_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 5 mM

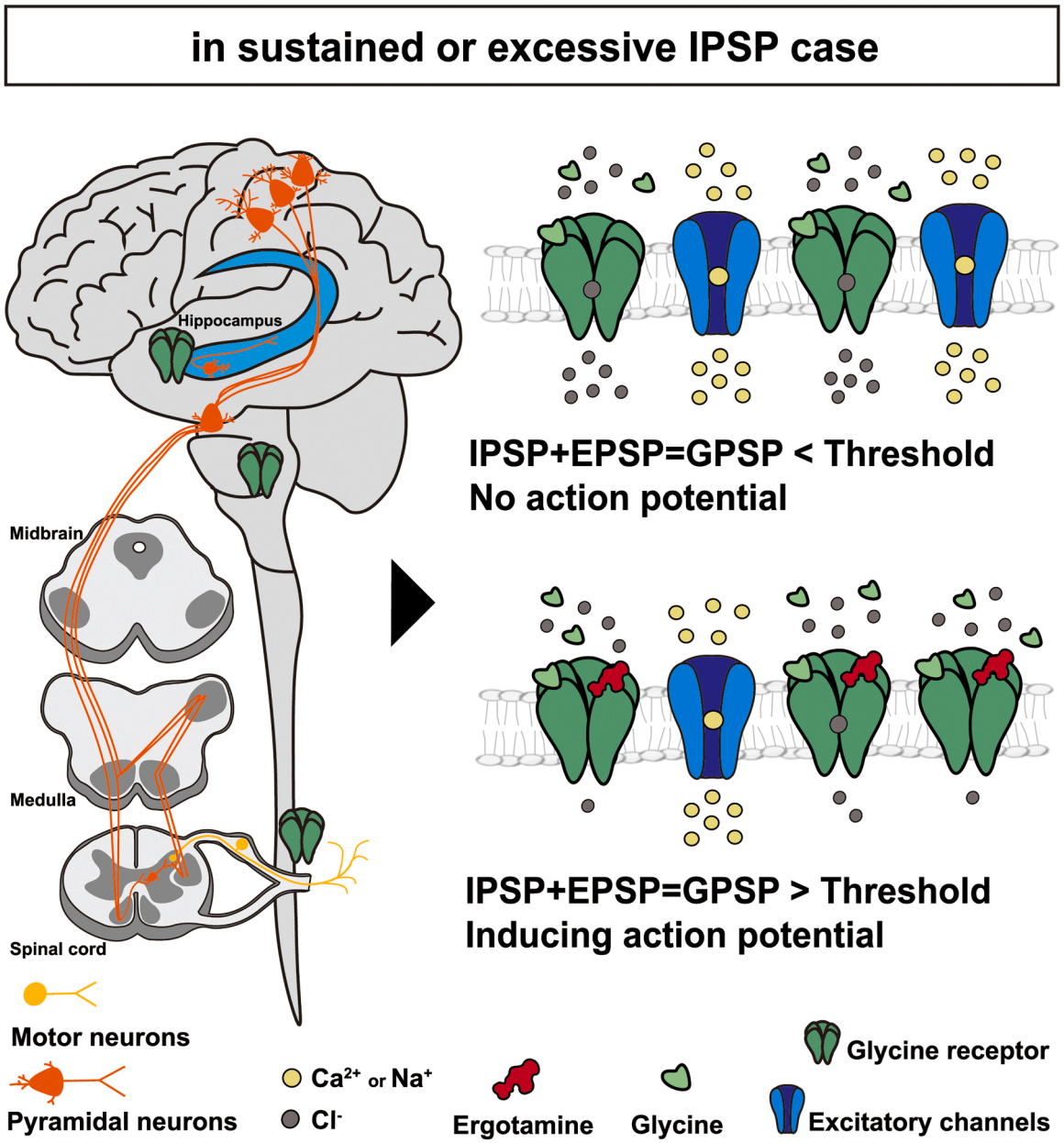

Fig. 1. Schematic Overview of Ergotamine Regulation through Glycine Receptors in Situations of Prolonged or Excessive IPSP. The left-central nervous system (CNS) diagram illustrates the expression sites of glycine receptors in the hippocampus's pyramidal neurons and pyramidal tract. While glycine receptors are conventionally known to be expressed in the spinal cord and brainstem, this image highlights their expression in the hippocampus and the pyramidal tract (encompassing the corticospinal tract and corticobulbar tract). These regions share a commonality in the presence of pyramidal neurons, and the diagram on the right elucidates the mechanism of glycine receptors expressed in pyramidal neurons, modulating signal transmission. Ultimately, the failure to form action potentials may lead to functional impairment. In situations characterized by persistent or excessive Inhibitory Postsynaptic Potentials (IPSP), ergotamine demonstrates the potential to improve functional deficits.

HEPES, pH 7.5) containing 0.1 mg/mL penicillin and streptomycin (Merck, Germany) at 16 °C. All incubation solutions were changed daily. Complementary RNAs were prepared as described in our previous study [48,49] and then pulled from glass capillary tubing (15–20 μm in diameter) by using a 10 nL micro-injector (VWR Scientific, CA, U.S.A). Electrophysiological experiments were performed within 5–6 days of oocyte isolation.

2.3. Molecular protein-ligand docking studies

Glycine receptor protein structures were obtained from a protein data bank (PDB ID: 3JAE). The Cryo-EM structure of glycine receptor and the 3D model of ergotamine-receptor interaction were conducted using Autodock Tools (version 4.2.6, La Jolla, CA, USA) from The Scripps Research Institute. The interaction structure was analyzed using multiple docking simulations and considered the intermolecular energy, internal energy, binding energy, kI, torsional energy, unbound extended energy, cluster RMS, Ref RMS, crystal structures, inhibition constant, and energy minimization. After considering various factors, mutation candidates were selected by analyzing amino acid residues at frequently repeated positions. The glycine receptor protein and ergotamine complex were also analyzed using Ligplot (version 4.5.3, EMBL-EBI, Hinxton, Cambridgeshire, UK) and Pymol (version 2.6.0, Schrödinger Inc., New York, NY, USA).

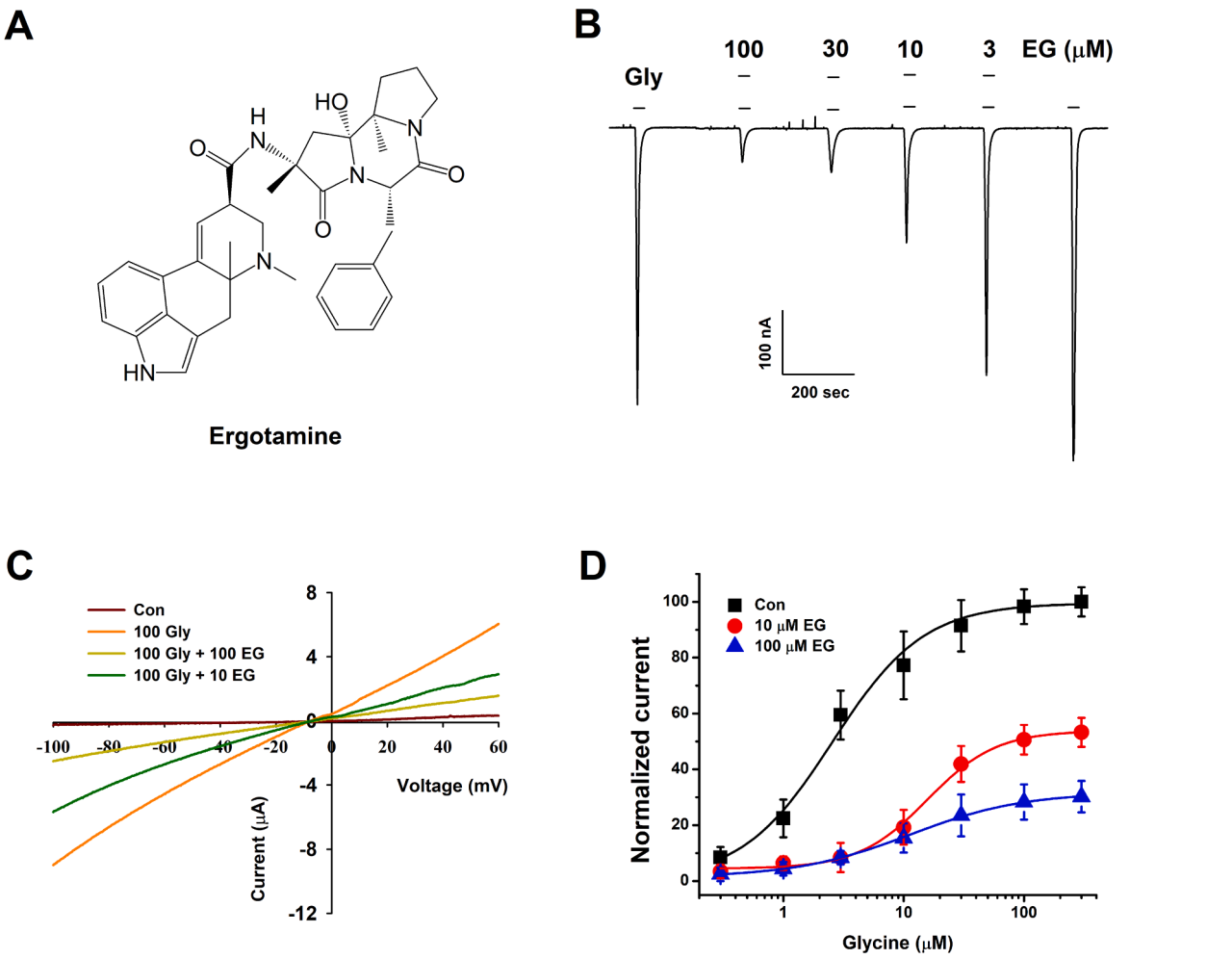

Fig. 2. Chemical structure and inhibitory effects of ergotamine on glycine receptor α1 in Xenopus oocytes. (A) This is the secondary structure of ergotamine. (B) Running two-electrode voltage-clamp recording in oocytes expressing the glycine receptor α1, glycine (100 μM) evoked a large inward current (I_{Gly}) at a holding potential of −80 mV for the indicated times. Ergotamine (100 μM) were supplemented into recording solution for 1 min (bar in figure). The I_{Gly} current treated by co-application of ergotamine (EG) and glycine (Gly) was inhibited. In the presence of ergotamine (10 μM and 30 μM) with the consistence concentration of glycine (100 μM), the inhibition effect was showed reversibly. Tracings are representative of six to eight separate oocytes from four different frogs. (C) Current–voltage relationships of I_{Gly} on ergotamine-stimulated inhibition in oocytes. The representative current–voltage relationship was obtained using 1 s-duration voltage ramps from −100 to + 60 mV at a holding potential of −80 mV. Voltage ramps were applied before and after the application of 100 μM glycine in the presence or absence of ergotamine (10 and 100 μM). Each point represents the mean ± S.E.M. (n = 6–8 oocytes from four different frogs). (D) Competition test between glycine and ergotamine for binding with glycine receptors α1. Exposing with the absence (■ Con) or presence of ergotamine (● 10 μM and ▲100 μM) for 1 min and then evoked with glycine from 0.3 to 100 μM, glycine-induced inward currents were voltage clamped at a holding potential of −80 mV for indicated times. Each point represents the mean ± S.E.M. (n = 6–8/group). The additional Imax, IC_{50}, and Hill coefficient values are summarized in Table 1.

2.4. Mutation and in vitro transcription of glycine receptor α1 channel cDNAs

Using *Pyrococcus furiosus* DNA polymerase (QuikChange XL site-directed mutagenesis kit (Stratagene, USA), we mutated a single amino acid. Following the manufacturer's instructions, an extension of the target domain was amplified via polymerase chain reaction (PCR) using the appropriate primers and then transformed into *Escherichia coli* strain DH5α. Final mutation products were selected by PCR and validated by DNA sequence analysis. Selected mutants were linearized and transcribed. Final cRNAs products were resuspended reach at 1 μg/μL in RNAse-free water and stored at −80 °C until use.

2.5. Data recording

An oocyte clamp (Warner Instruments) was used to record data from two-electrode voltage-clamp experiments in a perfusion chamber at room temperature. ND96 solution was prepared and administered with ergotamine and glycine during recording experiments. Oocytes were placed into the chamber with ND96 solution flowing at a rate of 2 mL/min. Recordings were initiated before the onset of desensitization to ensure accurate measurement of peak glycine-evoked currents. The perfusion system was designed to deliver glycine or glycine + EG at a constant flow rate of 2 mL/min, with the solution maintained in the chamber for 1 min before washout. To minimize the effects of desensitization, we systematically adjusted the washout interval through repeated trials, allowing sufficient recovery time between applications. This approach ensured that the observed currents were not significantly affected by progressive receptor desensitization. Furthermore, our experimental results demonstrated that EG did not alter desensitization kinetics or induce desensitization during prolonged glycine application, indicating that its modulatory effects on glycine receptors were independent of desensitization mechanisms. Two electrodes filled with 3 M KCl (electrolyte solution, 0.2–0.7 MΩ resistance) were used to probe each oocyte. Experiments used a holding potential of −80 mV to record current and −100 to + 60 mV within 300-ms for ramping experiments

and measuring the voltage relationship of glycine receptor α1. All data were collected and analyzed with Digidata 1320 (Molecular Devices, Sunnyvale, CA, USA) and pCamp 9 software (Molecular Devices).

2.6. Data analysis

Concentration-response curves to investigate the effect of ergotamine on glycine receptor-mediated currents (I_{Gly}) were constructed by plotting the maximum response value against ergotamine concentration. The plot was obtained by fitting the data to the Hill equation: $y/y_{max} = [A]_n/([A]_n + [IC_{50}]_n$ (Origin Pro 8.0 software, Origin, Northampton, USA), where y is the peak current at a given concentration of ergotamine; y_{max} is the maximal peak value; IC_{50} is the half maximal inhibitory concentration of ergotamine on I_{Gly}; [A] is the concentration of ergotamine, and n is the interaction coefficient. All values were presented as the mean ± standard error of the mean (SEM). Differences between groups were measured by one-way ANOVA and the Turkey-Kramer post-test. A value of $P < 0.01$ was considered statistically significant.

3. Results

3.1. Effect of ergotamine on glycine receptor α1-mediated currents in Xenopus oocytes

Glycine (100 μM) was added to the bath solution to stimulate a large inward current into oocytes expressing glycine receptor α1 cRNAs. Glycine exhibited a strong effect on I_{Gly}, indicating that the receptor was expressed and functional in Fig. 2B. Addition of various concentration of ergotamine alone had no effect on I_{Gly} at −80 mV holding potential in glycine receptor α1 cRNA-injected oocytes. Similar results were observed in H_2O-injected control oocytes (data not shown). Interestingly, addition of ergotamine and glycine significantly reduced I_{Gly} current compared with glycine alone (Fig. 2B, n = 6–8 oocytes from each of four frogs). The inhibition on I_{Gly} in response to ergotamine and glycine addition was reversible. The peak I_{Gly} current to different concentrations of ergotamine (3, 10, 30, and 100 μM) with 100 μM glycine was 25 ± 8.6 %, 56 ± 4.5 %, and 87.6 ± 5.6 %. A transient increase in glycine current amplitude was observed after EG washout, which exhibited a slight run-up tendency in Figs. 2B, 5A and B. This suggests that the effect may be attributed to recording stability rather than a biphasic modulation of glycine receptors by EG. While run-up phenomena can occur due to gradual changes in membrane properties or receptor responsiveness over time, the overall effect was minimal and did not significantly affect the interpretation of our results.

3.2. Pharmacological mechanism of ergotamine suppression of I_{Gly} in glycine receptor α1 channels

To examine whether ergotamine works by blocking ion channel opening, we investigated the inhibition effect of co-application of ergotamine and glycine with voltage ramps ranging from −100 mV to + 60 mV every second. Ergotamine (10 or 100 μM) with or without 100 μM glycine was added to the bath solution. After submaximal activation of glycine receptor α1 with 100 μM glycine, the rectification of inward and reduction of conductance were not observed, suggesting that the current-voltage relationship was linear in glycine receptor α1 channels. Adding ergotamine (10 or 100 μM) and 100 μM glycine caused the current-voltage relationship to respond in a voltage insensitive manner: 39.9 ± 4.8 % or 71.9 ± 4.1 % at −100 mV and 42.3 ± 6.2 % or 74.6 ± 5.4 % at + 60 mV, respectively (n = 6–8 oocytes from each of four frogs). The reversal potential was approximately 0 mV for application of ergotamine and glycine or glycine alone at negative voltage (Fig. 2C). These data indicate that the electrophysiological mechanism by which ergotamine affects I_{Gly} current is that glycine evokes current by Cl^- flux through glycine receptor α1 unrelated to ergotamine. To elucidating the mechanism of whether ergotamine suppression in glycine receptor α1-expressing oocytes occurs within the glycine binding site, the response of I_{Gly} currents was investigated using 0–100 μM glycine with or without ergotamine (10 or 100 μM). I_{Gly} current suppression levels in response to ergotamine were evaluated in the same cell. As shown in Fig. 2D, ergotamine (10 or 100 μM) did not notably increase the slope of the glycine-evoked current, indicating that a non-competitive relation between glycine and ergotamine exists. The IC_{50} values in response to 100 μM glycine or 100 μM glycine plus ergotamine (10 or 100 μM) were 59.8 ± 2.9 μM and 34.1 ± 4.0 μM respectively (n = 6–8 oocytes from each of four frogs). The I_{max} values, Hill coefficients, and IC_{50} values are shown in Table 1.

3.3. Virtual docking of ergotamine to wild-type and mutated glycine receptor α1

To visualize how ergotamine interacts with the glycine receptor α1 channel, homology modeling of wild-type and mutant glycine receptor α1 channels were constructed (Fig. 3). Docking scores and average ligand RMSD values are shown in Table 2 for all predicted docking sites of glycine receptor α1 and ergotamine. Predicted complex #1 exhibits the best-fit docking site with the lowest binding energy (−8.54 kcal/mol), lowest intermolecular energy (−7.24 kcal/mol), and a reasonable RMSD value (0.21 nm < 0.25 nm). In contrast, although predicted complex #2 has a similar binding energy (−7.51 kcal/mol), the intermolecular energy value of putative complex group #2 was much higher than putative complex #1 (−5.24 kcal/mol versus −7.24 kcal/mol). The alignment of the predicted binding mode of ergotamine in the putative ligand binding pockets of six mutated versions of predicted complex #1 (S104, D107, K111, F124, T129, D130) is shown in Fig. 4B. The binding interfaces between ergotamine and the six putative mutant sites in glycine receptor α1 were compared with the interface between ergotamine and the putative ligand binding pocket of the wild-type receptor in Fig. 4C and D. The virtual docking models suggest that the D130 and F124 residues are two potential binding sites that allow ergotamine to enter the glycine receptor α1 channel pore and interacting with the various receptor residues via hydrogen bond formation. Our analysis highlighted residues F124 and D130 as acceptable target docking sites for investigating the mechanism of ergotamine effects in additional experiments.

3.4. Effect of ergotamine on glycine-evoked currents in various mutations

We used electrophysiology to investigate the binding of positively charged inhibitors to versions of the glycine receptor α1 ion channel that are mutated at F124 and D130 to predict the location of the most likely ergotamine binding site. The cDNAs were mutated at locations presumed to be binding sites, amplified by PCR, and then injected into oocytes. At 2–3 days post-injection, electrophysiology studies of the receptors were conducted at −80 mV holding potential, and the concentration of glycine was varied from 0 to 300 μM in the presence of ergotamine. Representative traces of I_{Gly} stimulated by 100 μM glycine with and without ergotamine (30 or 100 μM) from three mutants (single mutation at F124A or D130A or a double mutation at F124A and D130A) of the glycine receptor α1 are shown in Fig. 5A–C, respectively. Plots

Table 1
Competition test between glycine and ergotamine for binding to glycine receptors α1.

	I_{max}	EC_{50}	n_H
Control	99.6 ± 2.4	2.6 ± 0.4	1.1 ± 0.3
10 μM EG	53.9 ± 2.3	15.7 ± 1.2	1.2 ± 0.2
100 μM EG	31.7 ± 0.5	11.2 ± 0.6	0.9 ± 0.2

Values represent means ± S.E.M. (n = 6–8/group). Half-maximal effective concentration (EC_{50}); Hill's coefficient (n_H); Maximum response (I_{max}) value as determined as described in Materials and methods.

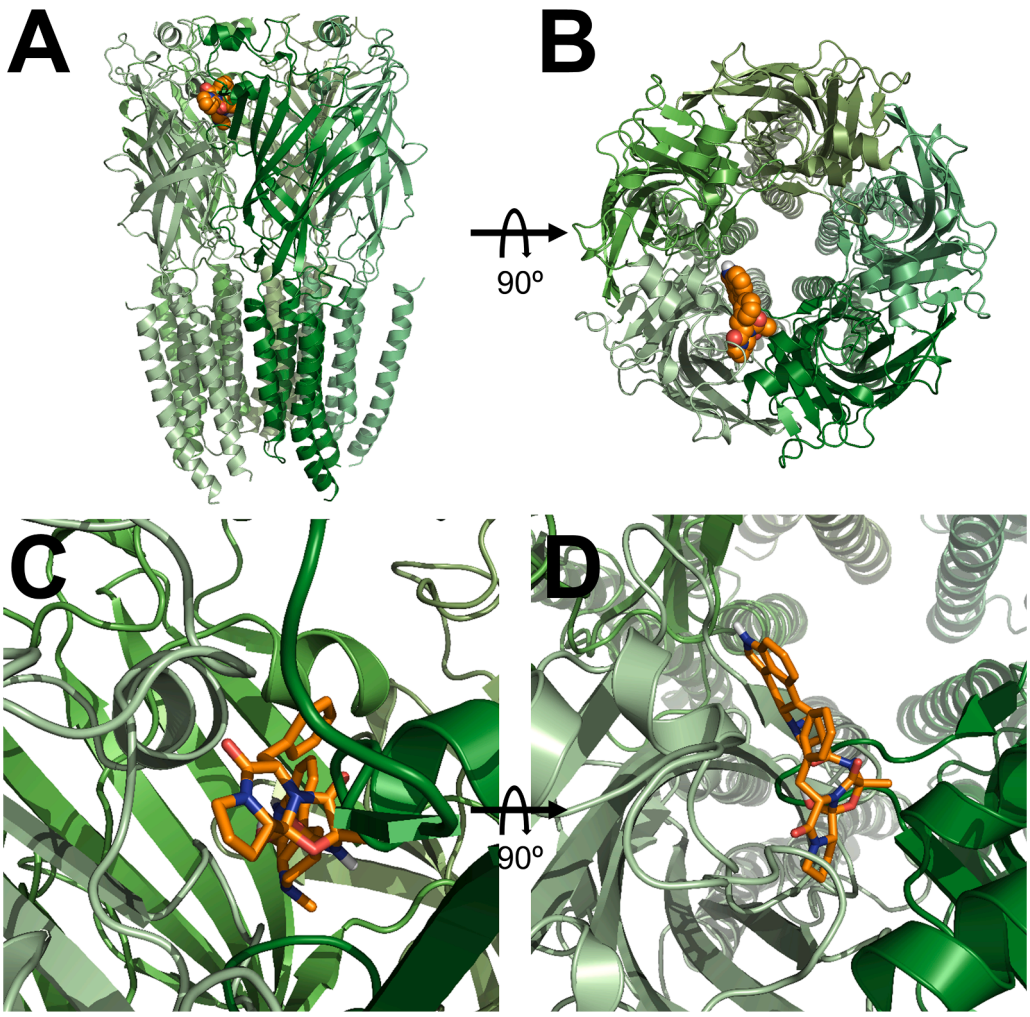

Fig. 3. Computational molecular modeling of ergotamine docked to glycine α1 channel. A and C Side views of the docked ergotamine in complex with glycine α1 channel. B and D Top views of docking model. The PDB 3JAE model, which is a glycine-bound state, was used and it was found that ergotamine binds to the upper (N-terminal) of the ECD.

Table 2
The predicted docking sites and their binding energy of glycine receptor α1 and ergotamine.

	Binding energy	KI (mM)	Intermolecular energy	Internal energy	RMSD (nm)	Binding residues
#1	−8.54	0.24	−7.24	−1.21	0.21	S104, D107, K111, F124, T129, D130
#2	−7.51	0.84	−5.24	−1.35	0.19	P61, K62, Y65, Y85, D108, K109
#3	−7.22	0.95	−4.56	−1.35	0.19	R57, L60, P61, L121, F122, I125, F126

*RMSD (root-mean-square deviation of atomic positions), Equilibrium dissociation concentration (KI), Binding energy, Intermolecular energy, Internal energy (kcal/mol).

showing the effects of ergotamine on glycine-evoked currents on oocytes expressing wild-type and mutant glycine receptor α1 channels are shown in Fig. 5D. The intensity of ergotamine's effect (I_{Gly}) on each receptor in rank order (high to low) was as follows: wild type (87.1 %), mutant α1 F124A (49.0 %), mutant α1 D130A (35.7 %), double mutant α1 F124A and D130A (12.8 %) (Table 3 and Table S-1). Comparisons between the responses of the wild-type and each of the three mutants, regarding ergotamine's-inhibitory effect on values of I_{max}, IC_{50}, and the Hill coefficient are presented in Table 3. Notably, the IC_{50} value of the double mutant α1 F124A and D130A was 59.1 ± 15.3 μM, and the Hill coefficient value was 1.9 ± 0.6. These data suggest that the F124 and D130 residues provide the best fit for ergotamine docking and the glycine receptor α1 channel to be in a near-native configuration. Additionally, to analyze changes in the EC_{50} value for glycine in the double mutant, we performed a concentration-response analysis. In the wild-type, the EC_{50} value was 2.6 ± 0.4 μM, while in the double mutant, it was 2.9 ± 1.6 μM, showing no significant difference. These results indicate that ergotamine's inhibitory effect is independent of glycine binding.

4. Discussion

ROS can directly modify the structure of glycine receptor through oxidative modifications of amino acid residues. This can lead to changes in receptor conformation, affecting their gating properties, ion conductance, and overall functionality. For instance, the oxidation of cysteine residues in glycine receptor can result in the formation of disulfide bonds, altering the receptor's structure and potentially impairing

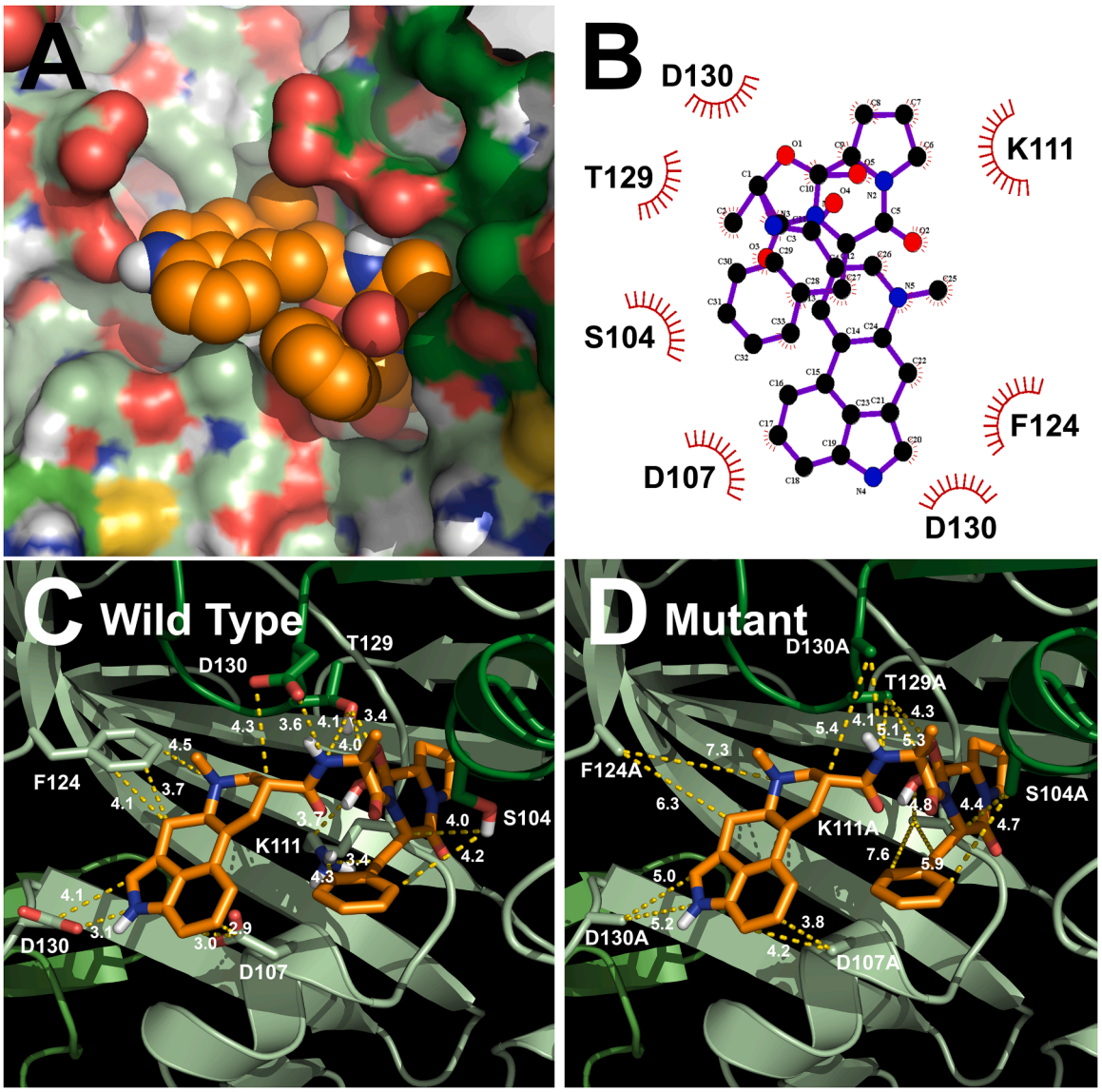

Fig. 4. Virtual docking of ergotamine to wild-type glycine α1 receptor and mutations. (A and B) Binding pocket and docking results of ergotamine and glycine receptor α1 channel. (A) Ergotamine located in binding pocket in extracellular area. (B) 2D schematic presentation of the predicted binding mode of ergotamine in the ligand binding pocket. Ligands and important residues are shown. (C and D) Pose view analysis and binding interface of ergotamine of wild-type (C) and mutant channels (D), whose mutations can disturb the interaction with ergotamine at varying degrees. The dotted lines show the distance between atoms as Å unit.

its function [24]. Oxidative stress can cause lipid peroxidation in the cell membrane, leading to changes in membrane fluidity and integrity [50]. Since glycine receptor are membrane-bound proteins, alterations in the lipid environment can affect their stability and function. Disrupted membrane integrity can impair the proper localization and clustering of glycine receptor, which is crucial for efficient synaptic transmission. Oxidative stress can activate various intracellular signaling pathways, such as the mitogen-activated protein kinase pathway, which can lead to the phosphorylation of glycine receptor or associated proteins [24]. Phosphorylation can modulate glycine receptor function, either enhancing or reducing their activity, and can affect receptor trafficking, synaptic localization, and degradation. High levels of oxidative stress can impair the release of glycine from presynaptic neurons, reducing the availability of the neurotransmitter to bind to glycine receptor. This can diminish inhibitory signaling and contribute to neuronal excitotoxicity. Oxidative stress is a common feature of neurodegenerative diseases like Alzheimer's, Parkinson's, and Amyotrophic Lateral Sclerosis (ALS). Dysfunctional glycine receptor due to oxidative stress can contribute to the pathophysiology of these diseases by disrupting inhibitory neurotransmission and promoting neuronal damage. Changes in glycine receptor function due to oxidative stress have been implicated in neuropathic pain conditions. Reduced inhibitory glycinergic transmission can lead to increased neuronal excitability and pain perception. Oxidative stress-induced modifications of glycine receptor may contribute to the development of epilepsy. Impaired glycinergic inhibition can result in uncontrolled neuronal firing and seizure activity. Currently, most studies on the glycine receptor focus on factors that increase its activity, such as tetrahydrocannabinol (THC) or cannabinoids to enhance IPSPs and reduce grand postsynaptic potentials (GPSPs), which transmit pain signals to the brain [12,51–54]. The results showing significant effects in patients [15] with mutated glycine receptors confirm that this remains an appealing approach for developing novel analgesics. And also oxidative stress can activate various intracellular signaling pathways that can lead to the phosphorylation of glycine receptor. However, studies on neurological dysfunctions caused by sustained and excessive IPSPs is lacking. Notably, receptors responsible for cognitive memory, such as NMDAR, AMPA, and α7 nAChR, are well studied [55,56], however, investigations into how glycine receptors

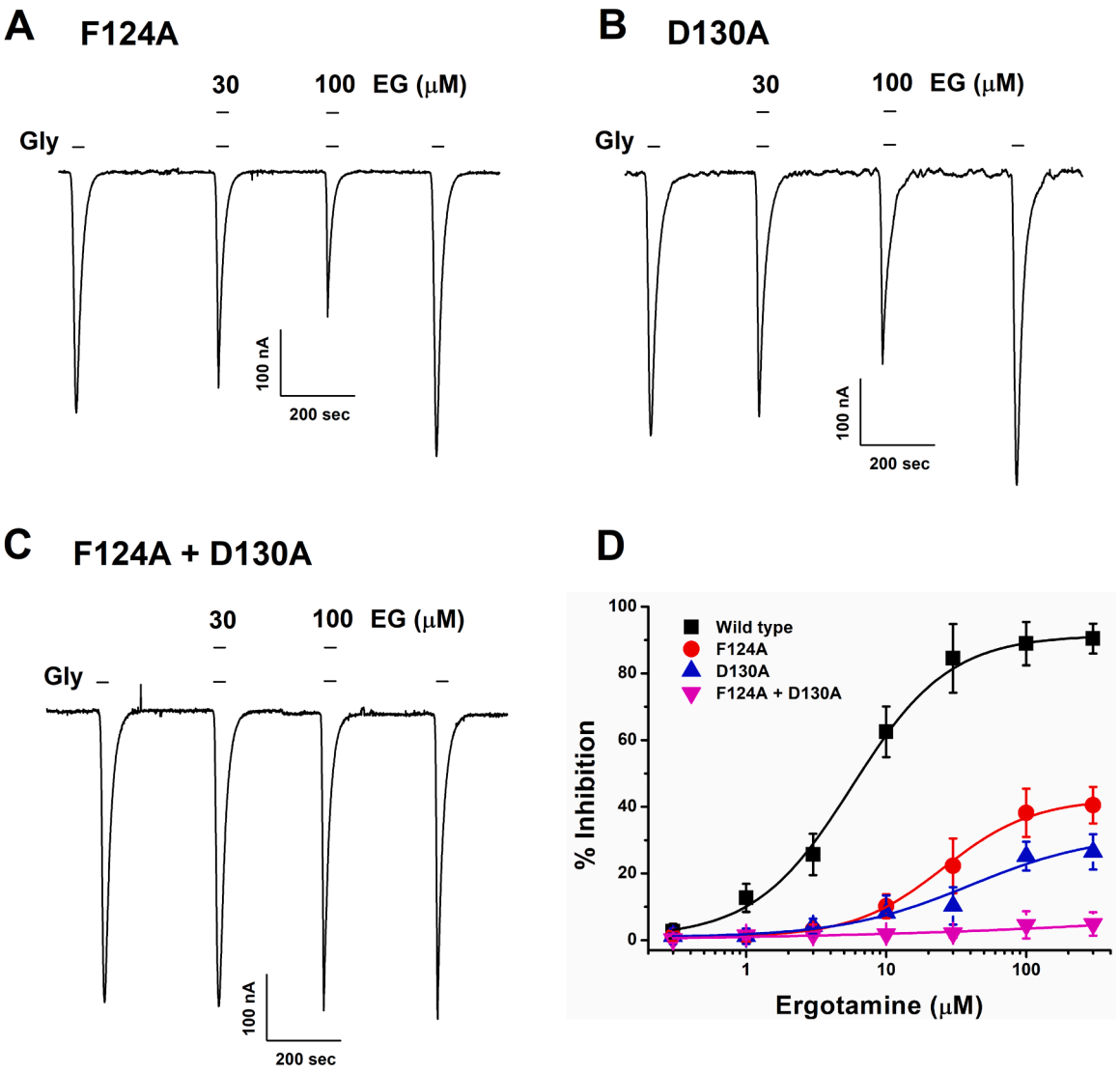

Fig. 5. Effects of ergotamine on I_{Gly} in wild-type and various mutant glycine receptor α1 channels. (A-C) Representative traces on I_{Gly} stimulated by glycine 100 μM with and without ergotamine (30 and 100 μM) in α1 F124A (A), α1 D130A (B) and α1 F124A and D130A (C). Traces are representative of six to eight separate oocytes from four different frogs. (D) Concentration-dependent curves of ergotamine on glycine–evoked inward current from oocytes expressing wild-type and various mutant glycine receptor α1 channels. Ergotamine reduced I_{Gly} in a concentration-dependent manner in wild-type receptors. The effect of ergotamine on I_{Gly} was presented following the rank order of potency: α1 wild >> α1 F124A > α1 D130A > α1 F124A and D130A.

Table 3
Effect of ergotamine on glycine-evoked current of wild-type glycine receptor α1 and its various mutants.

	I_{max}	IC_{50}	n_H
α1 wild	91.5 ± 2.2	5.7 ± 0.7	1.2 ± 0.2
α1 F124A	42.4 ± 1.5	25.7 ± 2.9	1.3 ± 0.1
α1 D130A	32.1 ± 8.8	28.1 ± 9.7	1.1 ± 0.3
α1 F124A + D130A	NA	NA	NA

Values represent means ± S.E.M. (n = 6–8/group). NA (Not Available). Half-maximal inhibitory concentration (IC_{50}); Hill's coefficient (n_H); Maximum response (I_{max}) value as determined as described in Materials and methods.

regulate subsequent EPSPs remain limited. Although this theoretical approach focuses on electrophysiological mechanisms, it suggests that inhibiting IPSPs while maintaining the same quantity of EPSPs increases the overall balance of GPSPs, allowing post-synaptic neurons to generate action potentials that trigger unique functions. Moreover, our understanding of how the pyramidal tract controls voluntary muscle function remains unclear. Pain is a complex condition, involving multiple mechanisms; therefore, it's challenging to dismiss antagonists as potential candidates for analgesic development. Recent studies report that strychnine, despite being a toxin, prevents inflammation via the ADAM17-TNFR1-NF-κβ pathway [24]. Although our study does not provide evidence regarding ergotamine's inhibition of inflammation through the ADAM17-TNFR1-NF-κβ pathway, it doesn't rule out its potential for inflammatory pain [57]. Above all, antagonism by ergotamine when IPSPs are excessive presents a new approach toward cognitive improvement and motor dysfunction [50]. Here, we presented evidence of ergotamine as a novel antagonist of the glycine receptor and elucidated its mode of action.

We expressed α1 glycine receptors in *Xenopus* oocytes to investigate the how ergotamine interacts with the receptor. It is well known that glycine receptor expressed in motor neurons of the mammalian brainstem and spinal cord are each regulated by corticobulbar and corticospinal tracts [50]. Studies in rats have confirmed that glycine receptors are expressed in pyramidal neurons and interneurons of hippocampus CA1 crucial for memory consolidation and receive inputs from CA3 neurons via Schaffer collaterals [6]. This finding was validated using the specific antagonist, strychnine and confirmed through staining. An

important point to note here is that the foundational cells in these key areas are all pyramidal neurons. Although our experimental results did not demonstrate the glycine receptor-ergotamine interaction in mammals, using *Xenopus* oocytes was suitable for confirming the molecular basis of the interaction. While Xenopus oocytes provide a widely accepted and well-controlled system for studying ion channel function and pharmacology, there are inherent limitations in extrapolating findings from this system to mammalian neuronal physiology. One key consideration is that the glycine EC_{50} values observed in Xenopus oocytes may differ from those in mammalian neurons due to differences in receptor expression levels, post-translational modifications, membrane lipid composition, and intracellular signaling environments. These factors can influence receptor sensitivity, gating kinetics, and drug interactions, potentially affecting the physiological relevance of our findings. Therefore, while our study offers valuable molecular insights into the modulatory effects of ergotamine on glycine receptors, further validation in mammalian neuronal systems, such as primary neuronal cultures or in vivo models, is essential to fully elucidate the physiological and pharmacological implications of our results.

We performed our molecular analysis by first identifying the optimal concentration of glycine (100 μM) for investigating how ergotamine operates in conditions of excessive IPSPs. Ergotamine (10 μM) inhibited 59.8 ± 2.9 % of the current stimulated by 100 μM glycine. Additionally, we examined the current-voltage relationship in ramp experiments and observed that the reversal point remained unchanged, with conductance varying in proportion to voltage. This indicates that currents are generated by single channels.

Glycine receptors have different binding pockets for agonists and antagonists. Glycine's binding sites are reported to be L151, F183, T228, and F231 [34]. In our experimental results, ergotamine's potential binding sites appear to be F124 and D130 in loop E. This is consistent with the results we observed with TEVC. The specific antagonist strychnine is reported to bind to G160, Y161, K200, and Y202 [18]. Cannabinoids (potentiators) are reported to bind to S324, and THC is reported to bind to S320 [52]. In summary, our findings with ergotamine revealed a new pocket location that differs from other known pockets. It is located at the upper part of the extracellular domain. Ergotamine binding in this new pocket also triggers a conformational change, reducing conductance by directionally narrowing the pore in the transmembrane domain.

Ergotamine is currently used as a medication for cluster headaches and migraines and is primarily known as an agonist of serotonin receptors. Except for the 5-HT3a subtype, the members of the serotonin receptor family are G protein-coupled receptors (GPCRs). Although ergotamine acts as an agonist on most subtypes, it's reported to have an antagonistic effect via the 5-HT1 and 5-HT2 subtypes [42]. However, our findings reveal that ergotamine does not interact with the ligand-gated channel, 5-HT3a, which is similar to the glycine receptor. When used at high doses, side effects include vasoconstriction, increased heart rate, and elevated blood pressure. This effect of ergotamine could be due to excessive antagonism affecting the role of IPSPs mediated by the glycine receptor, which regulates the EPSPs that signal muscle contractions. These findings indicate that ergotamine's effects on glycine receptors occur not only in the brainstem and spinal cord but also in the hippocampus.

In the current study, we describe the structural and electrophysiological functions of glycine receptors that bind ergotamine at the molecular and cellular levels. Previously reported data strongly implied that ergotamine binds within the membrane as reflected by the voltage-dependence of co-application experiments. Ergotamine might act on the ion channel pore and co-application induces a notable increase of the open state of the ion channel. Second, anionic ergotamine molecules could form favorable electrostatic interactions within the anion-selective pore. Our study is the first to report an interaction between ergotamine and glycine receptors. Our study reveals that ergotamine functions as a novel antioxidant of glycine receptors, highlighting its previously unrecognized role in modulating receptor activity. We investigated the structural and electrophysiological properties of glycine receptors that interact with ergotamine at the molecular and cellular levels. Previous findings suggested that ergotamine binds within the membrane, as indicated by the voltage-dependent effects observed in co-application experiments. This binding likely influences the ion channel pore, leading to a significant increase in the open-state probability of the receptor. Additionally, the anionic nature of ergotamine enables favorable electrostatic interactions within the anion-selective pore, further stabilizing its effect on glycine receptors. Our study is the first to demonstrate a direct interaction between ergotamine and glycine receptors, providing new insights into its antioxidant properties. These findings suggest that ergotamine's modulation of glycine receptor activity could be relevant for conditions related to cognitive dysfunction, inflammatory pain, and pyramidal tract disorders. The molecular mechanisms elucidated in this study contribute to a deeper understanding of ergotamine's potential therapeutic applications beyond its known pharmacological effects.

Ethics approval and consent to participate

The animal experiments were approved by the Animal Ethical and Welfare Committee (Approval No. CNU IACUC-YB-2016–07).

CRediT authorship contribution statement

Myungmi Moon: Project administration, Methodology, Formal analysis. **Jeongyeon Yun:** Project administration, Methodology, Investigation. **Minsu Pyeon:** Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gihyun Lee:** Software, Resources, Project administration, Data curation, Conceptualization. **Junho H. Lee:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Sanung Eom:** Funding acquisition, Conceptualization. **Jaehui Yang:** Data curation, Conceptualization. **Jihwon Yun:** Writing – review & editing, Data curation, Conceptualization. **Hye Duck Yeom:** Supervision, Software, Resources, Project administration, Conceptualization. **Mee-Hyun Lee:** Project administration, Methodology, Funding acquisition, Conceptualization.

Data availability

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: GIHYUN LEE reports financial support was provided by National Research Foundation of Korea. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2025.03.028.

Appendix B. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.csbj.2025.03.028.

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