Salicylate Induced GABA<sub>A</sub>R Internalization by Dopamine D1-Like Receptors Involving Protein Kinase C (PKC) in Spiral Ganglion Neurons

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Background: Sodium salicylate (SS) induces excitotoxicity of spiral ganglion neurons (SGNs) by inhibiting the response of γ-aminobutyric acid type A receptors (GABA<sub>A</sub>Rs). Our previous studies have shown that SS can increase the internalization of GABA<sub>A</sub>Rs on SGNs, which involves dopamine D1-like receptors (D1Rs) and related signaling pathways. In this study, we aimed to explore the role of D1Rs and their downstream molecule protein kinase C (PKC) in the process of SS inhibiting GABA<sub>A</sub>Rs.

Material/Methods: The expression of D1Rs and Gabra2 on rat cochlear SGNs cultured in vitro was tested by immunofluorescence. Then, the SGNs were exposed to SS, D1R agonist (SKF38393), D1R antagonist (SCH23390), clathrin/dy-namin-mediated endocytosis inhibitor (dynasore), and PKC inhibitor (Bisindolylmaleimide I). Western blotting and whole-cell patch clamp technique were used to assess the changes of surface and total protein of Gabra2 and GABA-activated currents.

Results: Immunofluorescence showed that D1 receptors (DRD1) were expressed on SGNs. Data from western blotting showed that SS promoted the internalization of cell surface GABA<sub>A</sub>Rs, and activating D1Rs had the same result. Inhibiting D1Rs and PKC decreased the internalization of GABA<sub>A</sub>Rs. Meanwhile, the phosphorylation level of Gabra2 S327 affected by PKC was positively correlated with the degree of internalization of GABA<sub>A</sub>Rs. Moreover, whole-cell patch clamp recording showed that inhibition of D1Rs or co-inhibition of D1Rs and PKC attenuated the inhibitory effect of SS on GABA-activated currents.

Conclusions: D1Rs mediate the GABA<sub>A</sub>R internalization induced by SS via a PKC-dependent manner and participate in the excitotoxic process of SGNs.

Keywords: Gabra2 Protein, Rat • Ototoxicity • Endocytosis • Phosphorylation • Receptors, Dopamine • Sodium Salicylate

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Background

Sodium salicylate (SS) is the active ingredient of aspirin, which can cause temporary hearing loss and tinnitus. Studies have shown that SS has a direct effect on auditory neurons from the periphery to the center, including neurons in the cochlear spiral ganglion [1], dorsal cochlear nucleus [2,3], inferior colliculus [4], medial geniculate body [5], and auditory cortex [6], and spiral ganglion neurons (SGNs) have been considered to be the primary targets of SS [1]. The damage of SS to SGNs is due to excitotoxicity, the mechanism of which involves the excitatory-inhibitory imbalance caused by the increased excitatory response mediated by N-methyl-D-aspartate receptors (NMDARs) and the reduced inhibitory response mediated by γ-aminobutyric acid type A receptors (GABA Rs). For example, SS raised the expression of NMDARs [7] and potentiated the NMDAR currents of SGNs [8,9]. On the other hand, SS lessened the surface expression of GABA R on SGNs [10,11] and reversibly inhibited the GABA (GABA R agonist)-activated currents [10]. Our previous studies have shown that inhibition of NMDARs decreased the suppressive effect of SS on GABA Rs, indicating that the interaction between NMDARs and GABA Rs also leads to a weakened GABA R response [10]. However, it is not fully understood how the downstream signaling effects of SS induce changes in GABA R function.

D1Rs have been detected on SGNs [11,12]. SS can promote the mRNA expression of D1Rs in SGNs [13]. Other research has shown that D1Rs can interact with GABA Rs and reduce the GABA-evoked currents in the middle spinachous neurons of the neostriatum [14]. Our previous studies have reported that SS can decrease the surface expression of the GABA R α2 subunit of SGNs, and inhibition of D1Rs blocks this effect, indicating that D1Rs mediates the effect of SS on GABA Rs [11]. However, how D1Rs mediate the inhibitory effect of SS on GABA Rs remains to be elucidated.

Our previous studies have found that SS can decrease the function of GABA Rs by increasing the GABA Rs endocytosis on SGNs [10,11]. Similar to the endocytosis of other receptors, GABA R endocytosis occurs primarily via a clathrin/dynamin-dependent pathway, which is mainly regulated by the phosphorylation of GABA R β and γ2 subunits [15].

In the cerebral cortex, the majority of functional GABA R subtypes contain the γ2 subunit [18]. The γ2 subunit is essential for the postsynaptic clustering of GABA Rs [19]. The main phosphorylation site of γ2 S/L is S327, which is regulated by PKC and calcineurin [16]. The signaling cascades activated by D1Rs involve PKA, PKC, and calcium/calmodulin-dependent protein (CaMKII) [17]. It was reported that D1Rs in guinea pig cochlea increased the glutamate receptor 1 (Glur1) phosphorylation via PKA-dependent signaling, but beyond PKA there may be other pathways involved, such as PKC and CaMKII [20]. Valdés-Baizabal et al found that D1- and D2-like receptors modulated voltage-gated sodium current by PKA and PKC pathways, respectively [21]. In neostriatal neurons, D1Rs activation reduces GABA R currents through PKA-mediated signaling [14]. However, very little information is available on the roles of PKC pathways in the regulation of GABA Rs by D1Rs in SGNs.

Hence, we propose that D1Rs regulate the GABA R internalization through PKC-mediated phosphorylation to mediate the effects of SS in SGNs. In the present work, western blotting was used to examine the effect of SS on the expression of GABAR2 when activating or inhibiting D1Rs, inhibiting receptor endocytosis, or inhibiting PKC. Whole-cell patch clamp was used to detect the effect of SS on GABA response after inhibiting D1Rs or PKC, to further clarify the possible interaction between D1Rs and GABA Rs in the mechanism of SS-induced ototoxicity to SGNs.

Material and Methods

Primary Culture of SGNs

SGN cultures were obtained from the cochleae of Sprague-Dawley rats that were 3-5 days old and of both sexes. Briefly, rats were decapitated, then the modioluses were quickly removed from the cochleae in 0°C Hank’s balanced salt solution (HBSS) under a microscope (Olympus, Japan) to obtain SGN-containing tissues. The tissues were then torn into small pieces and incubated in 0.25% trypsin-EDTA (Gibco, USA) at 37°C for 10 min. The SGNs pellet was obtained following 5-min centrifugation at 1000 rpm. The supernatant was removed, then the pellet was resuspended and gently triturated in Neurobasal medium (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 2% B27 (Sigma, USA). Cells were plated on 35-mm culture dishes coated with poly-D-lysine and maintained in a humidified incubator (Thermo, USA) at 37°C with 5% CO₂.

Cell Treatment

After 48 h of culture, SGNs were exposed to several groups of drugs for the western blotting assay (as listed in Table 1).

The doses chosen were based on our previous studies and the literature. In the groups containing dynasore or Bis I, SGNs were pretreated with these 2 drugs for 30 min, then each group of drugs was applied to incubate the SGNs for 1 h.

Immunofluorescence

SGNs cultured for 48 h were fixed in 4% paraformaldehyde at room temperature for 20 min, then washed with phosphate.
buffered Bsa1ne (PBS) and permeabilized with 0.2% Triton X-100 for 10 min. After blocking by 5% goat serum for 30 min, SGNs were incubated with primary antibodies overnight at 4°C: mouse or rabbit anti-βIII-tubulin (a neuronal marker) antibodies (1: 100, Abcam, USA), rabbit anti-dopamine D1 receptors (DRD1) antibody (1: 100, Abbcom, USA), and mouse anti-GABAR2 antibody (1: 100, Millipore, USA). The secondary antibody was a fluorescently labeled secondary antibody (1: 500, EarthOx, USA) in the dark at room temperature. Bands were visualized with an Odyssey infrared scanner (LI-COR, USA) and analyzed via Image J software. Flotillin-1 and βII-tubulin were used as the internal reference for surface protein and total protein, respectively. Relative expression of target protein=gray value of the target band/gray value of internal reference band ×100%. The data are presented as % of control.

### Western Blot Analysis

Surface proteins were extracted using a Mem-PER™ Plus Membrane Protein Extraction Kit (Thermo, USA). Total proteins were obtained with cells homogenized in ice-cold RIPA buffer plus protease inhibitor and phosphatase inhibitor for 30 min. The protein concentration was determined using a BCA Protein Concentration Assay Kit (Thermo, USA). Protein samples (60 μg) were loaded in 10% SDS-PAGE gels and transferred onto the polyvinylidene fluoride (PVDF) membranes. After blocking with 5% BSA for 1.5 h, membranes were incubated with primary antibodies (1: 1000 dilution): rabbit anti-flotillin-1 antibody (Affinity Biosciences, USA), rabbit anti-βII-tubulin antibody (Abcam, USA), rabbit anti-GABAR2 antibody (Abcam, USA), and rabbit anti-GABAR2 p-S327 antibody (Thermo, USA) at 4°C overnight. After washing 3 times for 5 min each with TBST, the membranes were incubated with fluorescently labeled secondary antibody (1: 500, EarthOx, USA) in the dark at room temperature. Bands were visualized with an Odyssey infrared scanner (LI-COR, USA) and analyzed via Image J software. Flotillin-1 and βII-tubulin were used as the internal reference for surface protein and total protein, respectively. Relative expression of target protein=gray value of the target band/gray value of internal reference band ×100%. The data are presented as % of control.

### Table 1. Drug treatment groups.

| Inhibiting endocytosis | Inhibiting PKC |
|------------------------|----------------|
| Ctrl                   | Ctrl           |
| SKF                    | SKF            |
| SKF+SCH                | SKF+SCH        |
| Dynasore               | Bis I          |
| SKF+Dynasore           | SKF+Bis I      |
| SKF+SCH+Dynasore       | SKF+SCH+Bis I  |
| SS                     | SS             |
| SS+SKF                 | SS+SKF         |
| SS+Dynasore            | SS+Bis I       |
| SS+SKF+Dynasore        | SS+SKF+Bis I   |
| SS+SKF+SCH+Dynasore    | SS+SKF+SCH+Bis I |

Ctrl = control; SKF = SKF38393, D1R agonist, 20 µM, MCE, USA; SCH = SCH23390, D1R antagonist, 20 µM, MCE, USA; Dynasore = clathrin/dynamin-mediated endocytosis inhibitor, 80 µM, Sigma, USA; SS = 5 mM, Sigma, USA; Bis I = Bisindolylmaleimide I, selective PKC antagonist, 4 μM, MCE, USA.

#### Statistical Analysis

All statistical analyses were performed by SPSS 25.0 software. Data are presented as mean±standard deviation (SD). For normally distributed data, one-way ANOVA was employed for comparison between groups, and the pairwise comparison was performed with the Tukey test. P<0.05 was considered statistically significant.

### Results

#### Expression of DRD1 and GABAR2 on SGNs

After primary culture for 48 h, SGNs were round or elliptical with a surrounding halo, high refraction, and long bipolar axons. SGNs were stained with primary antibodies against...
βIII-tubulin, DRD1 (Figure 1A), and GABARγ2 (Figure 1B). The merged images (overlap is shown in yellow) revealed that DRD1 and GABARγ2 were expressed on SGNs.

Downregulation of GABA_A R Surface Expression Induced by D1R Agonist and/or SS Was Completely Prevented by Endocytosis Inhibitor

Exposure to D1R agonist SKF38393 (20 µM) for 1 h significantly reduced the surface GABARγ2 to 48.57% of control (P<0.05, compared with the control group, Figure 2A) but did not change the total GABARγ2, indicating that D1R agonist increased the internalization of GABA_A Rs. The surface GABARγ2 in SKF38393+SCH23390 (D1R antagonist, 20 µM) group showed no difference compared with the control group (P>0.05, Figure 2A), indicating that SKF38393-induced inhibition on surface GABA_A R expression was specific to the D1Rs.

Treatment with 5 mM SS or SS+SKF38393 for 1 h significantly reduced the surface GABARγ2 levels to 47.15% and 44.76% of control, respectively (P<0.05, compared with the control group, Figure 2B), and the total GABARγ2 levels were unchanged (P>0.05, compared with the control group, Figure 2B), indicating that inactivating the D1Rs pathway partially reversed the internalization of GABA_A Rs and D1Rs positively mediated the inhibitory effect of SS on surface GABA_A Rs.

To confirm that the decrease of surface GABARγ2 caused by SS or D1R agonist was indeed due to increased GABA_A R internalization, clathrin/dynamin-mediated endocytosis inhibitor dynasore was used to block receptor endocytosis [22]. Administration of 80 µM dynasore alone for 1 h did not affect the surface and total protein levels of GABARγ2 (P>0.05, Figure 2A). However, in the presence of dynasore, SS and/or SKF38393 no longer decreased the surface levels of GABARγ2 as compared with the control group (P>0.05, Figure 2A, 2B), proving that the decreased GABARγ2 surface expression was due to increased GABA_A R internalization. All the data are showed in the Supplementary Table 1.

PKC Antagonist Partially Blocked D1R Agonist- and/or SS-induced GABA_A R Internalization

D1R activation can trigger PKC [17]. PKC can regulate the internalization of GABA_A Rs by affecting the phosphorylation of the GABA_A γ2 subunit [16]. To examined whether PKC plays a role in D1R-mediated GABA_A R internalization induced by SS, SGNs were treated with the cell-permeable PKC antagonist Bis I (4 µM) to inhibit PKC. The results showed that Bis I alone did not influence surface or total protein levels of GABARγ2 (P>0.05, Figure 3A). In the SKF38393+Bis I group, the surface group, Figure 2B), higher than the SS group (P<0.05), and the total GABARγ2 levels were unchanged (P>0.05, compared with the control group, Figure 2B), indicating that inactivating the D1Rs pathway partially reversed the internalization of GABA_A Rs and D1Rs positively mediated the inhibitory effect of SS on surface GABA_A Rs.

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Figure 2. GABARγ2 protein expression in the absence or presence of endocytosis inhibitor (dynasore, Dyn). (A) The surface expression of GABARγ2 (normalized to flotillin-1) was significantly decreased by SKF as compared with the control group, and this effect was completely reversed by SCH and Dyn. The total protein expression of GABARγ2 (normalized to βIII-tubulin) in these groups showed no significant difference. (B) The surface expression of GABARγ2 was significantly decreased by SS and SS+SKF as compared with the control group, which was partially reversed by SCH and completely reversed by Dyn. The total protein expression of GABARγ2 in these groups was not significantly different. Data are presented as mean±SD. All experiments n=4, * P<0.05, vs the control group, # P<0.05, vs the SKF group, & P<0.05 vs the SS group, by one-way ANOVA and Tukey test. Odyssey 3.0.23 (LI-COR, USA) and SPSS 25.0 (USA) were used for the creation of the figures.
Figure 3. Expression of GABARγ2 proteins before and after PKC inhibitor application. (A) The inhibitory effect of SKF on the surface expression of GABARγ2 was partially reversed by Bis I. Quantitative analysis of GABARγ2 total protein expression showed no considerable difference in these groups. (B) The inhibitory effect of SS, SS+SKF on the surface levels of GABARγ2 was partially reversed by SCH and Bis I. GABARγ2 total protein expression showed no significant difference in these groups. Data are presented as mean±SD. All experiments n=4, * P<0.05, vs the control group, # P<0.05, vs the SKF group, & P<0.05 vs the SS group, by one-way ANOVA and Tukey test. Odyssey 3.0.23 (LI-COR, USA) and SPSS 25.0 (USA) were used for the creation of the figures.
GABARγ2 was reversed to 70.81% of control, significantly higher than in the SKF38393 group (P<0.05, Figure 3A), suggesting that activation of D1Rs increased GABA_R internalization in a PKC-dependent manner. No significant difference was shown in the surface GABARγ2 expression in the SKF38393+SCH23390+Bis I group as compared to the control group (P>0.05, Figure 3A). As shown in Figure 3B, the surface levels of GABARγ2 in the SS+Bis I and SS+SKF38393+Bis I groups were reversed to 60.32% and 60.48% of control, respectively, significantly higher than in the SS group (P<0.05), indicating that blockade of PKC partially reversed the GABA_R internalization induced by SS or SS+SKF38393, thus further illustrating that the D1R-mediated promotion of GABA_R endocytosis induced by SS was PKC-dependent. Moreover, there was no significant difference in surface GABARγ2 expression between the SS+SKF38393+SCH23390+Bis I group and the control group (P>0.05, Figure 3B), suggesting that the suppression of surface GABARγ2 expression by SS was completely abolished under simultaneous inhibition of D1Rs and PKC. All the data are showed in the Supplementary Table 2.

SS Raised PKC-Mediated Phosphorylation at γ2 S327 by D1Rs

The GABARγ2 subunit has been shown to be phosphorylated by PKC at S327 [16]. Thus, we wanted to determine whether S327 was involved in D1R-mediated GABA_R internalization.

Administration of SKF38393 significantly increased the phosphorylation level of S327 to 174.49% of control (P<0.05) compared with the control group (Figure 4A). Treatment with SCH23390 together with SKF38393 completely abolished the effect of SKF38393 on S327 (P>0.05) compared with the control group (Figure 4A). Bis I alone did not affect the p-S327 (P>0.05, compared with the control group (Figure 4A), but treatment with SKF38393+Bis I significantly reduced the phosphorylation level of S327 to 142.12% of the control (P<0.05) compared with the SKF38393 group (Figure 4A), suggesting that inhibiting PKC partially reversed the effect of SKF38393 on p-S327. There was no significant difference in phosphorylation level of S327 between the SKF38393+SCH23390+Bis I group and the control group (P>0.05, Figure 4A).
Exposure of SGNs to SS significantly increased the phosphorylation level of S327 to 185.48% of control (P<0.05) compared with the control group (Figure 4B). SS+SKF38393 also significantly raised the phosphorylation level of S327 to 193.28% of control (P<0.05) compared with the control group (Figure 4B).

The level of p-S327 in the SS+SKF38393+SCH23390 group was 141.82% that of control, which was lower than in the SS group (P<0.05, Figure 4B), suggesting that the effect of SS on p-S327 was partially reversed by inactivating D1Rs, and D1Rs positively mediated the phosphorylation effect of SS on S327. Pairwise comparison showed no significant difference in the phosphorylation level of S327 in the SS and SS+SKF38393 groups, indicating that co-application of SS and D1R agonist had no additive phosphorylation effect on S327 (P>0.05, Figure 4B). Moreover, the p-S327 levels in the SS+Bis I and SS+SKF38393+Bis I groups were 135.70% and 139.59%, respectively, which were significantly lower than in the SS group (P<0.05, Figure 4B), suggesting that the phosphorylation of S327 induced by SS or SS+SKF38393 was partially blocked by inhibition of PKC. These results demonstrated that SS induced phosphorylation at S327 through D1Rs involving the PKC pathway. Additionally, the phosphorylation level of S327 in the SS+SKF38393+SCH23390+Bis I group was not significantly different compared with the control group (P>0.05), indicating that co-inhibition of D1Rs and PKC completely abolished the phosphorylation at S327 induced by SS (Figure 4B).

All the data are showed in the Supplementary Table 3.
Inhibition of D1Rs or PKC Attenuated the Suppression of GABA-Activated Currents by SS

Inward currents induced by GABA (500 µM) were recorded on SGNs (Figure 5A). As shown in Figure 5A and 5B, SKF38393 significantly reduced GABA currents by 67.56% as compared to the control group (500 µM GABA) (P<0.05). SS also significantly decreased the GABA-activated currents by 69.57% as compared to the control group (P<0.05). There was no statistically significant difference between the inhibition of SKF38393 and SS on GABA currents (P>0.05), which was in line with the effect of SKF38393 and SS on the surface GABA_R expression. To observe the effect of SS on GABA currents when antagonizing D1Rs, SS+SCH23390 was applied, which decreased the GABA currents by 38.77% as compared to the control group (P<0.05), and blockade of D1Rs significantly reversed the inhibitory effect of SS on GABA response by 30.80% (P<0.05) compared to the SS group. To determine if the depression of GABA currents by SKF38393 was specific to the D1Rs, SCH23390 was applied to abolish the effect of SKF38393, decreasing GABA-evoked currents induced by SS by 45.50% as compared to the control group (P<0.05), and the inhibition of SS on GABA currents was reversed by 24.07% (P<0.05) compared to the SS group. However, there was no significant difference between the currents induced by SS+SCH23390 and SS+SKF38393+SCH23390 (P>0.05). With Bis I in the intracellular solution, SS+SKF38393+SCH23390 reduced the GABA currents by 35.86% as compared to the control group (P<0.05), and co-inactivating D1Rs and PKC further reversed the inhibitory effect of SS on GABA response by 33.71% (P<0.05) compared to the SS group. These data suggest that D1Rs and PKC were involved in the process of SS suppressing GABA-activated currents and supported the results of western blotting.

Discussion

SS can cause neuronal excitotoxicity by inhibiting GABA_R function [10,23], but the molecular mechanism is not fully understood. Research showed that the cochlear response of the D1 knockout mice was enhanced, and the auditory brainstem response (ABR) threshold of all frequencies was increased by 5-20 dB [12], indicating that D1Rs play an important role in auditory function. Our previous studies have shown that D1R antagonists can weaken the effect of SS on GABA_R expression in SGNs, suggesting that D1Rs can mediate the effect of SS on GABA_Rs by interacting with GABA_Rs [11,13]. In this work, the expression of DRO1 on SGNs was detected by immunofluorescence, which was in agreement with the reports of Maison et al [12], Wei et al [11], and Inoue et al [24].

SS Promotes the Internalization of GABA_Rs Through D1Rs

There have been few studies on the mechanism by which SS or D1R activation causes the decrease of cell surface GABA_R expression. For example, SS might reduce GABA function through direct binding to GABA_Rs, but the site of action was not clear [25]. In the present study, we observed that stimulation of D1Rs downregulated the surface expression of GABA_R2 but did not influence the total GABA_R2 protein, suggesting that D1Rs activation can increase GABA_R internalization. SS also decreased the surface levels of GABA_R2 without affecting the total GABA_R2, which was in line with reports about the influence of SS on α2 and β3 subunits [10,11], suggesting that the internalization of GABA_Rs might be increased. According to the results of western blotting and electrophysiology experiments, after inactivating D1Rs, the suppression of surface GABA_R expression and GABA-activated currents by SS was weakened, suggesting that dopaminergic signaling plays a role in the mechanism by which SS inhibits GABA_Rs.

Our previous studies have shown that the interaction between NMDARs and GABA_Rs mediates the inhibitory effect of SS on GABA_Rs [10]. In this work, we found there was also an interaction between D1Rs and GABA_Rs, which was involved in the SS-induced inhibition of GABA_Rs.

We further observed that blocking endocytosis with dynasore completely abolished the suppressive effects of D1R activation on surface GABA_R expression, demonstrating that D1R activation promoted the GABA_R internalization via a clathrin-dynamin-dependent pathway. Dynasore treatment also abolished the inhibition of surface GABA_R expression by SS, demonstrating that SS eventually led to increased internalization of GABA_Rs. Because D1Rs were involved in the pathway of SS regulating GABA_Rs, it can be inferred that SS increased the internalization of GABA_Rs via dopaminergic signaling. Few previous studies have mentioned the mechanism of SS or D1R activation affecting the trafficking of GABA_Rs. Chen et al found that D3 receptor activation in the nucleus accumbens increased GABA_R internalization through a clathrin-dependent pathway [26]. Graziane et al showed that D4 receptors inhibited GABA_R function by preventing GABA_R externalization through the actin/cofilin/myosin pathway [27]. Our work reported for the first time that SS and D1Rs regulate GABA_R internalization through a clathrin/dynamin-mediated mechanism in SGNs.

There have been several studies revealing the time course of GABA_R endocytosis. For example, brain-derived neurotrophic factor (BDNF) [28], Mg²⁺-free condition [29], and diazepam [30] can trigger GABA_R internalization at between 15 and 60 min. Our present data from western blotting revealed that GABA_Rs on the surface of SGNs were significantly internalized after...
treatment with SS or D1R agonist for 1 h, and the time course observed was consistent with other findings.

**D1Rs-Mediated GABA<sub>R</sub> Internalization is Modulated by PKC**

GABA<sub>R</sub> internalization is regulated by several protein kinases, including PKA, PKC, calmodulin-dependent protein kinase II (CaMKII), Fyn, and Src [31]. Research has shown that stimulation of D1Rs can activate PKC [17]. PKC activation can induce GABA<sub>R</sub> internalization [32] and inhibit GABA<sub>R</sub> function by reducing GABA currents [33,34]. Therefore, we observed the changes in GABA<sub>R</sub> internalization after inhibiting PKC. Our data from western blotting shown that the reduction of surface GABA<sub>R</sub> caused by D1R activation was partially reversed by PKC inhibition, indicating that in SGNs, D1Rs activation triggered a signaling pathway that concluded with PKC activation, which could modulate GABA<sub>R</sub>. Inhibition of PKC also attenuated SS-induced GABA<sub>R</sub> internalization, suggesting that the mechanism by which D1Rs mediate the effect of SS on GABAs involves the PKC signaling pathway. A previous study reported that inhibition of PKC partially blocked the ethanol-induced GABA<sub>R</sub> internalization in cultured cerebral cortical neurons [35]. In hippocampus neurons, BDNF-induced α1-GABA<sub>R</sub> internalization was completely disrupted by PKC inhibition [36]. Moreover, in our study, inhibition of PKC only partially blocked GABA<sub>R</sub> internalization induced by SS or D1R activator, suggesting that there might be other mechanisms of cellular signaling downstream of D1R activation mediating the SS-induced GABA<sub>R</sub> internalization in SGNs. For example, D1 receptors in the striatum decreased the GABA<sub>R</sub> response through PKA-dependent regulation [14]. SS might increase the GABA<sub>R</sub> internalization via CaMKII [10]. Therefore, the failure of PKC inhibition to completely block the D1R-mediated internalization of GABA<sub>R</sub> in the present study may be due to the regulation of multiple signaling pathways by D1Rs.

Moreover, our data from whole-cell patch clamp recording showed that compared with inhibiting D1Rs, PKC inhibitor further reversed the suppressive effect of SS on GABA response, also supporting the view that PKC is involved in SS-induced GABA<sub>R</sub> internalization, and inhibition of PKC enhanced the GABA<sub>R</sub> function. Brandon et al found that inhibition of PKC markedly increased GABA currents in A293 cells and Xenopus oocytes transfected with GABA<sub>R</sub> [33]. In prefrontal cortical neurons, PKC inhibitor blocked the inhibitory effect of (-)-2,5-dimethoxy-4-iodoamphetamine on GABA-activated currents [37]. In HEK293 cells, PKC blockade abolished the suppression of Orexin-A on GABA response [38]. Taken together, these data suggest that PKC activity is negatively correlated with GABA<sub>R</sub> function.

**The Subject of SS Regulation Mediated by D1Rs is γ2 S327**

PKC phosphorylation sites on GABA<sub>R</sub> include β3 S408/409 and γ2 S327 [16]. We observed that the D1R agonist markedly increased the phosphorylation level of γ2 S327, which was partially reversed by inhibiting PKC, indicating that D1R activation increased the PKC-mediated phosphorylation at S327. The SS-induced enhancement of phosphorylation level of S327 was partially blocked by inactivating D1Rs or inhibiting PKC, further proving that D1R activation mediated the effect of SS by affecting the PKC-dependent phosphorylation at S327. So, it can be speculated that SS caused the interaction between D1Rs and GABA<sub>R</sub>, thus increasing the phosphorylation of 327 by the PKC pathway, thereby promoting the internalization of GABA<sub>R</sub>. As observed in other research, inhibition of CaMKII blocked the phosphorylation of β3 S383 and decreased the SS-induced GABA<sub>R</sub> internalization in SGNs [10], and inhibition of PKC in embryonic rat cortical neurons prevented the phosphorylation of β3 S408/409 and γ2L S327/343, increasing the amplitude of GABA currents [33]. These results all support our finding that the phosphorylation level of GABA<sub>R</sub> was positively correlated with the degree of GABA<sub>R</sub> internalization and negatively correlated with cell surface GABA<sub>R</sub> expression and function.

However, some studies produced conflicting results. Kittler et al revealed that in cortical neurons, the dephosphorylated γ2 subunits bind to the μ2 subunit of AP2 (AP2-μ2), causing GABA<sub>R</sub> to endocytose and reducing their surface expression, while the increased phosphorylation of related sites prevents the binding of γ2 subunits to AP2-μ2, blocking receptor endocytosis and increasing the GABA<sub>R</sub> surface levels [39]. Potentiation of GABA responses by tetrahydro-deoxycorticosterone was reduced after inhibiting PKC in HEK293 cells [40]. Diazepam dephosphorylated the γ2 S327 by activating calci-neurin in rat cortical neurons, which led to increased GABA<sub>R</sub>.
internalization [30]. These results show that the regulation of GABA\(_R\)s by PKC was not completely the same, and the phosphorylation at S327 also produced a different effect on GABA\(_R\) surface expression and function. The reasons for this may include the following: (1) GABA\(_R\)_s composed of different subunits have functional heterogeneity and lead to different phosphorylation effects [41]; and (2) PKC has a selective effect on neurons [42,43]. However, the exact cause remains to be further explored.

## Conclusions

We demonstrate that SS increases the PKC-mediated phosphorylation at \(\gamma\)2 S327 through D1Rs, thus triggering GABA\(_R\) internalization through a clathrin/dynamin-dependent endocytosis pathway and resulting in suppressed GABA\(_R\) surface levels, eventually leading to reduced GABA\(_R\)-mediated inhibition (Figure 6). Our discovery is meaningful for understanding how tinnitus develops and the possible role of dopaminergic signaling in the generation or modulation of tinnitus. However, since the inhibitory effect of SS on GABA\(_R\) does not occur exclusively through the mediation of D1Rs and PKC, more pathways need to be explored in further studies.

## Declaration of Figures’ Authenticity

All figures submitted have been created by the authors, who confirm that the images are original with no duplication and have not been previously published in whole or in part.

## Supplementary Tables

### Supplementary Table 1. Data of Figure 2, GABAR\(\gamma\)2 protein expression in the absence or presence of dynasore.

| Groups           | Surface GABAR\(\gamma\)2 (mean±SD) | Surface Flotillin-1 (mean±SD) | Total GABAR\(\gamma\)2 (mean±SD) | Total \(\beta\)III-tubulin (mean±SD) | Relative expression as % of control (mean±SD) |
|------------------|-----------------------------------|-------------------------------|-----------------------------------|-------------------------------------|-----------------------------------------------|
| Ctrl             | 138.47±29.11                      | 196.57±11.34                 | 100.95±13.51                     | 197.86±4.28                        | 100±0.00                                      |
| SKF              | 72.09±19.13                       | 209.35±10.51                 | 101.34±5.24                      | 200.21±20.37                       | 48.57±7.12                                    |
| SKF+SCH          | 141.36±21.36                      | 194.75±5.37                  | 102.69±9.03                      | 206.96±10.07                       | 103.79±5.69                                   |
| Dynasore         | 139.43±15.05                      | 199.91±7.64                  | 107.51±9.91                      | 206.27±15.85                       | 99.77±13.03                                   |
| SKF+Dynasore     | 123.07±22.19                      | 186.04±14.32                 | 116.57±6.47                      | 199.87±14.63                       | 94.44±7.24                                   |
| SKF+SCH+Dynasore | 125.56±7.11                       | 188.29±17.55                 | 107.77±8.66                      | 208.61±11.38                       | 94.57±13.83                                   |
| Ctrl             | 103.04±17.51                      | 112.93±11.65                 | 119.81±21.26                     | 159.16±33.16                       | 100±0.00                                      |
| SS               | 52.76±20.82                       | 121.77±12.96                 | 126.28±10.11                     | 131.89±34.01                       | 47.15±11.11                                   |
| SS+SKF           | 54.89±19.24                       | 135.92±18.99                 | 119.76±7.12                      | 136.72±36.37                       | 44.76±7.18                                    |
| SS+SKF+SCH       | 101.31±16.04                      | 146.16±22.45                 | 120.72±24.93                     | 134.21±36.61                       | 76.56±1.43                                    |
| SS+Dynasore      | 127.41±24.80                      | 122.01±21.27                 | 106.53±16.60                     | 130.01±30.71                       | 115.19±22.57                                  |
| SS+SKF+Dynasore  | 127.53±21.77                      | 123.06±32.65                 | 117.21±7.79                      | 140.48±39.40                       | 114.30±7.02                                  |
| SS+SKF+SCH+Dynasore | 136.35±14.02               | 130.11±28.63                 | 105.01±11.70                     | 131.24±37.08                       | 115.52±9.36                                  |
Supplementary Table 2. Data of Figure 3, GABAR\(\alpha2\) protein expression in the absence or presence of Bis I.

| Groups         | Density (mean±SD) | Relative expression as % of control (mean±SD) |
|----------------|-------------------|-----------------------------------------------|
|                | Surface GABAR\(\alpha2\) | Surface Flotillin-1 | Total GABAR\(\alpha2\) | Total βIII-tubulin | Surface GABAR\(\alpha2\) | Total GABAR\(\alpha2\) |
| Ctrl           | 112.98±11.30       | 161.74±20.64       | 97.21±8.68               | 203.93±23.46       | 100±0.00                   | 100±0.00                   |
| SKF            | 44.52±10.28        | 167.23±24.58       | 87.09±14.43              | 199.78±14.07       | 37.80±4.93                 | 91.58±2.72                 |
| SKF+SCH        | 103.46±13.36       | 162.63±22.54       | 87.56±20.05              | 192.37±11.39       | 91.43±13.03                | 95.01±10.76                |
| Bis I          | 94.45±7.95         | 157.94±22.50       | 90.96±19.57              | 192.76±9.11        | 85.61±3.17                 | 98.08±17.81                |
| SKF+Bis I      | 75.75±6.36         | 151.41±11.66       | 91.72±21.93              | 198.74±5.85        | 70.81±6.81                 | 96.22±4.95                 |
| SKF+SCH+Bis I  | 98.24±3.38         | 146.92±8.57        | 97.84±14.15              | 201.31±19.52       | 96.17±16.48                | 101.81±7.07                |
| Ctrl           | 119.83±11.36       | 173.48±26.59       | 96.25±6.55               | 186.92±14.93       | 100±0.00                   | 100±0.00                   |
| SS             | 34.02±5.21         | 204.94±26.63       | 85.39±6.59               | 191.88±3.13        | 23.94±3.11                 | 86.26±15.32                |
| SS+SKF         | 28.16±16.97        | 193.17±10.75       | 84.08±3.66               | 186.42±12.18       | 21.16±7.81                 | 87.40±18.54                |
| SS+SKF+SCH     | 95.78±4.22         | 200.90±26.48       | 85.32±3.34               | 181.09±10.15       | 69.20±8.91                 | 91.47±22.03                |
| SS+Bis I       | 83.56±5.94         | 199.48±24.65       | 87.11±4.61               | 186.47±1.82        | 60.32±8.30                 | 90.77±13.90                |
| SS+SKF+Bis I   | 82.89±5.87         | 198.91±23.94       | 94.89±13.26              | 182.13±24.46       | 60.48±23.36                | 101.21±27.66               |
| SS+SKF+SCH+Bis I | 118.98±15.05       | 196.13±17.81       | 102.71±11.84             | 178.71±16.77       | 88.26±22.59                | 111.68±22.99               |

Supplementary Table 3. Data of Figure 4, GABAR\(\alpha2\) p-S327 protein expression in the absence or presence of Bis I.

| Groups         | Density (mean±SD) | Relative expression as % of control (mean±SD) |
|----------------|-------------------|-----------------------------------------------|
|                | GABAR\(\alpha2\) p-S327 | Total βIII-tubulin | GABAR\(\alpha2\) p-S327 |
| Ctrl           | 122.49±29.52      | 203.93±23.46       | 100±0.00                   |
| SKF            | 209.16±20.65      | 199.78±14.07       | 174.49±19.42               |
| SKF+SCH        | 128.83±18.51      | 192.37±11.39       | 111.57±10.49               |
| Bis I          | 102.42±29.59      | 192.76±9.11        | 88.58±15.32                |
| SKF+Bis I      | 109.24±11.63      | 186.47±1.82        | 135.70±31.09               |
| SKF+SCH+Bis I  | 145.26±20.81      | 201.31±19.52       | 120.14±16.92               |
1. Wei L, Ding D, Salvi R. Salicylate-induced degeneration of cochlear spiral ganglion neurons-apoptosis signaling. Neuroscience. 2010;168(1):286-99

2. Zugaza J, Ceballos CC, Leão RM. High doses of salicylate reduces glycineergic inhibition in the dorsal cochlear nucleus of the rat. Hear Res. 2016;332:188-98

3. Zugaza J, Leão RM. Enhancement of endocannabinoid-dependent depolarization induced suppression of excitation in glycineergic neurons by prolonged exposure to high doses of salicylate. Neuroscience. 2018;376:72-79

4. Wang HT, Luo B, Huang YN, et al. Sodium salicylate suppresses serotonin-induced enhancement of GABAergic spontaneous inhibitory postsynaptic currents in rat inferior colliculus in vitro. Hear Res. 2008;236(1-2):42-51

5. Su YY, Luo B, Jin Y et al. Altered neuronal intrinsic properties and reduced synaptic transmission of the rat’s medial geniculate body in salicylate-induced tinnitus. PLoS One. 2012;7(10):e46969

6. Wang HT, Luo B, Zhou KQ, et al. Sodium salicylate reduces inhibitory postsynaptic currents in neurons of rat auditory cortex. Hear Res. 2012;292(1-2):77-83

7. Gao M, Fang XY, Feng S, et al. Salicylate enhances expression and function of NMDA receptors in cochlear spiral ganglion neurons. Journal of Otolaryngology. 2012(1):9-14

8. Ruel J, Chabbert C, Nouvian R, et al. Salicylate enables cochlear arachidonic-acid-sensitive NMDA receptor responses. J Neurosci. 2008;28(29):7313-23

9. Peng BG, Chen S, Lin X. Aspirin selectively augmented N-methyl-D-aspartate types of glutamate responses in cultured spiral ganglion neurons of mice. Neurosci Lett. 2003;341(1):21-24

10. Qin J. et al: Dual role during vesicle formation. Trends Cell Biol. 2006;16(12):607-9

11. Wei TJ, Chen HY, Huang X, et al. [A study on toxic effects of sodium salicylate on rat cochlear spiral ganglion neurons: dopamine receptors mediate expression of NMDA receptors and GABA receptors]. Sheng Li Xue Bao. 2017;69(3):285-90 [in Chinese]

12. Maison SF, Liu XP, Eatock RA, et al. Dopaminergic signaling in the cochlea: Receptor expression patterns and deletion phenotypes. J Neurosci. 2012;32(1):344-55

13. Huang X, Chen HY, Wei TJ, et al. [The sodium salicylate affects the expression of NMDA receptor and GABA receptor subunits in spiral ganglion neurons of the cochlea through DA receptor.] Lin Chung Er Bi Yan Hou Tou Jing Yi Za Zhi. 2017;31(20):1993-98 [in Chinese]

14. Flores-Hernandez J, Hernandez S, Snyder GL et al. D(1) dopamine receptor activation reduces GABA(A) receptor currents in neostriatal neurons through a PKA/DARPP-32/PP1 signaling cascade. J Neurophysiol. 2000;83(5):2996-3004

15. Mele M, Leal G, and Duarte CB. Role of GABAAR trafficking in the plasticity of inhibitory synapses. J Neurochem. 2016;139(4):838-47

16. Mele M, Chen RD, Duarte CB. Alterations in GABA(A) receptor trafficking and synaptic dysfunction in brain disorders. Front Cell Neurosci. 2019;13:77

17. Chapell R, Bueno OF, Alvarez-Hernandez X, et al. Activation of protein kinase C induces gamma-aminobutyric acid type A receptor internalization in Xenopus oocytes. J Biol Chem. 1998;273(49):32595-601

18. Brandon NL, Delmas P, Kitterl JT, et al. GABA(A) receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. J Biol Chem. 2000;275(49):38565-62

19. Chou WH, Wang D, McMahon T, et al. GABA(A) receptor trafficking is regulated by protein kinase C epsilon and the N-ethylethacolimide-sensitive factor. J Neurosci. 2010;30(42):13955-65

20. Kumar S, Suryanarayanan A, Boyd KN, et al. Ethanol reduces GABA(A) alpha1 subunit receptor surface expression by a protein kinase C-gamma dependent mechanism in cultured cerebral cortical neurons. Mol Pharmacol. 2010;77(5):793-803

21. Meistrell SR, Espinoza S, Cauden N, et al. Ethanol modulation of GABA(A) receptors in rat CA1 and dentate gyri. J Neurochem. 2011;115(9):2808-21

22. Beaufort N, Espinoza S, Cauden N, et al. Ethanol modulation of GABA(A) receptors expressed in the rat hippocampus. J Neurochem. 2008;107(2):433-44

23. Feng J, Cai X, Zhao J, Yan Z. Serotonin receptors modulate GABA(A) receptor function through a phospho-endocytosis mechanism in nucleus accumbens. J Neurosci. 2006;26(9):2513-21

24. Graziane NM, Yuen EY, Yan Z. Dopamine D4 receptors regulate GABA(A) receptor trafficking via an actin/cofilin/myosin-dependent mechanism. J Biol Chem. 2009;284(13):8329-36

25. Joshi S, Kapur J. Slow intracellular accumulation of GABA(A) receptor delta subunit is modulated by brain-derived neurotrophic factor. Neuroscience. 2009;164(2):507-19

26. Cho YI, Kim H, Kim WI, et al. Trafficking patterns of NMDA and GABA(A) receptors in a Mg2+-free cultured hippocampal neuron model of status epilepticus. Epilepsy Res. 2017;136:143-48

27. Nicholson MW, Sweeney A, Peke E, et al. Diazepam-induced loss of inhibitory synapses mediated by PLCdelta/Ca(2+)/calcineurin signalling downstream of GABA(A) receptors. Mol Psychiatry. 2018;23(9):1851-67

28. Mele M, Costa RO, Duarte CB. Alterations in GABA(A) receptor trafficking and synaptic dysfunction in brain disorders. Front Cell Neurosci. 2019;13:77

29. Chapell R, Bueno OF, Alvarez-Hernandez X, et al. Activation of protein kinase C induces gamma-aminobutyric acid type A receptor internalization in Xenopus oocytes. J Biol Chem. 1998;273(49):32595-601

30. Brandon NL, Delmas P, Kitterl JT, et al. GABA(A) receptor phosphorylation and functional modulation in cortical neurons by a protein kinase C-dependent pathway. J Biol Chem. 2000;275(49):38565-62

31. Chou WH, Wang D, McMahon T, et al. GABA(A) receptor trafficking is regulated by protein kinase C epsilon and the N-ethylethacolimide-sensitive factor. J Neurosci. 2010;30(42):13955-65

32. Kumar S, Suryanarayanan A, Boyd KN, et al. Ethanol reduces GABA(A) alpha1 subunit receptor surface expression by a protein kinase C-gamma-dependent mechanism in cultured cortical neurons. Mol Pharmacol. 2010;77(5):793-803

33. Meistrell SR, Espinoza S, Cauden N, et al. Ethanol modulation of GABA(A) receptors in rat CA1 and dentate gyri. J Neurochem. 2011;115(9):2808-21

34. Beaufort N, Espinoza S, Cauden N, et al. Ethanol modulation of GABA(A) receptors expressed in the rat hippocampus. J Neurochem. 2008;107(2):433-44

35. Feng J, Cai X, Zhao J, Yan Z. Serotonin receptors modulate GABA(A) receptor function through a phospho-endocytosis mechanism in nucleus accumbens. J Neurosci. 2006;26(9):2513-21

36. Poisbeau P, Cheney MC, Browning MD, Mody I. Modulation of synaptic inhibition by phospho-dependent sequestration of amygdala and hippocampal GABA(A) receptors via different tyrosine receptor kinase B-mediated phosphorylation pathways. Neuroscience. 2011;176:72-85

37. Bartley N, Chen RD, Duarte CB. Alterations in GABA(A) receptor trafficking and synaptic dysfunction in brain disorders. Front Cell Neurosci. 2019;13:77

38. Mao L, Heidt SA, Ressler KJ. Rapid brain-derived neurotrophic factor-dependent sequestration of amygdala and hippocampal GABA(A) receptors via different tyrosine receptor kinase B-mediated phosphorylation pathways. Neuroscience. 2011;176:72-85

39. Bartley N, Chen RD, Duarte CB. Alterations in GABA(A) receptor trafficking and synaptic dysfunction in brain disorders. Front Cell Neurosci. 2019;13:77

40. Adams JM, Thomas P, Smart TG. Modulation of neurotransmitter potentiation by protein kinases at synaptic- and extrasynaptic-type GABA(A) receptors. Neuropharmacology. 2015;88:63-73

41. Houston CM, Smart TG. CaMk-II modulation of GABA(A) receptors expressed in HEK293, NG108-15 and rat cerebellar granule neurons. Eur J Neurosci. 2006;24(9):2504-14

42. Piao Ouwen, Brownhie MD, Mody I. Modulation of synaptic GABA(A) receptor function by PKA and PKC in adult hippocampal neurons. J Neurosci. 1999;19(2):674-83

43. Jeng SC, Vigneswar B, Lambert JJ. Phosphorylation influences neurotransmitter modulation of synaptic GABA(A) receptors in rat CA1 and dentate gyrus neurons. Neuropharmacology. 2003;45(6):873-83