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Potentiation of the Glycine Response by Bisphenol A, an Endocrine Disrupter, on the Substantia Gelatinosa Neurons of the Trigeminal Subnucleus Caudalis in Mice

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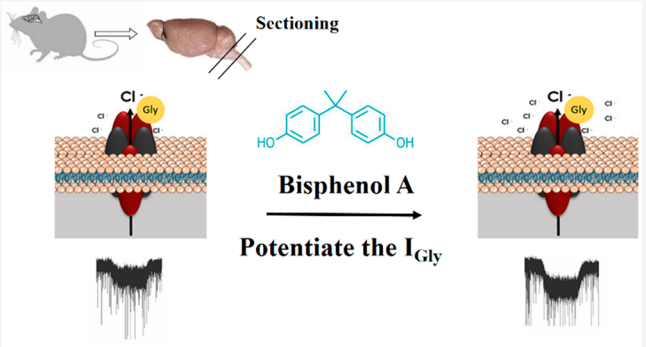
ABSTRACT: Lamina II, also called the substantia gelatinosa (SG) of the medullary dorsal horn (the trigeminal subnucleus caudalis, Vc), is thought to play an essential role in the control of orofacial nociception because it receives the nociceptive signals from primary afferents, including thin myelinated Aδ- and unmyelinated C-fibers. Glycine, the main inhibitory neurotransmitter in the central nervous system, plays an essential role in the transference of nociceptive messages from the periphery to higher brain regions. Bisphenol A (BPA) is reported to alter the morphological and functional characteristics of neuronal cells and to be an effector of a great number of ion channels in the central nervous system. However, the electrophysiological effects of BPA on the glycine receptors of SG neurons in the Vc have not been well studied. Therefore, in this study, we used the whole-cell patch-clamp technique to determine the effect of BPA on the glycine response in SG neurons of the Vc in male mice. We demonstrated that in early neonatal mice (0−3 postnatal day mice), BPA did not affect the glycine-induced inward current. However, in the juvenile and adult groups, BPA enhanced the glycine-mediated responses. Heteromeric glycine receptors were involved in the modulation by BPA. The interaction between BPA and glycine appears to have a significant role in regulating transmission in the nociceptive pathway.



INTRODUCTION

During a lifetime, many chemicals enter the human body through contact with food, air, water, and other environmental sources. Among these chemicals, a large number are toxic because they disrupt the endocrine system. Therefore, there has been growing interest in the influence of endocrine-disrupting chemicals on the health of the animals and humans.[1,2] Of these, bisphenol A (BPA) is one of the most frequently encountered monomers. It is used to manufacture polycarbonate plastics, epoxy resins, and methacrylate resins in dental composites and sealants.[3,4] The extensive, ubiquitous utilization of plastic materials exposes humans to high levels of BPA.[5] BPA is easily taken into the human body through ingestion, inhalation, absorption, and other routes, producing detrimental toxicological health effects.[4,6,7] When entering the body, BPA affects many organ systems, such as reproductive and developmental functions, hormonal activity, carcinogenesis, metabolism, and the immune system.[8−12]

One of the effects of BPA is the alteration of morphological and functional characteristics of neuronal cells in the central nervous system (CNS). Prenatal exposure to BPA disrupts the morphology of the hippocampal CA1 neurons during development.[13] BPA induces memory impairment related to decreases in the production of acetylcholine and glutamate uptake in the hippocampal and cortical regions.[14,15] Prenatal and neonatal exposure to BPA influences central dopaminergic neurotransmission, altering sensitivity to morphine.[16] Recent studies have also suggested the ability of BPA to inhibit the sodium and calcium channels in dorsal root ganglion neurons, altering the excitability of nociceptive afferent fibers, which has toxicological consequences to the CNS.[17,18]

One of the main functions of the CNS is receiving sensory information, including orofacial sensory signals. The trigeminal system is one of the primary components responsible for the sensory function in the orofacial region. Pain sensation from the orofacial region to the trigeminal subnucleus caudalis (Vc) is transferred by sensory fibers consisting of thin myelinated Aδ- and unmyelinated C-fiber,[19,20] which innervate in lamina I and much of lamina II of the Vc.[21,22] The Vc, also known as the medullary dorsal horn, has a similar structure and function

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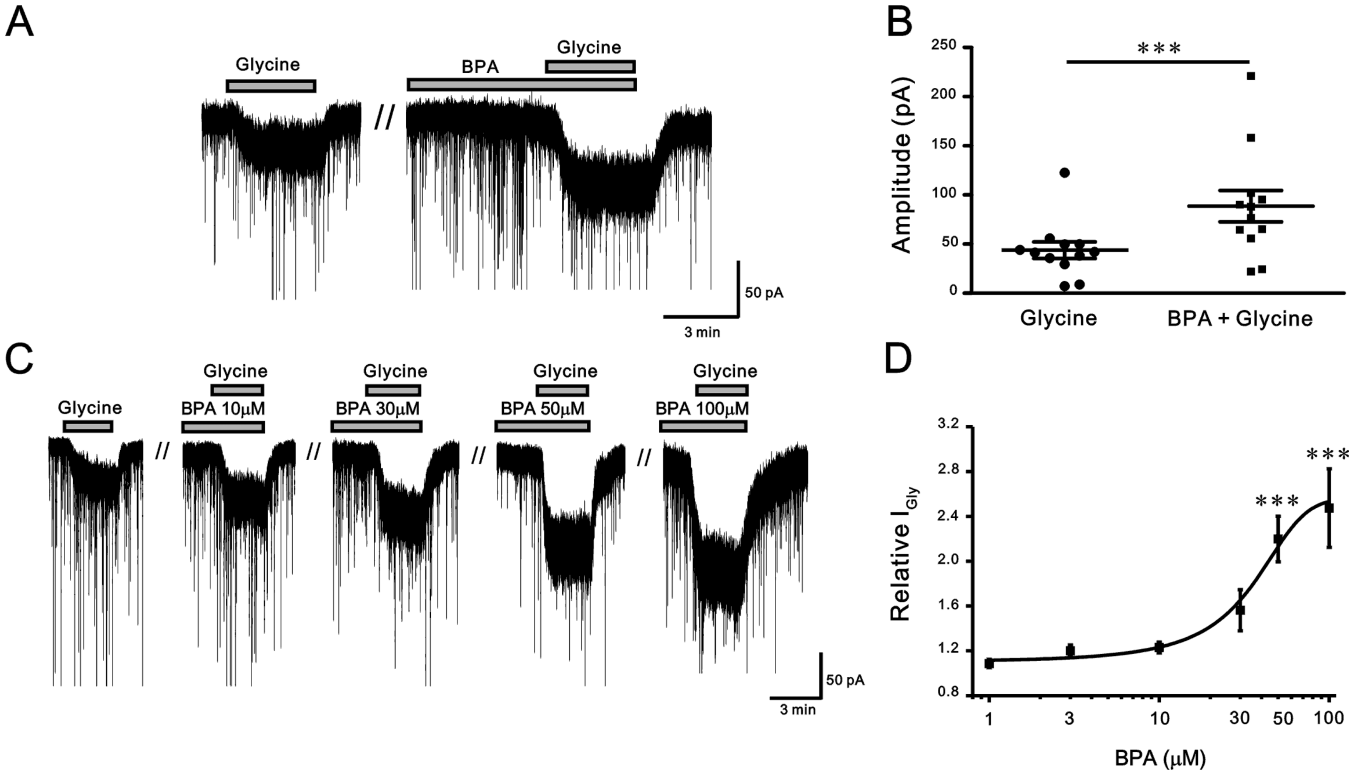

Figure 1. Potentiation of glycine-induced current (I_{Gly}) by bisphenol A (BPA). (A) Representative trace shows the glycine (30 μM)-induced inward current was increased by the simultaneous application with BPA (50 μM) on the substantia gelatinosa neuron of a juvenile male mouse. (B) Dot plot compares the mean I_{Gly} alone and in the coapplication of BPA (n = 12, from eight mice, *** $p < 0.001$, paired t-test) (in each dot plot, the number of the dot is number of the cell, the middle long horizontal bar is the mean value, and two shorter horizontal bars are the up and down SEM values). (C) Representative trace illustrates the change of the glycine (30 μM)-induced inward current to different concentrations of BPA (10, 30, 50, and 100 μM). (D) Curve figure shows the mean relatively of I_{Gly} increased in line with the increase in BPA concentrations (*** $p <$ 0.001, one way ANOVA, Scheffe's post hoc test).

to the spinal dorsal horn and is a key element in nociceptive transference.[23] For this reason, lamina II, also called the substantia gelatinosa (SG), of the Vc is considered a significant location in the pathway of orofacial nociceptive encoding to the higher brain centers.[24]

Glycine, a crucial inhibitory neurotransmitter in the CNS, is present in the SG neurons in high concentrations.[25,26] After release from the presynaptic terminals, glycine activates the postsynaptic glycine receptors to open ligand-gated ion channels, which allows the permeation of chloride ions into the cytoplasm. The inhibitory glycinergic transmission has been recognized to contribute an essential function in the transference of nociceptive signals from the periphery to the higher brain regions.[27,28] In addition to glycine, γ-aminobutyric acid (GABA) is also an inhibitory neurotransmitter, colocalized with glycine in the same SG neurons. This finding supports glycine and GABA acting as cotransmitters with principal roles in modulating nociception.[26,29] Recent studies have demonstrated the effects of BPA on GABA functions,[30,31] but the direct modulations of BPA on glycine receptors are not yet understood. Therefore, in this study, we examined the interaction between BPA and glycine receptors of the SG neurons by using the whole-cell patch-clamp method.

MATERIALS AND METHODS

Animal and Brain Slice Preparation. We carried out the experiments on Institute of Cancer Research (ICR) male mice (Damul Science, Suwon, Korea). The experimental conditions were strictly controlled to limit the effects of BPA exposure of the mice during organogenesis.[32] The mice were housed in a stable environment (temperature range: 23−26 °C) in stainless steel cages with access to food and water *ad libitum* and an automatic 12 h light/dark cycle (lights on at 06:00). There were three mice in one cage, except the pups were kept with their mothers (often 8−12 pups per litter) until 21 postnatal days. The food was supplied by DBL Co., Ltd. (Korea). Our experimental mice were fed with a non-purified diet which consisted of 20.5% crude protein, 3.5% crude fat, 8% crude fiber, 8% ash, 0.5% calcium, and 0.5% phosphorus (RodFeed, DBL, Korea). The water was supplied by the clean tap and contained in polycarbonate bottles covered by rubber stoppers. The animal bedding was made from aspen which was supplied by Tapvei, Tapvei Estonia OÜ (Estonia). The experimental protocol on living animals was approved by the Institutional Animal Care Use Committee of Jeonbuk National University (CBNU 2017−0082).

The method to prepare the brain slices was similar to that in our previous study.[33] ICR mice with suitable experimental ages were chosen randomly (early neonatal: 0−3 postnatal days, juvenile: 7−20 postnatal days, and adults: 45−60 postnatal days). First, the mice were decapitated, and then their brains were removed quickly and immersed in ice-cold bicarbonate-buffered artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, D-(+)-Glucose, 1.4 NaH_2PO_4, 25 $NaHCO_3$, and 0.5 sodium ascorbate (pH 7.3−7.4, bubbled with 95% O_2 and 5% CO_2). Ultrapure water from Water Purification Systems, Dream Mega (MDM CO., Ltd., Korea) was used to make the ACSF. Coronal slices (180−200 μm in thickness) containing the Vc were cut with a vibratome (VT1200S, Leica Biosystems, Wetzlar, Germany) in ice-cold ACSF. The brain slices were kept in oxygenated ACSF at room temperature for at least 1 h before performing the electrophysiological recordings.

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Electrophysiology. We used an upright microscope (BX51WI, Olympus, Tokyo, Japan) with Nomarski differential interference contrast optics to observe the slices. Each random brain slice was moved into the recording chamber, immersed, and continuously perfused with oxygenated ACSF at a flow speed of 4−5 mL/min. The SG (lamina II) on the slice was detected as a translucent band along the lateral edge of the slice, just medial to the spinal trigeminal tract. The SG neurons were found in the SG regions and were randomly chosen to perform the electrophysiological recordings.

Pipets were pulled from the thin-walled borosilicate glass-capillary tubing (PG52151-4, WPI, Sarasota, FL, USA) on a Flaming/Brown puller (P-97; Sutter Instruments Co., Novato, CA, USA). The patch pipet solution was prepared with the following (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 4 MgATP, and 10 EGTA (adjusted to pH 7.3 with KOH). Before patching, the resistance of the recording pipets was in the 4−6 MΩ range. After a gigaohm seal was formed between the pipet and the SG neuron, negative pressure was performed to cause the cell membrane patch to rupture, then electrical measurements were carried out using the whole-cell patch-clamp recording mode with an Axopatch 200B (Molecular Devices, San Jose, CA, USA). All experiments were performed at room temperature. During each recording, the holding potential was maintained at −60 mV to record the currents. Changes in the membrane currents of the cells were acquired using a Digidata 1322A interface (Molecular Devices) connected to a desktop computer. The electrophysiological signals were filtered (2 kHz, Bessel filter of Axopatch 200B) before being digitized at a speed of 1 kHz. The acquisition and subsequent analysis of the acquired records were done with Clampex 10.6 software (Molecular Devices).

At different stages of the experiments, the investigators were blinded to the experimental groups. All experiments were performed at the same time of day to avoid the effect of circadian rhythms on neuronal nociception.

Chemicals. Glycine (≥99%), BPA (≥99%), picrotoxin (≤100%), ginkgolide B (≥90%), and all the chemicals for ACSF were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stocks of all drugs were prepared according to their solubility in distilled water, apart from BPA, picrotoxin, and ginkgolide B, which were made in dimethyl sulfoxide (DMSO, final concentration 1‰). The stock chemicals were diluted to the final concentrations in ACSF immediately before use and then applied to the neurons via bath application.

Data and Statistical Analysis. The acquired traces were plotted by using Origin 8 software (OriginLab Corp., Northampton, MA, USA). For dose response study, we used this formula for curve fitting:

$$y = A_1 + \frac{A_2 - A_1}{1 + 10^{(\log x0 - x)p}}$$

where A_1 is bottom asymptote, A_2 is top asymptote, $\log_x 0$ is center, and p is Hill slope.

EC_{50} is the concentration which induces the half-maximal response and the values of EC_{50} were computed by

$$EC_{50} = 10^{\log x0}$$

All values are represented as the mean ± SEM. A paired t-test and one way ANOVA test were applied to compare the amplitudes of the mean inward currents between the different groups. A p-value <0.05 was defined as the level of statistically significance. The value "n" in the figures indicates the number of SG neurons.

RESULTS

The data were obtained from 60 SG neurons that belonged to 33 male ICR mice.

Potentiation of the I_{Gly} Response by BPA. To investigate the modulation of the glycine-induced inward current (I_{Gly}) by BPA, we observed what happened when we applied glycine and BPA together to the SG neurons of ICR juvenile mice at 7−20 postnatal days (Figure 1). After the successive application of glycine (30 μM), an inward current was created and then, when washed out, the glycine current was reversed. Then, the neurons were pretreated with 50 μM BPA alone for approximately 5 min. At this concentration, BPA did not induce any detectable membrane current change. However, when BPA was applied simultaneously, I_{Gly} was potentiated (Figure 1A). As shown in the dot plot, the mean amplitudes of I_{Gly} alone and in the presence of BPA were 43.7 ± 8.39 pA and 88.6 ± 15.9 pA, respectively (n = 12, from eight mice, p < 0.001, Figure 1B). BPA at 50 μM significantly enhanced the glycine currents when coapplied extracellularly.

Afterward, we also checked the effect between BPA and glycine at different doses of BPA ranging 10−100 μM. The representative trace indicates the clear BPA concentration dependency of I_{Gly} (Figure 1C). There was a gradual increase in the I_{Gly} in proportion to the increase in BPA concentrations, with an EC_{50} of 33.5 pA (Figure 1D). The EC_{50} value was estimated by curve-fitting using Origin 8 software.

Age-Dependent Effect of BPA on the Glycine Response. When the effect between BPA and glycine was investigated at different ages (early neonatal, 0−3 postnatal days; juvenile, 7−20 postnatal days; and adult mice, 45−60 postnatal days), we found an interesting result. The modulation of BPA on glycine was age-dependent in the ICR mice (Figure 2). We compared the mean relative amplitudes and each relative amplitude was calculated by dividing the absolute amplitude with that of the I_{Gly} alone in the same cell. In the early neonatal group, there was no significant difference between the glycine (30 μM)-induced inward current alone and that in the presence of BPA (50 μM) (n = 6, from four mice, p > 0.05). In contrast, 50 μM BPA increased the I_{Gly} to 220 ± 20% (n = 12, from eight mice, p < 0.001) and to 139 ± 10% (n = 5, from four mice, p < 0.05) in the controls in the juvenile and adult groups, respectively.

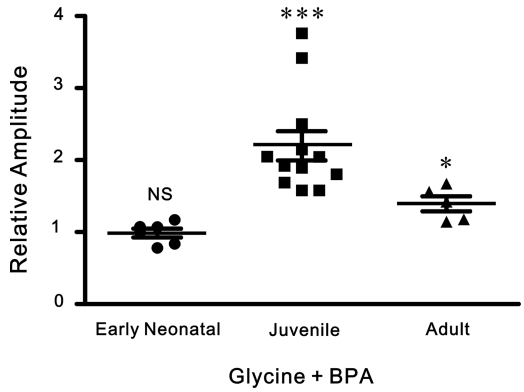

Figure 2. Age-dependent effect. Comparison of the relative glycine (30 μM)-induced inward current (I_{Gly}) in the coapplication of BPA (50 μM) between early neonatal (n = 6, from four mice), juvenile (n = 12, from eight mice), and adult groups (n = 5, from four mice) in ICR male mice. There is no significant difference between the mean relative I_{Gly} alone and in the presence of BPA in the neonatal mice. However, the mean relative I_{Gly} was increased in the simultaneous application of glycine and BPA in the juvenile and adult groups (* p < 0.05, *** p < 0.001, NS no significant, paired t-test) (in each dot plot, the number of the dot is number of the cell, the middle long horizontal bar is the mean value, and two shorter horizontal bars are the up and down SEM values).

Heteromeric Glycine Receptors Are Involved in the Potentiation of I_{Gly} by BPA. In the structural aspect, glycine receptors are composed of a combination of five different

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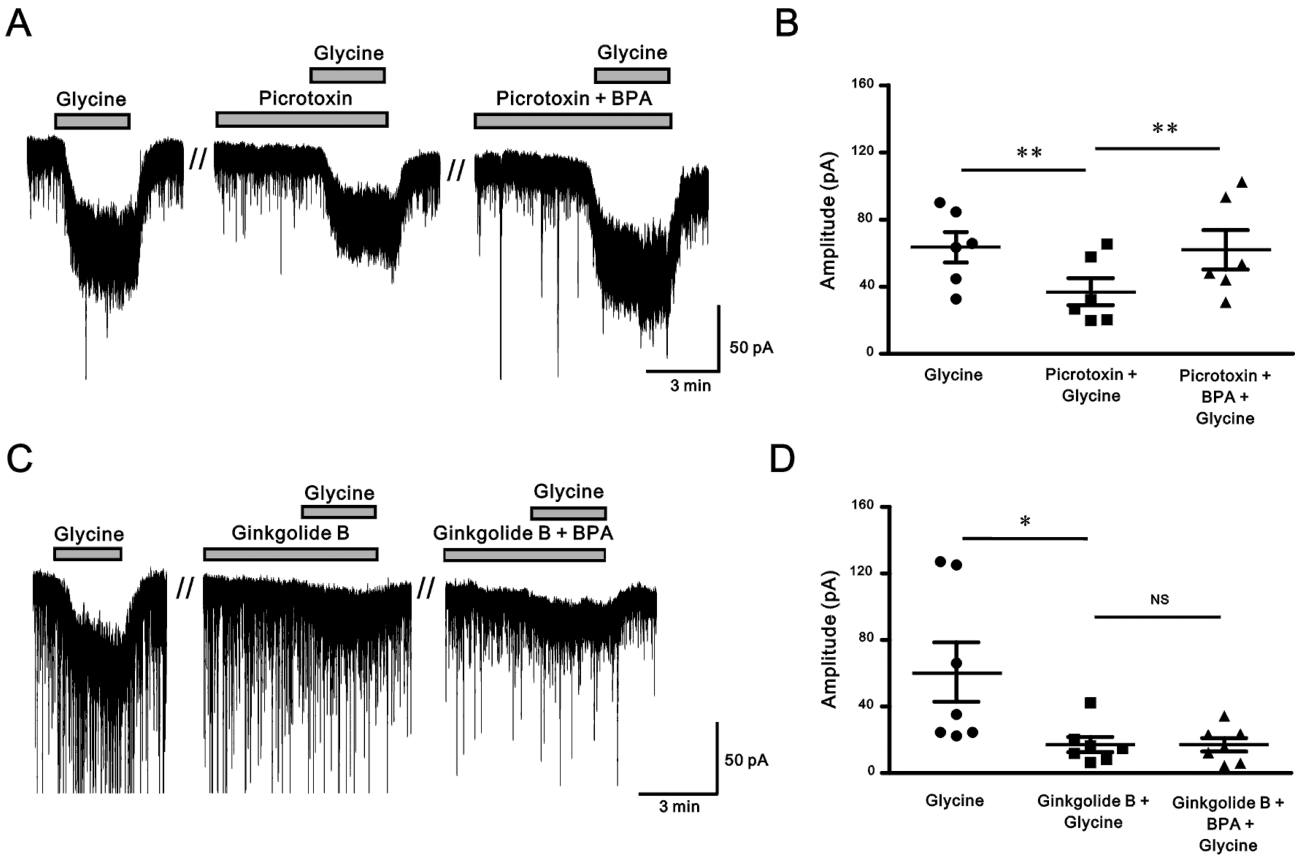

Figure 3. Subtype of glycine receptor is responsible for the potentiation of glycine-induced current (I_{Gly}) by BPA. (A) Representative trace illustrates that the I_{Gly} was blocked by picrotoxin (50 μM) and then increased in the coapplication of BPA (50 μM). (B) Dot plot showing the comparison of I_{Gly} alone, in the presence of picrotoxin and in the presence of both picrotoxin and BPA (n = 6, from five mice). (C) Glycine-induced inward current was inhibited by ginkgolide B (GB) but still not increased in the simultaneous application of BPA. (D) There is a significant difference between the mean I_{Gly} produced by glycine alone and glycine in GB, but there is no significant change between the mean I_{Gly} in GB and in the participation of both GB and BPA (n = 7, from seven mice) (* $p < 0.05$, ** $p < 0.01$, NS no significant, paired t-test) (in each dot plot, the number of the dot is number of the cell, the middle long horizontal bar is the mean value, and two shorter horizontal bars are the up and down SEM values).

transmembrane protein subunits, one β subunit and four α subunits (α1−α4). From this design, there are two ways to form different functional receptors, the homomeric configuration, including five α subunits, and the heteromeric configuration, including both α and β subunits.[34] It has been reported that picrotoxin selectively blocks homomeric glycine receptors and ginkgolide B (GB) is a powerful blocker of heteromeric receptors.[35,36] Therefore, in the next step of the experiment, we used 50 μM picrotoxin and 10 μM GB to determine which type of glycine receptor was responsible for the potentiation of I_{Gly} by BPA in the ICR juvenile mice (Figure 3).

First, we used a homomeric glycine receptor antagonist, picrotoxin. The representative trace showed that 30 μM glycine-induced inward current was blocked by 50 μM picrotoxin. After that, the unblocked I_{Gly} was still potentiated in the presence of 50 μM BPA (Figure 3A). As shown in the dot plot, the mean I_{Gly} alone was 63.5 ± 9.05 pA, then decreased to 37.1 ± 8.03 pA in the presence of picrotoxin. Later, it was increased significantly to 62 ± 11.8 pA by the coapplication of picrotoxin and BPA (n = 6, from five mice, $p < 0.01$, Figure 3B). This result indicates that the enhancement of I_{Gly} by BPA did not involve the homomeric glycine receptors.

In the next stage, the function of the heteromeric glycine receptors was analyzed by using GB (10 μM). Like the above experiments, the 30 μM glycine-induced inward current was also blocked by GB. However, as we expected, a later application of BPA did not enhance the I_{Gly} (Figure 3C). The dot plot illustrates that the mean I_{Gly} (60.7 ± 17.8 pA) was inhibited considerably in the presence of GB (17.1 ± 4.56 pA) (n = 7, from seven mice, $p < 0.05$). Nevertheless, there was no significant change between the mean I_{Gly} in GB and the coapplication of GB and BPA (17 ± 4.03) (n = 7, from seven mice, $p > 0.05$, Figure 3D). These results provide evidence that the heteromeric glycine receptors are responsible for the modulation of I_{Gly} by BPA.

■ DISCUSSION

Glycine, stored in small synaptic vesicles, is known to be the principal fast inhibitory neurotransmitter in the spinal cord.[37] The binding of glycine results in glycine receptor opening, which allows the influx of chloride ions through the cell membrane. The increase in the chloride conductance hyperpolarizes the postsynaptic cell and can inhibit the neuronal firing.[38] Many agents, such as alcohol, anesthetics, steroids, serotonin, Zn^{2+}, and tropeines, have been found to have an important role in modulating the function of glycine receptors.[33,39] BPA, a component of polycarbonate plastic utilized in food and beverage containers and in some dental material, is also an endocrine disruptor and has been

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demonstrated to be an effector of a great number of ion channels, such as voltage-gated Ca^{2+} channels, voltage-gated Na^{+} channels, large-conductance Ca^{2+}/voltage-activated K^{+} (BK) channels, nicotinic receptors, estrogen receptor, and GABA receptors.[17,31,40−43] To the best of our knowledge, this is the first report to identify a positive correlation between glycine and BPA in the SG neurons of the Vc in mice. Through electrophysiological study, we showed that BPA induced potentiation on the glycine receptor-created responses (except in the early neonatal 0−3 postnatal days) through activation of heteromeric glycine receptors.

The diversity of the glycine receptors is reflective of the complexity of the subunit structures and the complicated distribution of each subunit during brain development. In adult animals, the expression of the α1 subunits is abundant in the brain stem and the spinal cord and less in some regions of the thalamus, the hypothalamus, and the superior and inferior colliculi.[34] The α2 subunit is the most abundant isoform in the immature period (embryonic and neonatal), and in adulthood, it persists in some areas, such as the retina, the auditory brain stem nuclei, the hippocampus, the spinal cord, and layer VI of the cerebral cortex.[34,44] Like the α1 subunits, the distribution of the α3 subunits is similar, but it is lower during all developmental periods.[34,45] The expression of the α4 subunit is rare in the adult brain rat and is predominantly detected in the inner plexiform layer of the retina.[34,46] Finally, the glycine β subunits are found extensively throughout the CNS in adults. Remarkably, this kind of glycine subunit is detected in minor amounts in the embryonic period, but after birth, it increases considerably with time and persists at a high level in adults.[34,35] Changes in the isoform expression of glycine composition results in developmental changes in the function of this receptor.[47]

Neurons, in general, and SG neurons of the Vc, in particular, express both homomeric and heteromeric glycine receptors.[48] Like other regions of the CNS, the subunit composition of the glycine receptors in the spinal cord changes with age. In fetal and early neonatal cells, glycine receptors are predominantly expressed in the homomeric form. However, following neuronal development, homomeric glycine receptors are progressively replaced by heteromeric ones.[49,50] The data from our experiment gave strong evidence that the potentiation of BPA on glycine-induced inward currents was created via heteromeric glycine receptors. However, BPA failed to increase I_{Gly} in the early neonatal period of our experimental subjects. These results are consistent with the reported age-related changes in glycine subunit composition. This means that the β subunit might be required for the enhancement of the glycine response by BPA, which persists at a very low level in the early neonatal period. We found that in the juvenile group, BPA increased the I_{Gly} more than 2-fold and it was higher than that in the adult group (around 1.3-fold). This difference might have occurred because of changes in the proportion of heteromeric glycine expression between the two groups. In one study of age-related alterations in the glycine receptor in the rat hippocampus, glycine receptor α1 and β mRNA expression was the highest from 14 to 21 postnatal days and slightly decreased in 9-week-old rats. The levels of α2 and α3 were also very high in the 7- to 14-day postnatal period but decreased significantly at 9 weeks.[51] Furthermore, in the anteroventral cochlear nucleus of Fischer rats, compared to the young group, the mRNA expression of α1 and β decreased significantly in the aged group, whereas the α2 mRNA expression increased.[52] Further studies need to be conducted to evaluate changes in the glycine subunit receptor in the SG neurons at different development stages and determine which types of heteromeric glycine receptors are responsible for the enhancement of I_{Gly} by BPA.

BPA is a popular commercial chemical that has been studied in hundreds of animals and hundreds of *in vitro* mechanistic assays. The depth of BPA research is not only because of the huge production of this chemical (more than 4 million tons/year)[53] but also the ubiquitous exposure of humans to this chemical. In research conducted in the United States, BPA was detected in the urine of more than 95% of the population.[54] As a lipophilic compound, after absorption into the blood, BPA can cross the blood-brain barrier easily and accumulate in the brain and then impair the CNS function.[55,56] Therefore, it is important to evaluate the effects of BPA on the channels or receptors involved in neurotransmission in the brain. In a recent study, BPA was reported to regulate sodium currents in the dorsal root ganglion of mice by increasing the frequency of action potential firing, suggesting increased pain sensitivity.[57] Besides, BPA was also shown to potentiate the peak of the GABA-induced currents in acutely treated rat CA3 pyramidal neurons, which might influence the CNS.[31] As a cotransmitter with GABA, our experiments on the potentiation of BPA and glycine on SG neurons gave the same results, suggesting again the modulation effects of BPA on nociception.

Regarding the BPA concentration, we selected 50 μM for checking the potentiation of BPA on the glycine response. This concentration is within the range of the dosing levels of BPA used in previous studies.[17,31] Therefore, this selected concentration was considered to be suitable for the first research into the molecular mechanisms between BPA and glycine receptors. Besides, it was concluded that BPA was detectable in brain tissues. In one study of Geens et al., the mean concentration of BPA measured in the human brain was 0.91 ng/g and the highest concentration was up to 2.36 ng/g.[58] In rodent models, by feeding the pregnant mice 10 μg/mL of drinking water during their pregnancy, BPA in the brain of their 28-day-old offspring was measured to be around 20 ng/g.[59] To evaluate properly the relevant levels of BPA in brain tissues which affect human health, namely, the neurotransmitters, further investigation is needed.

The potentiation of BPA on GABA was demonstrated to involve the $GABA_A$ receptors, whose composition includes γ subunits.[30] In another study on the relationship between BPA and BK channels, the results showed that the activation of BPA on BK channels occurred through the α and β1 subunits.[43] To supplement the above abundant effects of BPA, we demonstrated another functional aspect of BPA to potentiate glycine receptors through the interaction with β subunits.

In brief, our study supplies new evidence that BPA can potentiate glycine-induced responses involving activation through the heteromeric glycine receptors. Glycine-mediated synaptic inhibition in the spinal cord, in general, and in the trigeminal subnucleus caudalis, in particular, has an essential role in processing sensory messages.[60] Therefore, glycine receptors are the main targets for many pharmacological agents involved in pain-relieving therapeutics.[61] Changes in the function of the glycine receptors affects the action of those drugs, as well as the excitability of the neurons. Exposure to BPA itself has been shown to alter the activity in the neural pathway related to the pain and nociception.[62] Taken together, the effect of BPA on the glycine receptors in the SG neurons

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provides additional knowledge about the modifiable role of BPA in the transmission of orofacial nociceptive information.

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Notes

The authors declare no competing financial interest.

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