Protein kinase A is responsible for the presynaptic inhibition of glycinergic and glutamatergic transmissions by Xenon in rat spinal cord and hippocampal CA3 neurons

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2. Running Title Page

a) Running Title  (55/60 characters including space)
PKA is responsible for the presynaptic inhibition by Xe

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c) The number of Text pages: 33,
The number of Tables: 0
Figures: 9
References: 49
The number of words in the
Abstract: 195,
Significant statement: 75
Introduction: 574
Discussion: 1496

d) Abbreviations:
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione disodium; D-APV, D-2-Amino-5-phosphonovaleric acid; EGTA, ethylene glycol tetraacetic acid; GABA, γ-aminobutylic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; KA, kainic acid; NMDA, N-methyl-D-aspartate; PPR, paired-pulse rate; PKA, protein kinase A; PKC, protein kinase C; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; Rf, failure rate; s(e)IPSCs, spontaneous (or action potential-evoked) inhibitory postsynaptic currents; s(e)EPSCs,
spontaneous (or action potential-evoked) excitatory postsynaptic currents; mIPSCs, spontaneous miniature inhibitory postsynaptic currents; SEM, standard error of the mean; \( \tau_{\text{Decay}} \), \( 1/e \) decay time constant; TTX, tetrodotoxin; \( V_H \), holding potential.

e) A recommended section assignment to guide the listing in the table of contents.

Section options are: Neuropharmacology
3. ABSTRACT (195/250 words)

The effects of a general anesthetic xenon (Xe) on spontaneous, miniature, and electrically-evoked synaptic transmissions were examined using the “synapse bouton preparation”, with which we can clearly evaluate pure synaptic responses and accurately quantify pre- and post-synaptic transmissions. Glycinergic and glutamatergic transmissions were investigated in rat spinal sacral dorsal commissural nucleus (SDCN) and hippocampal CA3 neurons, respectively. Xe presynaptically inhibited spontaneous glycinergic transmission, the effect of which was resistant to tetrodotoxin, Cd²⁺, extracellular Ca²⁺, thapsigargin (a selective sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase inhibitor), SQ22536 (an adenylate cyclase inhibitor), 8-Br-cAMP (membrane permeable cAMP analogue), ZD7288 (a HCN channel blocker), chelerythrine (a PKC inhibitor) and KN-93 (a CaMKII inhibitor), while being sensitive to PKA inhibitors (H-89, KT5720 and Rp-cAMPS). Moreover, Xe inhibited evoked glycinergic transmission, which was cancelled by KT5720. Like glycinergic transmission, spontaneous and evoked glutamatergic transmissions were also inhibited by Xe in a KT5720-sensitive manner. Our results suggest that Xe decreases glycinergic and glutamatergic spontaneous and evoked transmissions at the presynaptic level in a PKA-dependent manner. These presynaptic responses are independent to Ca²⁺ dynamics. We conclude that PKA can be the main molecular target of Xe in the inhibitory effects on both inhibitory and excitatory neurotransmitter release.
4. Significance Statement (75/80 words)

Spontaneous and evoked glycinergic and glutamatergic transmissions were investigated using the whole-cell patch clamp technique in rat spinal sacral dorsal commissural nucleus (SDCN) and hippocampal CA3 neurons, respectively. Xenon (Xe) significantly inhibited glycinergic and glutamatergic transmission presynaptically. As a signalling mechanism, protein kinase A (PKA) was responsible for the inhibitory effects of Xe on both glycine and glutamate release. These results may help understand how Xe modulates neurotransmitter release and exerts its excellent anesthetic properties.

5. Visual Abstract: None
6. INTRODUCTION (574/750 words)

Xe belongs to noble gases and is inert, showing little reactivity. Nevertheless, the anesthetic effects of Xe have long been the focus of attention, with advantages such as rapid induction and awakening, analgesic properties, stable circulatory dynamics during surgery, and brain protection (Goto et al., 1997; Goto et al., 1998; Homi et al., 2003; Holsträter et al., 2011). Despite its excellent anesthetic properties, the precise mechanism how Xe inhibits synaptic transmission still remains unclear.

Neural networks in the spinal cord, rather than in the brain, mainly regulate the potency of general anesthetics (Antognini and Schwartz, 1993). Glycine is a primary fast inhibitory neurotransmitter in the spinal cord and brainstem, and general anesthetics seriously modulate glycine-induced synaptic responses. According to Eckle et al., (2013), the functional roles of glycine receptors mediating the immobilising properties of volatile anesthetics are much more essential than those of the GABA_A receptor, another predominant inhibitory neurotransmitter receptors in the central nervous system (CNS).

We recently reported that nitrous oxide (N_2O), another inhalation anesthetic, predominantly inhibits spontaneous and action potential-evoked glycine release from presynaptic glycineric nerve endings in rat SDCN neurons (Nakamura et al., 2020). The N_2O also inhibits directly action potential-dependent transmission from single presynaptic glutamatergic boutons of rat hippocampal CA3 neurons (Wakita et al., 2015b). Next, we have reported that Xe inhibits spontaneous and evoked inhibitory and excitatory postsynaptic currents (sIPSCs/sEPSCs and eIPSCs/eEPSCs) in rat spinal SDCN (Kubota et al., 2020) and hippocampal CA3 neurons (Nonaka et al., 2019). In addition, Xe has been reported to interact with glutamate receptor subtypes such as NMDA, AMPA and KA receptors, especially inhibiting NMDA receptor subtype (Maclver, 2014; Hao et al., 2020). Recently, Kotani et al. (2023) also reported that Xe and N_2O inhibited largely spontaneous and evoked NMDA currents in rat hippocampal CA3 neurons. These previous studies using “synapse bouton preparation” (Akaike and Moorhouse, 2003) of isolated brain and spinal neurons especially indicate that Xe inhibits spontaneous, miniature, and evoked neurotransmissions by acting predominantly on presynaptic nerve terminals. At concentrations that inhibited release from glutamatergic, GABAergic, and glycineric nerve endings in rat SDCN and hippocampal CA3 neurons, Xe as well as N_2O had no effects on voltage-dependent Na^+ and Ca^{2+} currents where they could be measured (Nonaka et al., 2019; Kubota et al., 2020; Nakamura et al., 2020). On the other hand, Xe activates two-pore-domain potassium (K^+) leak channels such
as Twik Related Potassium Channel 1 Protein (TREK-1) (Gruss et al., 2004; Solt and Forman, 2007). While ionotropic synaptic transmission is the most important target of general anesthesia (Campagna et al., 2003; Zhou et al., 2012; Hao et al., 2020), effects on other receptors including metabolic glutamate receptors have not been reported. The specificity of the volatile and gaseous anesthetics including Xe only on ionotropic synaptic transmission may explain the excellent anesthetic properties. However, it is still unclear how Xe modulates neurotransmitter release in terms of the cellular mechanisms and signalling cascades.

Therefore, in this study using the “synapse bouton preparation” allowing us to investigate both pre- and post-synaptic functions without interference of surrounding cells, we investigated how and whether or not Xe differently modulates the second messenger pathways. In addition to the possible involvement of PKA as reported previously (Katsurabayashi et al., 2004), we also checked the involvement of PKC and CaMKII signalling. To make sure that it is not region-specific, presynaptic glycinergic and glutamatergic nerve endings of SDCN and CA3 neurons were investigated, respectively, based on our previous reports.
7. Materials and Methods

Ethical approval

All experiments were performed in accordance with the Guiding Principles for the Care and Use of Animals in the Field of Physiological Science of the Physiological Society of Japan and were approved by the Ethics Committee of Kumamoto Kinoh Hospital and Kumamoto Health Science University. All efforts were made to minimize animal suffering and reduce the number of animals used in this study.

Preparation

Wistar rats of either sex (11-17 days old, SLC, Shizuoka, Japan) were decapitated under deep anaesthesia with pentobarbital (50 mg/kg, i.p.). The spinal cord was dissected, and the lumbosacral region was transversely sliced at a thickness of 350 µm using a microtome (7000 smz; Campden Instruments Ltd., Leicester, England). Slices containing the SDCN were kept in an incubation medium (in mM; 124 NaCl, 5 KCl, 1.5 KH2PO4, 24 NaHCO3, 2 CaCl2, 1.3 MgSO4, and 10 glucose) saturated with 95% O2 and 5% CO2 at room temperature (22-25 °C) for at least 1 h before mechanical dissociation. For dissociation, slices were transferred into a 35 mm culture dish (Primaria 3801; Becton Dickinson, Rutherford, NJ, USA) containing the standard extracellular solution (in mM; 150 NaCl, 5 KCl, 2 CaCl2, 1 MgCl2, 10 glucose, and 10 HEPES, a pH of 7.4 with Tris-base). The SDCN region was identified using a binocular microscope (SMZ-1; Nikon, Tokyo, Japan). Details on mechanical dissociation can be found in a previous study (Akaike and Moorhouse, 2003). Briefly, mechanical dissociation was accomplished using a custom-built vibration device and a fire-polished glass pipette oscillating at approximately 50-60 Hz (0.3-0.5 mm) on the surface of the SDCN region. Slices were removed, and mechanically dissociated neurons were allowed to settle and left to adhere to the bottom of the dish for 15 min. A similar manipulation was performed for the mechanical dissociation of hippocampal CA3 neurons (Nonaka et al., 2019). The glycinergic synaptic transmission was recorded from acutely dissociated SDCN neurons (Figure 1-7), and the glutamatergic transmission was recorded from acutely dissociated CA3 neurons (Figure 8), respectively.

Electrophysiological recordings

Electrophysiological recordings were performed using a conventional whole-cell patch clamp technique with an amplifier (Multiclamp 700 B; Molecular Devices, Union City, CA, USA). Neurons were clamped at a $V_H$ of 0 mV and -60 mV for recording glycinergic and...
glutamatergic currents, respectively, except where indicated. The resistance of the patch pipettes to record glycinergic and glutamatergic currents filled with internal solution (in mM; 140 CsF, 10 CsCl, 2 EGTA, 2 ATP-Na₂, and 10 HEPES with the pH adjusted to 7.2 with Tris-base) was 2-6 MΩ. All liquid junction potential for recording Na, Ca and K currents (~ -11 mV, measured by exchanging the bath solution from the internal solution to the standard extracellular solution), series resistance (40%-70%), and pipette capacitance were compensated (Lee et al., 1979; Yatani et al., 1982; Inoue et al., 1986; Kaneda et al., 1989; Oyama and Akaike 1990; Rhee et al., 1996; Shin et al., 2008; Wakita et al., 2015a). The data were discarded when the series resistance changed > 15%. Neurons were observed under phase contrast on an inverted microscope (DMI3000; Leica, Nussloch, Germany). The membrane currents were filtered at 1-5 kHz (Multiclamp 700 B), digitized at 4-20 kHz (Digidata 1440; Molecular Devices), and stored on a computer equipped with pCLAMP 10.7 (Molecular Devices). When recording, 10 mV hyperpolarising step pulses (30 ms in duration) were periodically delivered to monitor access resistance.

Whole-cell currents induced by exogenous application of test solution with glycine, GABA or glutamate are the sum of all extrasynaptic and intrasynaptic receptor responses existing on postsynaptic cell membrane as described previously (Akaike et al., 2002; Akaike and Moorhouse, 2003). Briefly, stimulating pipettes were made from a theta glass electrode (2.0 mm outer diameter, 1.4 mm inner diameter) (TG200-4; Warner Instruments, New Haven, CO, USA) using a pipette puller (P-97; Sutter Instrument Co., Novato, CA, USA). The inner diameter of the tip of the theta pipette was less than 1 μm. Two silver wires were inserted into each barrel of the stimulating theta electrode filled with a standard extracellular solution. This stimulating electrode was placed near the surface of the proximal dendrites of both SDCN and CA3 neurons. Brief paired pulses (100 μsec duration, 70–90 μA) were applied by the stimulation pipette at a frequency of 0.1 Hz using a stimulator (SEN-7203, Nihon Kohden, Tokyo, Japan) equipped with an isolator unit (SS-701J, Nihon Kohden) to evoke two eEPSCs (eEPSC₁ and eEPSC₂).

To record glycinergic currents, the standard extracellular solution routinely contained 10 μM bicuculline (GABAₐ receptor antagonist), 20 μM CNQX (AMPA/KA receptor subtype antagonist), and 30 μM D-APV (NMDA receptor subtype antagonist). To record glutamatergic currents, the standard extracellular solution routinely contained 10 μM bicuculline. To record voltage-gated Na⁺ currents (I₉), a pipette (internal) solution containing 140 mM CsF, 10 mM CsCl, 2 mM EGTA, 2 mM ATP-Na₂, and 10 mM HEPES (pH 7.2 with Tris-base) was used, and neurons were held at a V_H of -120 mV. The external solution for
recording $I_{\text{Na}}$ contained 10 µM bicuculline, 30 µM CNQX, 30 µM D-APV, and 200 µM Cd$^{2+}$ to block ionotropic GABA$_A$ and glutamate receptors, and voltage-gated Ca$^{2+}$ channels. To record voltage-gated Ca$^{2+}$ currents ($I_{\text{Ca}}$), a pipette solution containing 140 mM CsMeHSO$_3$, 10 mM CsCl, 2 mM EGTA, 2 mM ATP-Mg, and 10 mM HEPES (pH 7.2 with Tris-base) was used and neurons were held at a $V_{\text{H}}$ of -60 mV. The external solution for recording $I_{\text{Ca}}$ contained 10 µM bicuculline, 30 µM CNQX, 30 µM D-APV, and 300 nM TTX to block ionotropic GABA$_A$ receptors, glutamate receptor subtypes, and voltage-gated Na channels. To record voltage-gated K$^+$ currents ($I_{\text{K}}$), a pipette solution containing 140 mM KMeHSO$_3$, 10 mM KCl, 2 mM EGTA, 2 mM ATP-Mg, and 10 mM HEPES (pH 7.2 with Tris-base) was used, and neurons were held at a $V_{\text{H}}$ of -80 mV. The external solution for $I_{\text{K}}$ contained 10 µM bicuculline, 30 µM CNQX, 30 µM D-APV, 300 nM TTX, and 200 µM Cd$^{2+}$. In recordings of voltage-gated ion channels, the patch pipettes with lower resistance of 0.8-1.2 MΩ was used and the liquid junction potential (-10 ~ -11 mV) was compensated.

**Data analysis**

The amplitudes of voltage-gated ion currents were calculated by subtracting the baseline from the respective peak amplitudes (pCLAMP 10.7; Molecular Devices). In cases where glycinergic eIPSCs and glutamatergic eEPSCs were not evoked by electrical stimulation, these sweeps had failed and were excluded from the averaged value of the eIPSC$_1$ amplitude. The paired-pulse ratio (PPR) was determined as the amplitude ratio (eIPSC$_2$/eIPSC$_1$) of eIPSC$_2$ and eIPSC$_1$. When neither eIPSC$_1$ nor eIPSC$_2$ was evoked, these sweeps were excluded from the average value of PPR. Glycinergic sIPSCs were detected automatically using template search methods with an amplitude threshold of 8 pA, and then confirmed manually (pCLAMP 10.7). The basal noise levels were lesser than 6 pA. The average values of the frequency, amplitude and decay time constant of all sIPSCs during the control period (5-10 min) were calculated for each recording, and the frequency and amplitude of the events during the application of Xe solution (3 min) were normalized to these values. The effect of Xe was quantified as a percentage change in sIPSCs or eIPSCs compared to the respective control values. The same treatments were performed for glutamatergic sEPSCs and eEPSCs of CA3 neurons. The inter-event intervals and amplitudes of many synaptic events obtained from the same neuron were examined by constructing cumulative probability distributions, which were then compared using the Kolmogorov-Smirnov (K-S) test with Stat View software (SAS Institute, Inc., Cary, NC, USA). Numerical values are presented as the mean ± standard error of the mean (SEM) using values normalized to the control. Significant
differences in the mean amplitude and frequency were tested using a Student’s paired two-tailed t-test or repeated measure (RM) ANOVA with absolute values rather than normalized ones. The statistical test method was defined in the relevant figure legends. Differences were considered statistically significant at p < 0.05. Numerical values are provided as the mean ± SEM. The n number in this study was the number of animal since we used one animal per experiment.

Drugs

The drugs used in this study were TTX, D-APV, CNQX, EGTA, bicuculline, strychnine, SQ22536, KT5720 (Sigma, St. Louis, MO, USA), and H-89, 8-Br-cAMP, Rp-cAMPS (MedChemExpress, Monmouth Junction, NJ, USA), and ZD7288 (Tocris, Bristol, UK). Other reagents for the test solutions were obtained from Wako Pure Chemicals (Osaka, Japan). The Xe gas mixture (70% Xe, 28.5% O2, and 1.5% CO2) was purchased from a gas supplier (Kumamoto Sanso Co., Kumamoto, Japan). We chose 70% Xe because this is the maximum clinically used concentration. To bath apply Xe in solution, the Xe gas mixture was bubbled into the extracellular test solution in a 5 mL glass test tube for 3-5 min, and then the test tube was tightly sealed with a parafilm as in previous studies (Haseneder et al., 2008; Georgiev et al., 2010; Yamamoto et al., 2012; Nonaka et al., 2019; Kubota et al., 2020). The pH of the extracellular solution bubbled with Xe gas mixture was 7.30 ± 0.02 (n = 4). In another set of experiments, we confirmed that the extracellular solution (pH 7.3) as a control did not affect the basal properties of sIPSCs and eIPSCs, such as frequency and amplitude (n = 3, data not shown). The Xe solution was applied using the ‘Y-tube system’ for rapid solution exchange with a perfusion rate of 0.9-1.0 mL/min (Murase et al., 1990). The tip of the Y-tube (0.2 mm inner diameter) was positioned 1.0 mm away from the patched neurons. During the gravity-driven application of the Xe solution, a pinhole was made in the parafilm seal to minimize the loss of Xe gas.
8. Results

Effect of Xe on glycinergic sIPSCs in acutely isolated SDCN neurons

Acutely isolated SDCN neurons were voltage-clamped at a $V_H$ of 0 mV, and sIPSCs were recorded in the presence of 10 µM bicuculline, 20 µM CNQX, and 30 µM D-APV. The outwardly directed synaptic currents were completely blocked by adding 3 µM strychnine, a selective glycine receptor antagonist ($n = 4$, data not shown), confirming glycine receptor-mediated sIPSCs. In these conditions, the application of 70% Xe significantly decreased the mean sIPSC frequency (67.1 ± 2.3% of the control; 0.61 ± 0.12 Hz for the control and 0.39 ± 0.07 Hz for the Xe condition, $n = 10$, $P = 0.0017$), without affecting the mean current amplitude (98.4 ± 2.9% of the control; 37.8 ± 4.5 pA for the control and 37.1 ± 4.5 pA for the Xe condition, $n = 10$, $P = 0.6015$) (Fig. 1A, B). Xe shifted the cumulative probability of inter-event intervals of sIPSCs to the right ($P = 0.0003$, K-S test), consistent with a decrease in sIPSC frequency. However, Xe did not affect the cumulative probability of sIPSC amplitude ($P = 0.9461$, K-S test). Xe also had no effect on the $\tau_{\text{Decay}}$ of glycinergic sIPSCs (100.1 ± 1.9% of the control; 11.8 ± 0.3 ms for the control and 11.7 ± 1.1 ms for the Xe condition, $n = 10$, $P = 0.8991$) (Fig. 1C). We also investigated the effect of Xe on exogenous glycine-induced membrane currents ($I_{\text{Gly}}$) in acutely isolated SDCN neurons. Xe significantly enhanced the $I_{\text{Gly}}$ induced by 3 µM glycine to 124.6 ± 9.7% of the control ($n = 9$, $P = 0.0351$). However, Xe did not affect $I_{\text{Gly}}$ induced by more than 10 µM glycine (Fig. 1D). Taken together, it is feasible that Xe acts presynaptically to decrease spontaneous glycine release onto SDCN neurons.

Both $\text{Ca}^{2+}$ influx and $\text{Ca}^{2+}$ release were not involved in the Xe-induced decrease of glycinergic sIPSC and mIPSC frequency.

To investigate the mechanisms underlying the Xe-induced decrease in sIPSC frequency, we next examined the effect of Xe (70%) on glycinergic mIPSCs, which were recorded in the presence of 300 nM TTX, a specific voltage-gated Na$^+$ channel blocker. The application of 300 nM TTX significantly decreased the sIPSC frequency (58.3% ± 4.9% of the control, $n = 7$, $P = 0.0020$, Fig. 2C). Xe still decreased the mIPSC frequency (71.0 ± 4.7% of the TTX condition, $n = 7$, $P = 0.0011$, Fig. 2 A, Ca). Application of 200 µM Cd$^{2+}$, a general voltage-gated Ca$^{2+}$ channel blocker, further decreased the frequency without affecting the amplitude of glycinergic mIPSCs (frequency: 67.1 ± 4.1% of the TTX control, $n = 7$, $P = 0.0021$, Fig. 2C). In the presence of TTX plus 200 µM Cd$^{2+}$, Xe continued to decrease the
mIPSC frequency (70.1 ± 3.9% of the TTX and Cd^{2+} conditions, n = 7, P = 0.0016, Fig. 2 B, Ca). In contrast, Xe did not affect the amplitude and decay time constant of glycinergic mIPSCs in the presence of TTX or Cd^{2+} (Fig. 2Cb). These results suggest that voltage-gated Na^{+} and Ca^{2+} channels are not involved in the Xe-induced decrease in the frequency of sIPSC and mIPSC. To rule out the involvement of voltage-gated ion channels, we directly examined the effects of Xe on three typical types of voltage-gated ion channels (I_{Na}, I_{Ca}, I_{K}), which are directly related to the regulation of presynaptic membrane potentials, in acutely isolated SDCN neurons. This is because glycinergic presynaptic terminals might originate from adjacent spinal area including the SDCN, as spinal dorsal neurons receive inhibitory synaptic inputs originated from adjacent area (Lu and Perl, 2005). As shown in Fig. 2 (D, E), Xe did not affect the voltage-gated Na^{+}, Ca^{2+}, and K^{+} currents in SDCN neurons. Taken together, the results suggest that Xe may decrease spontaneous glycine release independent of presynaptic voltage-gated Na^{+} and Ca^{2+} channels in acutely isolated SDCN neurons.

The probability of neurotransmitter release is proportional to the intra-terminal Ca^{2+} concentration ([Ca^{2+}]_{terminal}) (for review, Neher and Sakaba, 2008; Williams and Smith, 2018). Therefore, we examined whether Ca^{2+} influx from the extracellular space or Ca^{2+} release from presynaptic intra-axonal Ca^{2+} stores affects the Xe-induced decrease in mIPSC frequency. We first examined the effect of a nominal Ca^{2+}-free extracellular solution on the Xe-induced decrease in mIPSC frequency. Exposure of the Ca^{2+}-free extracellular solution to SDCN neurons greatly decreased both the frequency and amplitude of glycinergic mIPSCs (frequency: 67.7 ± 8.2% of the control, n = 8, P = 0.0087; amplitude: 64.4 ± 7.3% of the control, n = 8, P = 0.0028, Fig. 3 Aa, C). However, in the Ca^{2+}-free extracellular solution with TTX, Xe still decreased mIPSC frequency (67.9 ± 2.8% of the Ca^{2+}-free condition, n = 8, P = 0.0010) without changing the current amplitude (Fig. 3 Ab, C). We also examined the effect of thapsigargin, an inhibitor of sarcoplasmic/endoplasmic Ca^{2+} stores (Garavito-Aguilar et al., 2004), on the Xe-induced decrease in mIPSC frequency. The application of 1 μM thapsigargin greatly increased the basal frequency of glycinergic mIPSCs even in the Ca^{2+}-free extracellular solution with TTX (505.9 ± 70.6% of the Ca^{2+}-free condition, n = 7, P = 0.0046) and increased the amplitude (133.4 ± 15.3% of the Ca^{2+}-free condition, n = 7, P = 0.0225) (Fig. 3 Ba, C). Even in the presence of 1 μM thapsigargin, Xe again decreased mIPSC frequency (67.8 ± 1.4% of the thapsigargin condition, n = 7, P = 0.0037) without affecting the amplitude (Fig. 3 Bb, C). These results indicate that Ca^{2+} dynamics are not involved in Xe-induced presynaptic inhibition.
Cyclic AMP-PKA pathway involved in the Xe-induced decrease in glycinergic sIPSC frequency.

The above results suggest that neither the Ca$^{2+}$ influx across the plasma membrane nor Ca$^{2+}$ release from Ca$^{2+}$ stores is involved in the Xe-induced decrease in mIPSC frequency. These results indicate that Xe acts downstream of the Ca$^{2+}$ influx or Ca$^{2+}$ release to decrease the probability of the glycine release. Many factors are involved in the neurotransmitter release from presynaptic nerve terminals (Dittman and Ryan, 2019). Therefore, we examined whether the manipulation of presynaptic cAMP concentration affected the inhibitory effect of Xe on spontaneous glycine release. We examined the effect of SQ22536, an adenylyl cyclase (AC) inhibitor (Emery et al., 2013), on the Xe-induced decrease in sIPSC frequency. The application of 50 μM SQ22536 significantly decreased the basal frequency of glycinergic sIPSCs (87.2 ± 1.7% of the control, \(n = 8\), \(P = 0.0101\), Fig. 4 Aa, B). In the presence of 50 μM SQ22536, Xe decreased the sIPSC frequency (72.1 ± 4.1% of the SQ22536 condition, \(n = 8\), \(P = 0.0025\), Fig. 4 Ab, Ba). However, the SQ22536 treatment did not alter the sIPSC amplitude (Fig. 4Bb). Next, the effect of 8-Br-cAMP, a membrane-permeable cAMP analogue activating PKA (EC$_{50}$ < 10 μM, Hei et al., 1991), was tested on the Xe-induced decrease in sIPSC frequency. The application of 100 μM 8-Br-cAMP significantly increased the basal frequency of glycinergic sIPSCs (133.8 ± 13.3% of the control, \(n = 8\), \(P = 0.0134\), Fig. 4 Ca, D). In the presence of 100 μM 8-Br-cAMP, Xe still decreased the sIPSC frequency without affecting the amplitude (70.8 ± 5.1% of the 8-Br-cAMP condition, \(n = 8\), \(P = 0.0022\), Fig. 4 Cb, D).

Cyclic AMP affects hyperpolarisation-activated and cyclic nucleotide-gated cation (HCN) channels. Therefore, we further examined the effect of ZD7288, an HCN channel blocker (Milligan et al., 2006), on the Xe-induced decrease in sIPSC frequency. The application of 50 μM ZD7288 had little effect on the basal frequency of glycinergic sIPSCs (118.6 ± 13.8% of the control, \(n = 7\), \(P = 0.2085\), Fig. 4 Ea, F). However, in the presence of 50 μM ZD7288, Xe again decreased the sIPSC frequency (68.5 ± 4.8% of the ZD7288 condition, \(n = 7\), \(P = 0.0106\)) without affecting the amplitude (Fig. 4 Eb, F). These results suggest that Xe acts on the downstream of the cAMP pathway.

PKA is involved in the Xe-induced decrease in glycinergic sIPSC frequency.

Next, we examined whether PKA is involved in the inhibitory effect of Xe on spontaneous glycine release. We examined the effect of H-89, a PKA blocker (Chijiwa et al., 1990), on the Xe-induced decrease in sIPSC frequency. The application of 3 μM H-89
significantly increased the basal frequency of glycinergic sIPSCs (320.0 ± 77.5% of the control, \( n = 7, \ P = 0.0207 \)), with a significant decrease in the amplitude (Fig. 5 Aa, B). In the presence of 3 μM H-89, Xe failed to decrease the sIPSC frequency (99.4 ± 3.6% of the H-89 condition, \( n = 7, \ P = 0.3755 \), Fig. 5 Ab, B). We next examined the effect of KT5720, which is a selective PKA blocker that binds to ATP sites on the catalytic subunits of PKA (Davies et al., 2000; Murray, 2008), on the Xe-induced decrease in sIPSC frequency. The application of 1 μM KT5720 significantly increased the basal frequency of glycinergic sIPSCs (145.3 ± 20.9% of the control, \( n = 8, \ P = 0.0304 \)) without affecting the amplitude (Fig. 5 Ca, D). In the presence of 1 μM KT5720, Xe again failed to decrease the sIPSC frequency (96.9 ± 3.7% of the KT5720 condition, \( n = 8, \ P = 0.9446 \), Fig. 5 Cb, D). We further examined the effect of Rp-cAMPS, which binds to cAMP-binding sites on PKA to inhibit enzymatic activity (de Wit et al., 1984; Murray, 2008), on the Xe-induced decrease in sIPSC frequency. The application of 30 μM Rp-cAMPS significantly increased the basal frequency of glycinergic sIPSCs (119.1 ± 5.9% of the control, \( n = 9, \ P = 0.0180 \)) without changing the amplitude (Fig. 5 Ea, F). In the presence of 30 μM Rp-cAMPS, Xe again failed to decrease the sIPSC frequency (99.4 ± 2.9% of the Rp-cAMPS condition, \( n = 9, \ P = 0.6339 \), Fig. 5 Eb, F).

Both PKC and CaMKII were not involved in the Xe-induced decrease in glycinergic sIPSC frequency.

We also examined whether other protein kinases, such as PKC and CaMKII, are involved in the Xe-induced decrease in sIPSC frequency. Chelerythrine (3 μM), a PKC blocker (Herbert et al., 1990) itself, increased the frequency of sIPSCs, and Xe decreased the frequency without affecting the amplitude (Fig. 6 A, B). Similarly, KN-93 (3 μM), a CaMKII blocker (Sumi et al., 1991), also increased the frequency of sIPSCs. Moreover, this increased frequency was significantly decreased by adding Xe (Fig. 6 C, D). The CaMKII blocker did not affect the amplitude.

Xe decreased the action potential-dependent glycine release evoked by single bouton stimulation of SDCN neurons.

The release of neurotransmitters can be roughly distinguished into two types: action potential-dependent and action potential-independent. Therefore, we next examined the effects of Xe on action potential-dependent glycine release. The focal paired-pulse electrical stimulation of a single glycinergic nerve terminal (bouton) enables the elicitation of glycinergic eIPSCs (Akaike et al., 2002; Akaike and Moorhouse, 2003). Paired-pulse stimuli
were delivered with an interval of 100 ms to the surface of the proximal dendrites of isolated single SDCN neurons using a theta glass electrode. Paired-pulse stimuli evoked two eIPSCs (eIPSC₁ and eIPSC₂). The amplitude and failure rate (Rᵣ) of eIPSC₁ and PPR (eIPSC₂/eIPSC₁) were analyzed and calculated. Figure 7A shows a typical time course of the amplitude of eIPSC₁ (a) and PPR (b) obtained before, during, and after the application of 70% Xe in the absence and presence of 1 μM KT5720. Xe significantly decreased the mean eIPSC₁ amplitude to 63.5 ± 5.0% of the control (84.3 ± 17.5 pA for the control and 52.7 ± 10.2 pA for the Xe condition, n = 6, P = 0.0207, Fig. 7Aa, Ba). In addition to the decrease in eIPSCs, Xe significantly increased the Rᵣ of eIPSC₁ (147.7 ± 20.0% of the control; 0.55 ± 0.08 for the control and 0.74 ± 0.07 for the Xe condition, n = 6, P = 0.0028) and the PPR (185.5 ± 17.1% of the control; 0.98 ± 0.10 for the control and 1.81 ± 0.26 for the Xe condition, n = 6, P = 0.0094, Fig. 7Bb, c). These results indicate that Xe acts presynaptically, resulting from a decrease in the probability of action potential-dependent glycine release. The presynaptic effects of Xe on eIPSCs were completely blocked in the presence of 1 μM KT5720. After the blockade of PKA by 1 μM KT5720, Xe failed to change the amplitude (103.8 ± 5.8% of the control, n = 6, P = 0.3274) and Rᵣ (93.4 ± 4.5% of the control, n = 6, P = 0.0961) of eIPSC₁, and the PPR (92.3 ± 10.9% of the control, n = 6, P = 0.4166, Fig. 7A, B). In addition, Xe did not affect the τDecay of glycinergic eIPSCs (Fig. 7C).

Xe decreased the spontaneous and action potential-dependent glutamate release of CA3 neurons.

We have previously reported that Xe inhibits spontaneous and evoked glutamate release in CA3 pyramidal neurons (Nonaka et al., 2019). Therefore, we examined whether the Xe-induced presynaptic inhibition of glutamatergic transmission is also involved in PKA activity. Glutamatergic sEPSCs were recorded from “synapse bouton preparation” of acutely isolated CA3 pyramidal neurons at Vₜₜ of -60 mV (Fig. 8A). Under these conditions, the application of 70% Xe significantly decreased the sEPSC frequency to 61.7 ± 4.8% of the control (n = 9, P = 0.0047, Fig. 8Ba). Xe also decreased the mean amplitude of sEPSCs to 78.2 ± 5.0% of the control (n = 9, P = 0.0172, Fig. 8Bb), which might be due to the direct inhibition of postsynaptic AMPA/KA receptors, as shown in our previous study (Nonaka et al., 2019). However, in the presence of 1 μM KT5720, 70% Xe failed to affect the frequency (104.2 ± 8.6% of the KT5720 condition, n = 9, P = 0.7255) and amplitude (101.2 ± 8.4% of the KT5720 condition, n = 9, P = 0.6400) of sEPSCs (Fig. 8 Ba, b). The decay time constant of glutamatergic sEPSCs was not affected by Xe, despite the presence of KT5720 (Fig. 8Bc).
We also examined the effects of 70% Xe on the action potential-dependent glutamate release. Glutamatergic eEPSCs were triggered by focal electrical stimulation of presynaptic single terminals (bouton) attached to acutely isolated CA3 pyramidal neurons. Figure 8C shows a typical time course of the amplitude (a) and PPR (b) of eEPSC\(_1\) obtained before, during, and after the application of Xe in the absence (black) and presence (red) of 1 μM KT5720. Xe significantly decreased the mean eEPSC\(_1\) amplitude to 68.3 ± 3.8% of the control (264.1 ± 52.7 pA for the control and 175.5 ± 36.4 pA for the Xe condition, \(n = 8\), \(P = 0.0056\), Fig. 8 Ca, Da). Xe also significantly increased the \(R_f\) of eEPSC\(_1\) (135.5 ± 8.6% of the control; 0.51 ± 0.05 for the control and 0.67 ± 0.05 for the Xe condition, \(n = 6\), \(P = 0.0022\)) and the PPR (173.6 ± 19.3% of the control; 0.78 ± 0.06 for the control and 1.30 ± 0.11 for the Xe condition, \(n = 7\), \(P = 0.0058\), Fig. 8 C, Db,c). These results again indicate that Xe presynaptically decreases the probability of action potential-dependent glutamate release. In the presence of 1 μM KT5720, 70% Xe failed to decrease the eEPSC\(_1\) amplitude (87.7 ± 4.5% of the KT5720 condition, \(n = 8\), \(P = 0.0539\)), \(R_f\) (102.3 ± 4.1% of the KT5720 condition, \(n = 6\), \(P = 0.4761\)), and PPR (86.3 ± 5.4% of the KT5720 condition, \(n = 7\), \(P = 0.0642\)) of eEPSCs (Fig. 8Da-c). The decay time constant of eEPSCs was not affected by Xe despite the presence of KT5720 (Fig. 8Dd).
9. Discussion (1496/1500 words)

Xe acts presynaptically to inhibit spontaneous, miniature, and evoked glycine release.

Present evidences indicate that Xe acts presynaptically to inhibit glycine release. The whole-cell currents induced by exogenous application glycine (Fig. 9A lower panel), GABA or glutamate are the sum of all extrasynaptic (red circle, Fig. 9A) and synaptic (red ellipse, Fig. 9C) receptor responses existing on postsynaptic neurons. In Fig. 9C schematic illustration shows synaptic receptor right under each bouton (blue triangle). Glycinergic sIPSC (Fig. 9B lower panel) and glutamatergic sEPSCs are observed as spontaneous responses of many homogenous synapses (Fig. 9B). Focal single- or paired-pulse electrical stimulation was performed for glycinergic eIPSCs (Fig. 9C lower panel) and glutamatergic eEPSCs at single synaptic level (Akaike et al., 2002; Akaike and Moorhouse, 2003).

First, Xe decreased the mean frequency of glycinergic sIPSCs and mIPSCs without affecting \( \tau_{\text{Decay}} \). Xe also shifted the cumulative probability of sIPSC frequency to the right without affecting amplitude, suggesting that Xe acts presynaptically to decrease the probability of glycine release. Moreover, Xe did not change the sensitivity of the postsynaptic glycine receptors (Fig. 1Bb), since Xe did not affect \( I_{\text{Gly}} \) induced by more than 10 µM glycine (Fig. 1Db). Secondly, Xe decreased eIPSC\(_1\) amplitude and simultaneously increased PPR and \( R_f \) without affecting \( \tau_{\text{Decay}} \) of eIPSCs, supporting the presynaptic action of Xe on evoked glycine release. Thirdly, regarding the “synaptic bouton preparation” mechanically isolated SDCN neurons preserve functional presynaptic boutons without their parent neurons or surrounding cells, including glial cells (Akaike and Moorhouse, 2003). These results strongly indicate that Xe acts presynaptically to inhibit glycine release. Likewise, we have previously reported that Xe acts presynaptically to inhibit spontaneous and evoked glutamate and GABA release onto the spinal SDCN and hippocampal CA3 pyramidal neurons (Nonaka et al., 2019; Kubota et al., 2020).

In contrast, previous studies have shown that Xe has differential effects on neurotransmitter release in several central neurons. Xe (65%) did not reduce mEPSC frequency in rat spinal dorsal horn neurons (Haseneder et al., 2009). However, Xe (50%) inhibits the frequency of both mEPSCs and mIPSCs in rat spinal neurons (Georgiev et al., 2010; Yamamoto et al., 2012). The reasons for such different presynaptic effects of Xe on neurotransmitter release remain unclear. However, experimental conditions, for example, synaptic bouton preparation in our studies and slice preparations in other studies, might explain the discrepancy in presynaptic effects of Xe on neurotransmitter release. For example,
in slice preparation, a significant portion of Xe might be diffused before reaching patched neurons because of slow bath application. Whereas, in the synaptic bouton preparation using fast application technique within 20 ms by “Y-tube” system, the loss of Xe during application could be minimized. The higher concentration of Xe in our present study (70%) compared to other studies (50-65%) might also explain this discrepancy. However, this is not the case because our previous study reported that even 30% Xe significantly decreased spontaneous glutamate release in CA3 pyramidal neurons (Nonaka et al., 2019). Further studies are needed to confirm the presynaptic roles of Xe in synaptic transmission in brain slices or more intact preparations.

**Neither the Ca^{2+} influx nor the Ca^{2+} release was involved in the inhibition of miniature glycine release by Xe.**

The mechanisms underlying the Xe-mediated presynaptic inhibition of neurotransmitter release have not been studied in our previous studies (Nonaka et al., 2019; Kubota et al., 2020). Therefore, we attempted to reveal how Xe acts presynaptically to inhibit glycine release onto SDCN neurons. Even in the presence of TTX and Cd^{2+}, Xe still reduced sIPSC frequency, suggesting that voltage-gated Na^{+} and Ca^{2+} channels in presynaptic nerve endings were not involved in the Xe-induced decrease in sIPSC frequency. We also confirmed that Xe had no direct effect on currents mediated by voltage-gated Na^{+}, K^{+}, and Ca^{2+} channels, which are closely related to membrane potential and an increase in [Ca^{2+}]_{terminal} in presynaptic nerve terminals, though neurotransmitter release depends on an increase in [Ca^{2+}]_{terminal} (Angleson and Betz, 2001; Kirischuk and Grantyn, 2003). Xe did not directly modulate voltage-gated Na^{+} and Ca^{2+} channels in cardiac myocytes (Stowe et al., 2000). We also found that Xe decreased the sIPSC frequency even in the absence of extracellular Ca^{2+}, indicating that Xe-mediated inhibition is independent of Ca^{2+} influx from the extracellular space. Furthermore, the involvement of Ca^{2+} release from Ca^{2+} stores would be negligible because thapsigargin did not affect the Xe effects. These results show that Xe inhibits miniature glycine release in a Ca^{2+}-independent manner.

**PKA was predominantly involved in the Xe-mediated presynaptic inhibition of glycine release**

cAMP is a well-known modulator that regulates neurotransmitter release in a PKA-dependent or independent manner. We have reported that miniature release is regulated by cAMP, and the action potential-evoked release is regulated by PKA (Katsurabayashi et al.,
2004). Apparently, different results obtained in the current study might be related to the following: The increase in sIPSC frequency with 8-Br-cAMP, a membrane-permeable analog of cAMP (Fig. 4) and PKA inhibitors (KT5720, H-89, and Rp-cAMP) (Fig. 5) may be due to the accumulation of unused-cAMP, which may activate PKA in turn.

On the other hand, the inhibitory effect of Xe on mIPSCs, which are thought to be PKA-independent (Fig. 3Ba), suggests that Xe may also act on cAMP itself. However, the inhibitory effect of Xe on mIPSCs may have been masked by the significant increase in sIPSCs caused by the PKA inhibitors (Fig. 5). Consequently, the effect of Xe may be responsible for PKA-dominant rather than cAMP-dominant mechanisms.

Cyclic AMP is a positive modulator of HCN channels (He et al., 2014) as well, and Xe acts on neuronal HCN channels (Mattusch et al., 2015). However, HCN channels might not be involved in the inhibition of spontaneous glycine release by Xe, as Xe still reduced the sIPSC frequency even in the presence of ZD7288. In contrast, selective blockers of PKC and CaMKII had little effect on Xe-induced decrease in sIPSC frequency. However, the pharmacological blockade of PKA activity with PKA inhibitors (H-89, KT5720, and Rp-cAMPS) completely attenuated Xe-induced decrease in sIPSC frequency. We also found that Xe failed to change the eIPSC1 amplitude, Rf, and PPR of eIPSCs in the presence of KT5720, suggesting that PKA activity plays a pivotal role in the Xe-induced inhibition of both spontaneous and evoked glycine release. Although these PKA inhibitors act on ATP-binding (H-89 and KT5720) or cAMP-binding sites (Rp-cAMPS) to inhibit enzymatic activity (de Wit et al., 1984; Davies et al., 2000; Murray, 2008), further experiments using genetic tools such as PKA-knockout animals are needed to support our conclusions obtained from pharmacological experiments.

Although other protein kinases, PKC and CaMKII, are not involved in the Xe-mediated modulation of inhibitory glycinergetic synaptic transmission in SDCN neurons, either chelerythrine (PKC inhibitor) or KN-93 (CaMKII inhibitor) greatly increased sIPSC frequency. The mechanism of PKC or CaMKII inhibitors-induced increase in basal sIPSC frequency is not known and it might be due to side effects of inhibitors, as chelerythrine and KN-93 inhibit several types of voltage-gated K+ channels (Rezazadeh et al., 2006; Hegyi et al., 2015). Nevertheless, Xe decreased sIPSC frequency even in the presence of each inhibitor. In addition, the IC50 of chelerythrine is 0.66 μM (Herbert et al., 1990). The concentration of chelerythrine we used in this study was 3 μM which was pharmacologically considered sufficient to inhibit PKC activity by about 80% (Herbert et al., 1990). Likewise, the IC50 of KN-93 is 0.37 μM (Sumi et al., 1991). The concentration of KN-93 used in this study was 3
μM, which was pharmacologically considered sufficient to inhibit CaMKII activity by about 80% (Tokumitsu et al., 1990; Sumi et al., 1991). Therefore, these two kinases might not be involved in the Xe-mediated inhibition of spontaneous glycine release.

**PKA was also involved in the presynaptic inhibition of excitatory synaptic transmission by Xe.**

Xe-mediated modulation of glutamatergic fast spontaneous and evoked excitatory synaptic transmission (sEPSC and eEPSC) in CA3 pyramidal neurons was also examined. Xe failed to decrease sEPSC frequency, the amplitudes of sEPSCs and eEPSCs, the Rf and PPR of eEPSCs after the blockade of PKA activity with KT5720, suggesting that the presynaptic inhibitory action of Xe on glutamate release was also PKA-dependent. Although Xe inhibited AMPA/KA receptors in a non-competitive manner (Dinse et al., 2005; Nonaka et al., 2019). It would be of interest to examine whether postsynaptic AMPA/KA receptors are also modulated by PKA activity. This raises the possibility that PKA-dependent effects of Xe at central synapses are not limited to glycinergic and glutamatergic synapses. Nonetheless, it remains to be elucidated how PKA mediates the Xe-induced presynaptic inhibition of neurotransmitter release. It would be great interest to examine whether Xe directly or indirectly acts on PKA to modulate neurotransmitter release, since Xe is reported to modify lipid membrane and change the phase distribution of lipid raft mixtures (Weinrich & Worcester, 2013).

**Conclusion**

As a mechanism of Xe-mediated presynaptic inhibition of glycinergic and glutamatergic synaptic transmissions, PKA activity plays a central role. The precise mechanism of how Xe acts on PKA shall be investigated in the future.
10. Acknowledgements

We would like to thank Editage (www.editage.com) for English language editing.

11. Data Availability Statement

There are no datasets within this article.

12. Author contributions

Participated in research design: Jang, Nakamura, Nonaka, and Akaike.
Conducted experiments: Jang, Nakamura, and Nonaka.
Contributed new reagents or analytic tools: None.
Performed data analysis: Jang, Nakamura, Nonaka and Nagami.
Wrote or contributed to the writing of the manuscript: Jang, Nakamura, Noda, Kotani, Katsurabayashi, Nagami, and Akaike.
13. References


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ylbenzylamine), a calcium/calmodulin-dependent protein kinase II inhibitor, is a direct extracellular blocker of voltage-gated potassium channels. *J Pharmacol Exp Ther* **317**:292-299.


14. Footnotes

a) This work was supported by Grant-in-Aid from Kumamoto Health Science University to K.N. (18-02, 20-07), JSPS KAKENHI grant to K.N. [grand numbers: 21K08984], Grand-in-Aid from Kitamoto Hospital to N.K. and N.A., and Grant-in-Aid from Kumamoto Kinho Hospital to H.N. and N.A.

b) Conflicts of interest: No author has an actual or perceived conflict of interest with the contents of this article.
15. Figure legends

Figure 1. Effect of Xe on glycinergic sIPSCs in SDCN neuron.

A, A typical trace of glycinergic spontaneous inhibitory postsynaptic current (sIPSC) observed before, during, and after the application of 70% Xe in the presence of 10 μM bicuculline, 20 μM CNQX, and 30 μM D-APV. The lower recordings represent glycinergic sIPSCs with an expanded time scale at indicated regions.

B, Cumulative probability distribution for the inter-event interval (a) and current amplitude (b) of glycinergic sIPSCs. The plot includes 407 events for the control (black lines) and 218 events for the Xe condition (red lines). Inset columns represent the mean ± SEM from ten neurons (experiments), and the dotted lines in a and b represent the relative control of basal frequency and amplitude of sIPSCs, respectively. **; p < 0.01, n.s; not significant; t-test.

C, a, Superimposed and normalized traces of single glycinergic sIPSCs observed before (black) and during (red) the application of Xe with an expanded time scale. The traces were scaled by the amplitude. b, Cumulative probability distribution of the 1/e decay time constant (τ_{Decay}) of glycinergic sIPSCs. The plot includes 407 events for the control (black line) and 218 events for the Xe condition (red line). The inset represents the Xe-induced change in τ_{Decay} of glycinergic sIPSCs. Each column represents the mean ± SEM of the ten experiments. n.s; not significant; t-test.

D, a, Representative current traces of glycine-induced whole-cell currents (I_{Gly}) were elicited by the exogenous application of 3 μM (upper) and 100 μM (lower) glycine in the absence and presence of Xe. Glycine was applied every 2 min, and Xe was applied 1 min prior to glycine application. b, Xe-induced changes in I_{Gly} induced by various concentrations of glycine. Each point and error bar represent the mean ± SEM from five to 12 experiments. Note that Xe significantly potentiates I_{Gly} only by low concentrations of glycine (less than 10 μM). *; p < 0.05, n.s; not significant; t-test.

Figure 2. Effects of TTX and Cd^{2+} on Xe-induced decrease in mIPSC frequency in SDCN neuron.

A, A typical trace of glycinergic mIPSCs observed before, during, and after the application of Xe in the presence of 300 nM TTX. Insets represent glycinergic mIPSCs with an expanded time scale at indicated regions.
B, A typical trace of glycinergic mIPSCs observed before, during, and after the application of Xe in the presence of 300 nM TTX plus 200 µM Cd^{2+}. Insets represent glycinergic mIPSCs with an expanded time scale at indicated regions.

C, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic mIPSCs in the presence of 300 nM TTX or mIPSCs in the presence of 300 nM TTX plus 200 µM Cd^{2+}. Each column represents the mean ± SEM of seven experiments. **; p < 0.01, n.s; not significant; t-test ##; p < 0.01, n.s†; RM ANOVA.

D, Effects of Xe on voltage-gated Na^+ (a), Ca^{2+} (b), and K^+ (c) channel currents. The Na^+ currents were elicited by depolarising step pulses (up to -20 mV, 50 ms duration) at a V_H of -120 mV. The Ca^{2+} currents were elicited by depolarising step pulses (up to 0 mV, 50 ms duration) at a V_H of -60 mV. The K^+ currents were elicited by depolarising step pulses (up to +40 mV, 500 ms duration) at a V_H of -80 mV. Note that Xe did not affect the voltage-gated Na^+, Ca^{2+}, and K^+ channel currents.

E, Xe-induced changes in the current amplitude mediated by voltage-gated Na^+, Ca^{2+}, and K^+ channels in SDCN neurons. Each column represents the mean ± SEM from seven experiments for Na^+ currents, eight experiments for Ca^{2+} currents, and seven experiments for K^+ currents. n.s; not significant; t-test.

Figure 3. Effects of Ca^{2+}-free external solution and thapsigargin on Xe-induced decrease in mIPSC frequency in SDCN neuron.

A, a, A typical trace of glycinergic mIPSCs observed before and during the application of Ca^{2+}-free external solution in the presence of 300 nM TTX. b, A typical trace of glycinergic mIPSCs observed before, during, and after the application of Xe in the TTX plus Ca^{2+}-free external solution. Insets represent glycinergic mIPSCs with an expanded time scale at indicated regions.

B, a, A typical trace of glycinergic mIPSCs observed before and during 1 µM thapsigargin in the presence of 300 nM TTX and Ca^{2+}-free external solution. b, A typical trace of glycinergic mIPSCs observed before, during, and after the application of Xe in the TTX, Ca^{2+}-free external solution plus 1 µM thapsigargin. Insets represent glycinergic mIPSCs with an expanded time scale at indicated regions.

C, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic mIPSCs in the TTX plus Ca^{2+}-free external solution and TTX, Ca^{2+}-free external solution plus 1 µM thapsigargin. Each column represents the mean ± SEM of eight experiments. **; p < 0.01,
n.s; not significant; t-test, †; p < 0.01, n.s†; RM ANOVA.

Figure 4. Effects of SQ22536, 8-Br-cAMP, and ZD7288 on Xe-induced decrease in sIPSC frequency in SDCN neuron.

A, a, A typical trace of glycinergic sIPSCs observed before and during the application of 50 µM SQ22536, an adenylyl cyclase inhibitor. b, A typical trace of glycinergic sIPSCs observed before, during, and after the application of Xe in the presence of 50 µM SQ22536. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

B, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 50 µM SQ22536. Each column represents the mean ± SEM of eight experiments. *; p < 0.05, **; p < 0.01, n.s; not significant; t-test, †; p < 0.01, n.s†; RM ANOVA.

C, a, A typical trace of glycinergic sIPSCs observed before and during the application of 100 µM 8-Br-cAMP, a PKA activator. b, A typical trace of glycinergic sIPSCs observed before, during, and after the application of Xe in the presence of 100 µM 8-Br-cAMP. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

D, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 100 µM 8-Br-cAMP. Each column represents the mean ± SEM of eight experiments. *; p < 0.05, **; p < 0.01, n.s; not significant; t-test, †; p < 0.01, n.s†; RM ANOVA.

E, a, A typical trace of glycinergic sIPSCs observed before and during the application of 50 µM ZD7288, an HCN channel blocker. b, A typical trace of glycinergic sIPSCs observed before, during, and after the application of Xe in the presence of 50 µM ZD7288. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

F, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 50 µM ZD7288. Each column represents the mean ± SEM of seven experiments. **; p < 0.01, n.s; not significant; t-test, †; p < 0.01, n.s†; RM ANOVA.

Figure 5. Effects of H-89, KT5720, and Rp-cAMPS on Xe-induced decrease in sIPSC frequency in SDCN neuron.

A, a, A typical trace of glycinergic sIPSCs observed before and during the application of 3
µM H-89, a PKA inhibitor. b, A typical trace of glycinergic sIPSCs observed before, during, and after the application of Xe in the presence of 3 µM H-89. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

B, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 3 µM H-89. Each column represents the mean ± SEM of seven experiments. *; p < 0.05, **; p < 0.01, n.s; not significant; t-test, n.s†; RM ANOVA.

C, a, A typical trace of glycinergic sIPSCs observed before and during the application of 1 µM KT5720, a PKA inhibitor. b, A typical trace of glycinergic sIPSCs observed before, during, and after the application of Xe in the presence of 1 µM KT5720. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

D, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 1 µM KT5720. Each column represents the mean ± SEM of eight experiments. *; p < 0.05, **; p < 0.01, n.s; not significant; t-test, n.s†; RM ANOVA.

E, a, A typical trace of glycinergic sIPSCs observed before and during the application of 30 µM Rp-cAMPS, a PKA inhibitor. b, A typical trace of glycinergic sIPSCs observed before, during, and after the application of Xe in the presence of 30 µM Rp-cAMPS. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

F, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 30 µM Rp-cAMPS. Each column represents the mean ± SEM of nine experiments. *; p < 0.05, **; p < 0.01, n.s; not significant; t-test, n.s†; RM ANOVA.

Figure 6. Effects of chelerythrine and KN-93 on Xe-induced decrease in sIPSC frequency in SDCN neuron.

A, a, A typical trace of glycinergic sIPSCs observed before and during the application of 3 µM chelerythrine, a PKC inhibitor. b, A typical trace of glycinergic sIPSCs observed before, during, and after the application of Xe in the presence of 3 µM chelerythrine. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

B, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 3 µM chelerythrine. Each column represents the mean ± SEM of seven experiments. **; p < 0.01, n.s; not significant; t-test, **; p < 0.01, n.s†; RM ANOVA.

C, a, A typical trace of glycinergic sIPSCs observed before and during the application of 3 µM KN-93, a CaMKII inhibitor. b, A typical trace of glycinergic sIPSCs observed before,
during, and after the application of Xe in the presence of 3 µM KN-93. Insets represent glycinergic sIPSCs with an expanded time scale at indicated regions.

D, Xe-induced changes in the frequency (a) and amplitude (b) of glycinergic sIPSCs in the presence of 3 µM KN-93. Each column represents the mean ± SEM of eight experiments. *, p < 0.05; **, p < 0.01; n.s; not significant; t-test, n.s; RM ANOVA.

Figure 7. Effect of Xe on glycinergic eIPSCs in spinal SDCN neurons.

A, A typical time course of glycinergic eIPSC amplitude (a) and paired-pulse ratio (eIPSC2/eIPSC1; b) before, during, and after the application of Xe in the absence (black) and presence (red) of 1 µM KT5720. Insets (1' to 4') represent single traces in the numbered regions.

B, Xe-induced changes in the eIPSC1 amplitude (a), failure rate (Rf, b), and paired-pulse ratio (PPR, c) in the absence and presence of 1 µM KT5720. The eIPSC1 amplitude, Rf, and PPR obtained during the application of Xe were normalised to those obtained under the control condition (dotted line). Each column and error bar represents the mean ± SEM of six experiments. *, p < 0.05; **, p < 0.01, n.s; not significant; t-test, n.s; RM ANOVA.

C, a, Superimposed and normalised traces of single eIPSCs obtained in the control (black) and Xe (red) conditions with an expanded time scale in the absence (left) and presence (right) of 1 µM KT5720. b, Xe-induced change in τ Decay of glycinergic eIPSCs. Each column represents the mean ± SEM of six experiments. n.s; not significant; t-test, n.s; RM ANOVA.

Figure 8. Effects of Xe on glutamatergic sEPSCs and eEPSCs in hippocampal CA3 neurons.

A, Typical traces of glutamatergic sEPSCs observed before, during, and after the application of Xe in the absence (a) and presence (b) of 1 µM KT5720. All recordings were recorded in the presence of 10 µM bicuculline.

B, Xe-induced changes in the frequency (a), amplitude (b), and τ Decay (c) of glutamatergic sEPSCs in the absence and presence of 1 µM KT5720. The frequency, amplitude, and τ Decay of glutamatergic sEPSCs obtained during the application of Xe were normalised to those obtained under the respective control conditions (dotted lines). Each column and error bar represents the mean ± SEM of nine experiments. *, p < 0.05; **, p < 0.01, n.s;
not significant; t-test, n.s†; RM ANOVA.

C, Typical time course of glutamatergic eEPSC$_1$ amplitude (a) and paired-pulse ratio (eEPSC$_2$/eEPSC$_1$; b) before, during, and after the application of Xe in the absence (black) and presence (red) of 1 µM KT5720. Insets (1’ to 4’) represent single traces in the numbered regions.

D, Xe-induced changes in the eEPSC$_1$ amplitude (a), failure rate (R$_f$, b), paired-pulse ratio (PPR, c), and $\tau_{\text{Decay}}$ (d) of eEPSCs in the absence and presence of 1 µM KT5720. The eEPSC$_1$ amplitude, R$_f$, PPR, and $\tau_{\text{Decay}}$ obtained during the application of Xe were normalised to those obtained under the control condition (dotted lines). Each column and error bar represents the mean ± SEM of six to eight experiments. *; p < 0.05, **; p < 0.01, n.s; not significant; t-test, n.s†; RM ANOVA.

Figure 9. Schematic illustrations of three types of current recordings in SDCN neuron.

A, Conventional whole-cell current recording. The glycinergic whole-cell currents ($I_{\text{Gly}}$) induced by exogenous glycine (Gly) application are sum of all extra- and intrasynaptic (synaptic) Gly receptor responses on postsynaptic cell membrane. Red circles, extrasynaptic Gly receptors; filled blue triangles, nerve endings (boutons); synaptic Gly receptor (green ellipse) right under bouton. The lower panel; Typical current trace induced by rapid exogenous application of Gly (3 µM, black) and Gly (3 µM, red) + 70% Xe using Y-Tube method (quoted from Fig. 1Da).

B, Spontaneous inhibitory synaptic currents (sIPSCs) induced by Gly release from many homogenous glycinergic nerve endings without any stimulation. The lower panel; Typical sIPSCs without application of Gly in normal external solution (quoted from Fig. 1A).

C, Glycinergic inhibitory postsynaptic current (eIPSC) evoked by a paired-pulse electric focal stimulation of a bouton. The lower panel; A typical example of glycinergic eIPSCs induced by focal paired-pulse stimulation (Control, dark; 70% Xe, red, quoted from Fig. 7A).
**Figure 3**

(A) mIPSC

- TTX 10pA for 1 min, then Ca²⁺-free
- TTX and Ca²⁺-free (a') 20pA for 1 s
- TTX (a') and Ca²⁺-free (b')

(B) mIPSC

- TTX + Ca²⁺-free 10pA for 1 min, then Thapsigargin 1 μM
- TTX + Ca²⁺-free (a') 20pA for 1 s
- Thapsigargin (a') 1 μM, then Xe (b')

(C) Relative Freq.

- Control
- Ca²⁺-free
- Ca²⁺-free + Xe
- Ca²⁺-free + Thapsigargin
- Ca²⁺-free + Thapsigargin + Xe

- Relative Amp.

- Control
- Ca²⁺-free
- Ca²⁺-free + Xe
- Ca²⁺-free + Thapsigargin
- Ca²⁺-free + Thapsigargin + Xe

- n.s.
- *p < 0.05
- **p < 0.01
- ##p < 0.001
Figure 7

**A**

1' & 2'

3' & 4'

![Graph showing eIPSC with Xe and KT5720](image)

**B**

a) Relative Amp.

b) Relative R

c) Relative PPR

![Bar graphs showing relative values](image)

**C**

a) Paired pulse ratio

b) Relative T Decay

![Graphs showing paired pulse and T decay](image)
Figure 9

A. Whole-cell Rec. of SDCN neuron

B. Many homogenous synaptic Rec.

C. Single synaptic Rec.

Stim.

- Rec.
- Bouton
- Synaptic receptor
- Extrasynaptic receptor

Drugs

Gly 3μM
70% Xe

Control

70% Xe
0.5s
30pA
30ms

3 μm