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Salicylate Induced $GABA_AR$ 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 ($GABA_ARs$). Our previous studies have shown that SS can increase the internalization of $GABA_ARs$ 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 $GABA_ARs$.

Material/Methods: The expression of D1Rs and GABARγ2 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/dynamin-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 GABARγ2 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 $GABA_ARs$, and activating D1Rs had the same result. Inhibiting D1Rs and PKC decreased the internalization of $GABA_ARs$. Meanwhile, the phosphorylation level of $GABA_AR$γ2 S327 affected by PKC was positively correlated with the degree of internalization of $GABA_ARs$. 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 $GABA_AR$ internalization induced by SS via a PKC-dependent manner and participate in the excitotoxic process of SGNs.

Keywords: Gabrg2 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 ($GABA_ARs$). 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 $GABA_AR$ on SGNs [10,11] and reversibly inhibited the GABA ($GABA_AR$ agonist)-activated currents [10]. Our previous studies have shown that inhibition of NMDARs decreased the suppressive effect of SS on $GABA_ARs$, indicating that the interaction between NMDARs and $GABA_ARs$ also leads to a weakened $GABA_AR$ response [10]. However, it is not fully understood how the downstream signaling effects of SS induce changes in $GABA_AR$ 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 $GABA_ARs$ and reduce the GABA-evoked currents in the middle spinous neurons of the neostriatum [14]. Our previous studies have reported that SS can decrease the surface expression of the $GABA_AR$ α2 subunit of SGNs, and inhibition of D1Rs blocks this effect, indicating that D1Rs mediates the effect of SS on $GABA_ARs$ [11]. However, how D1Rs mediate the inhibitory effect of SS on $GABA_ARs$ remains to be elucidated.

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

In the cerebral cortex, the majority of functional $GABA_AR$ subtypes contain the γ2 subunit [18]. The γ2 subunit is essential for the postsynaptic clustering of $GABA_ARs$ [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 $GABA_AR$ currents through PKA-mediated signaling [14]. However, very little information is available on the roles of PKC pathways in the regulation of $GABA_ARs$ by D1Rs in SGNs.

Hence, we propose that D1Rs regulate the $GABA_AR$ 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 GABARγ2 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 $GABA_ARs$ 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 SGNs-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% CO_2.

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

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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+SKF+SCH	SS+SKF+SCH
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.

buffered Bsaline (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, Abcam, USA), and mouse anti-GABARγ2 antibody (1: 100, Millipore, USA). The secondary antibodies used in this study were Alexa Fluor-488 or 555 labeled goat anti-mouse or rabbit IgG (1: 300, CST, USA). Images were observed with a fluorescence microscope (Olympus, Japan) at 400× magnification.

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-βIII-tubulin antibody (Abcam, USA), rabbit anti-GABARγ2 antibody (Abcam, USA), and rabbit anti-GABARγ2 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 βIII-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.

Whole-Cell Patch Clamp Recording

SGNs cultured for 12 h were used for whole-cell patch clamp experiments. Microelectrodes were pulled from borosilicate glass capillaries using the P-97 electrode puller (Sutter, USA) with pipette resistances of 3 to 8 MΩ. Currents were recorded by the EPC10 system (HEKA, Germany). Extracellular solution containing (in mM): NaCl (140), KCl (2.8), $MgCl_2$ (2.0), $CaCl_2$ (1.0), Glucose (10.0), HEPES (10.0). Intracellular solution composed of (in mM): CsCl (140), $CaCl_2$ (0.1), $MgCl_2$ (2.0), Na_2-ATP (2.0), EGTA (1.1), HEPES (10.0). The pH of the extracellular and intracellular solution was adjusted to 7.2. The holding potential was maintained at -70 mV to form a whole-cell recording mode. A sodium channel current (I_{Na}) reversibly blocked by 0.3 μM tetrodotoxin (TTX) was induced and the sealed cell was confirmed as an SGN. Drugs, including GABA (500 μM), GABA+SKF38393 (20 μM), GABA+SS (5 mM), GABA+SS+SCH23390 (20 μM), and GABA+SS+SKF38393+SCH23390, were delivered via a microdosing system (ALA, USA) onto the cells (the time of application was about 3 s), and PKC inhibitor Bis I (4 μM) was added to the intracellular solution to detect the change in the GABA-activated currents. The currents were exported via Pulse 8.0 software. Data were collected and analyzed offline with HEKA Pulsefit 8.61 software. Plottings were performed with SigmaPlot 10.0 software. GABA response was calculated as current density=peak current/cell membrane capacitance (pA/pF).

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 GABARγ2 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

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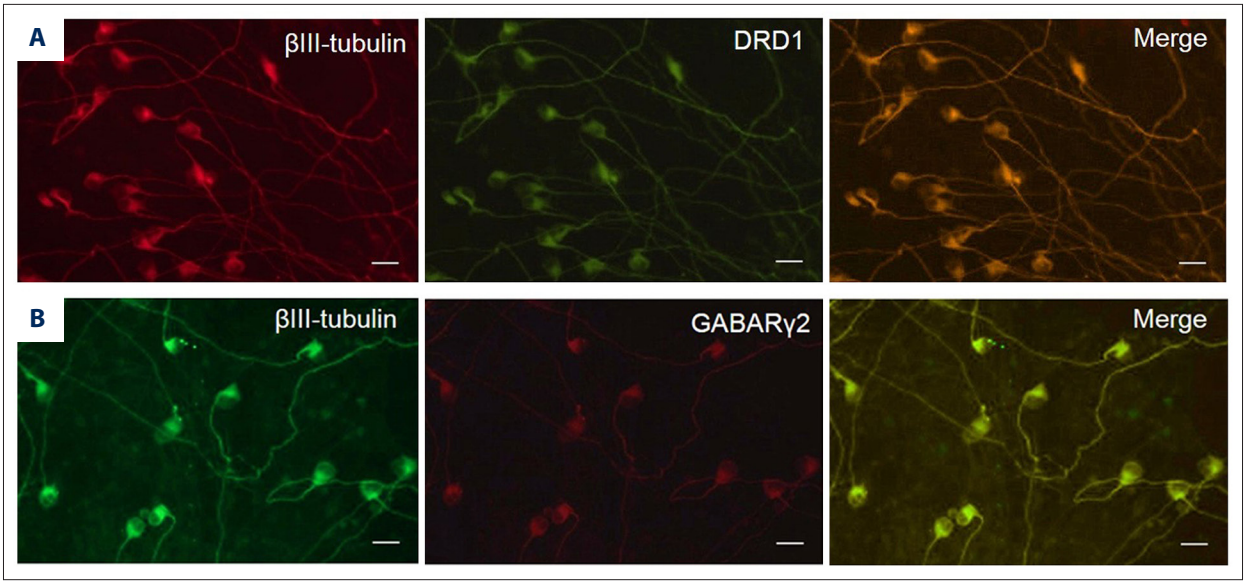

Figure 1. Expression of DRD1 and GABARγ2 on SGNs. (**A**) SGNs were labeled with βIII-tubulin (red) and DRD1 (green), 400×. (**B**) SGNs were labeled with βIII-tubulin (green) and GABARγ2 (red), 400×. Scale bar: 20 μM. Olympus CellSens Standard 1.17 (Japan) was used for the creation of the figures.

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 protein of GABARγ2 in these 2 groups showed no signs of changes as compared with the control group ($P>0.05$, **Figure 2B**), indicating that SS or SS+SKF38393 increased the internalization of GABA$_A$Rs. However, there was no significant difference in the surface GABARγ2 expression in the SS and SS+SKF38393 groups ($P>0.05$), suggesting that co-application of SS and SKF38393 had no additive effect on GABA$_A$R internalization. Further, in the SS+SKF38393+SCH23390 group, the GABARγ2 surface expression was significantly reversed to 76.56% of control ($P<0.05$, compared with the control 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.

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$_A$R 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

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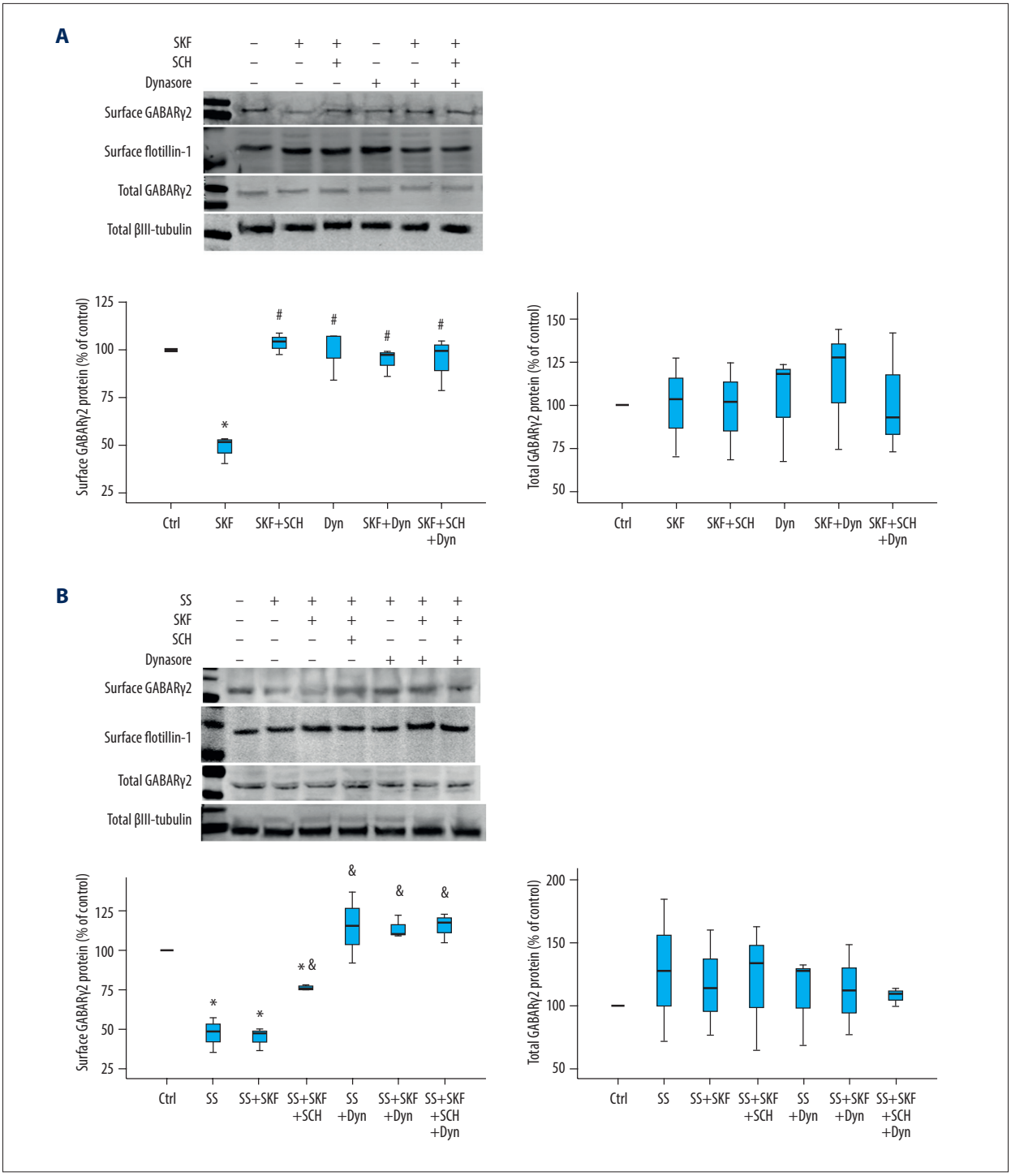

Figure 2. GABARγ20 protein expression in the absence or presence of endocytosis inhibitor (dynasore, Dyn). (**A**) The surface expression of GABARγ2 (normalized to flotillin-1) was significantly decreased by SKF as compared with the control group, and this effect was completely reversed by SCH and Dyn. 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.

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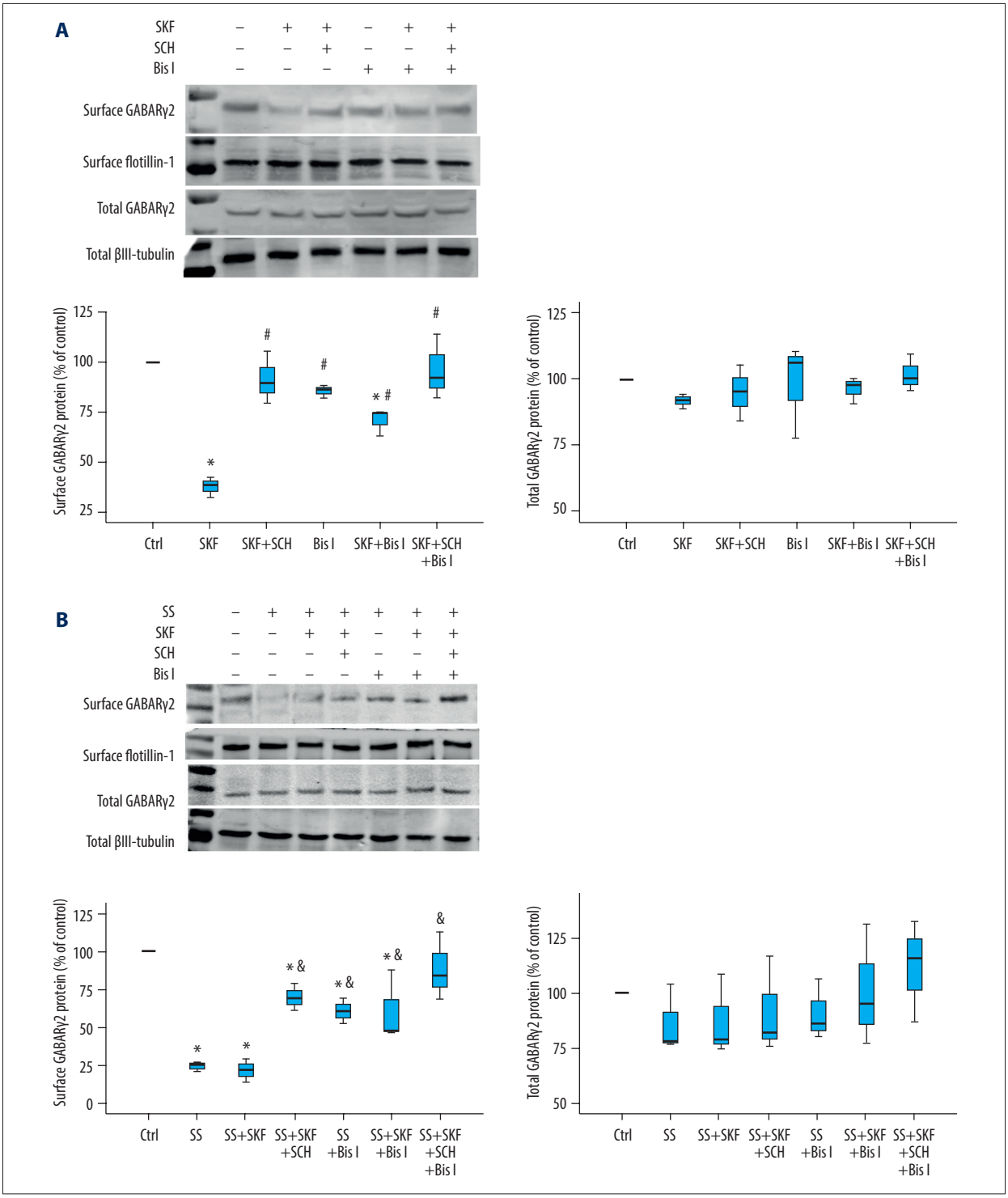

Figure 3. Expression of GABARγ2 proteins before and after PKC inhibitor application. (**A**) The inhibitory effect of SKF on the surface expression of GABARγ2 was partially reversed by Bis I. Quantitative analysis of GABARγ2 total protein expression showed no considerable difference in these groups. (**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.

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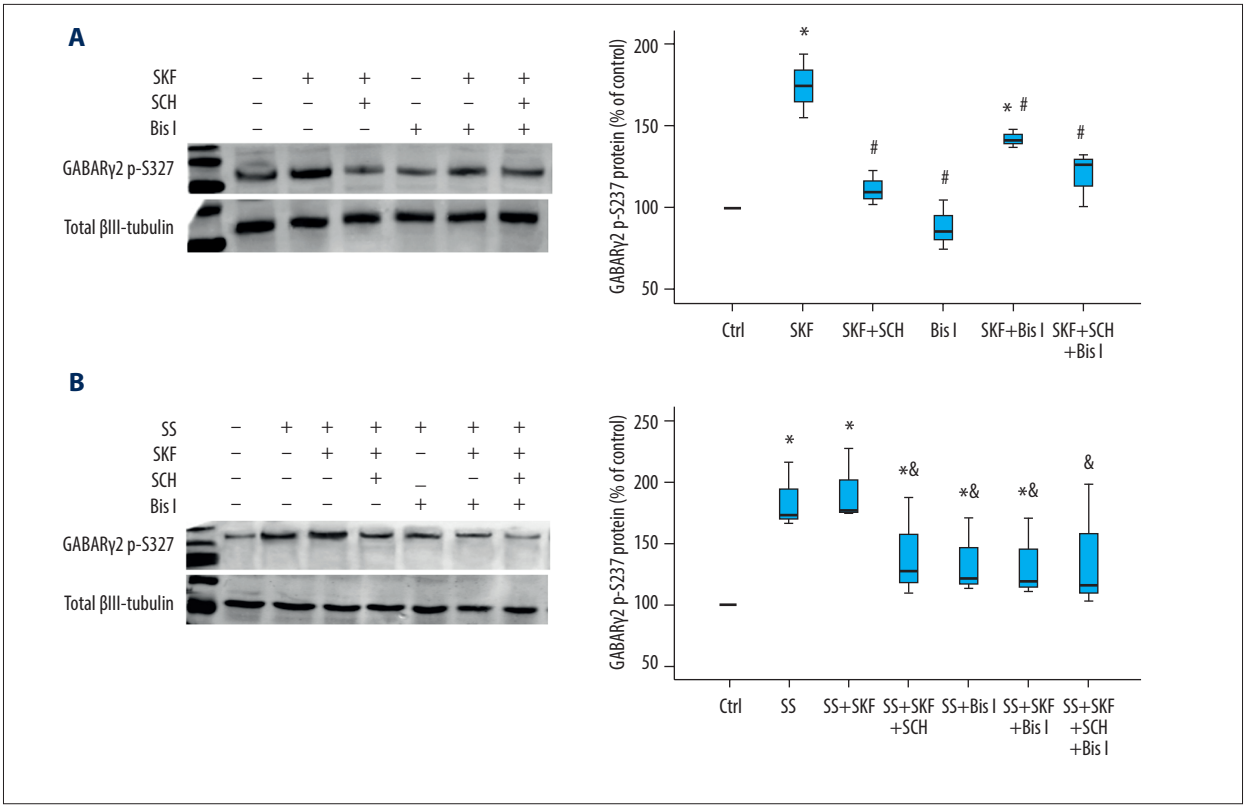

Figure 4. Phosphorylation of S327 of GABA$_A$R γ2 subunit in SGNs. (**A**) SKF markedly enhanced the phosphorylation levels of S327 (expression of the p-327 protein was normalized to βIII-tubulin) as compared to the control group, which was partially reversed by inhibiting PKC. (**B**) SS, SS+SKF markedly enhanced the phosphorylation levels of S327 as compared to the control group, which was partially reversed by inhibiting D1Rs or PKC. 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$_A$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$_A$R internalization induced by SS or SS+SKF38393, thus further illustrating that the D1R-mediated promotion of GABA$_A$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 GABA$_A$R γ2 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$_A$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 SKF3839+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**).

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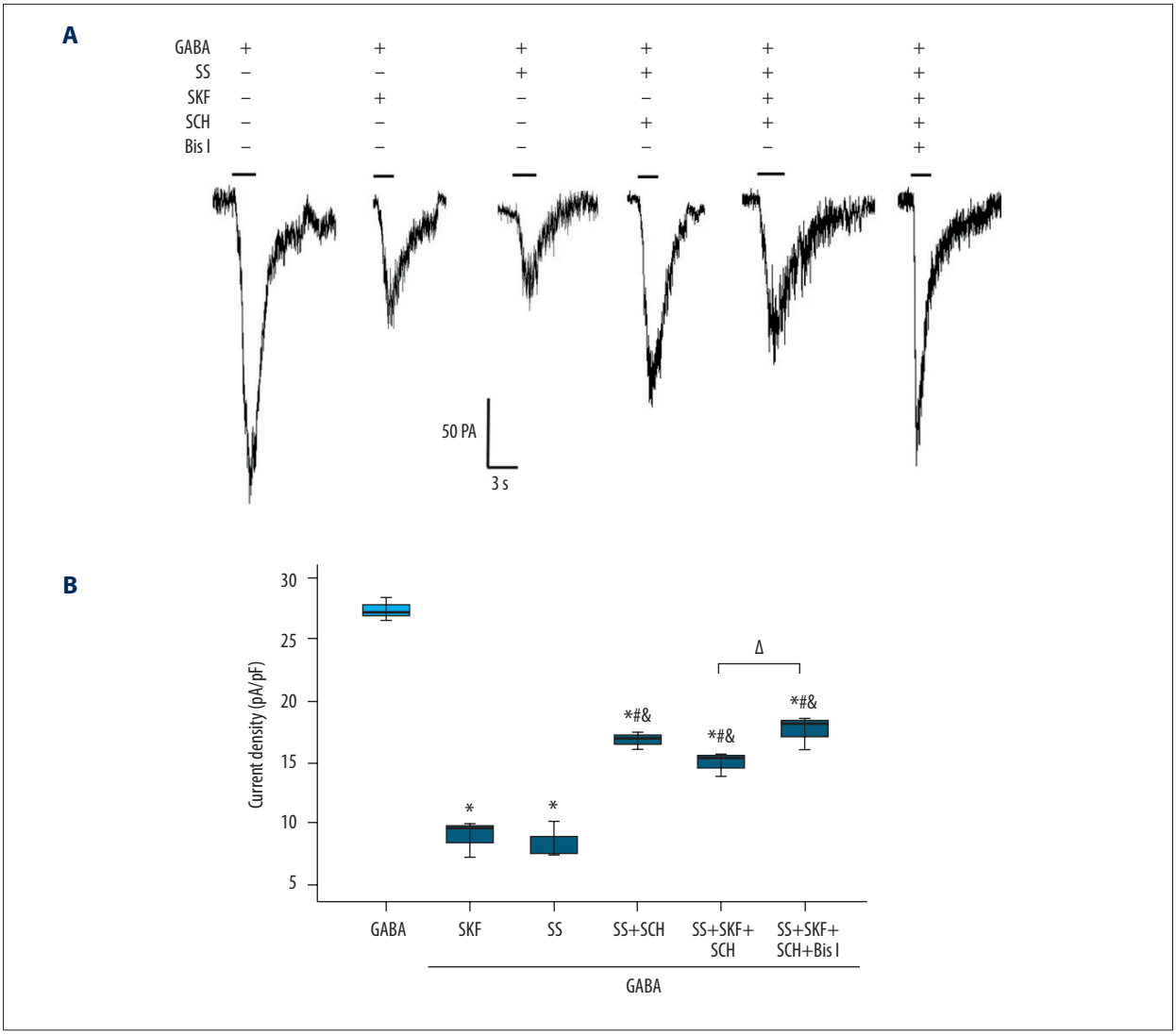

Figure 5. GABA-activated currents recorded in SGNs. (**A, B**) The suppression of 5 mM SS on GABA-activated currents was reduced by inhibiting D1Rs, then further reduced following co-inhibiting D1Rs and PKC. Data are presented as mean±SD. n=7. * P<0.05 vs GABA group, $^{\#}$ P<0.05, vs the SKF group, $^{\&}$ P<0.05 vs the SS group, $^{\Delta}$ P<0.05, SS+SKF+SCH group vs SS+SKF+SCH+Bis I group, by one-way ANOVA and Tukey test. Pulsefit 8.61 (HEKA, USA) and SPSS 25.0 (USA) were used for the creation of the figures.

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**.

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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$_A$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$_A$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 antagonist can weaken the effect of SS on GABA$_A$R expression in SGNs, suggesting that D1Rs can mediate the effect of SS on GABA$_A$Rs by interacting with GABA$_A$Rs [11,13]. In this work, the expression of DRD1 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$_A$Rs Through D1Rs

There have been few studies on the mechanism by which SS or D1R activation causes the decrease of cell surface GABA$_A$R expression. For example, SS might reduce GABA function through direct binding to GABA$_A$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$_A$Rγ2 but did not influence the total GABA$_A$Rγ2 protein, suggesting that D1Rs activation can increase GABA$_A$R internalization. SS also decreased the surface levels of GABA$_A$Rγ2 without affecting the total GABA$_A$Rγ2, which was in line with reports about the influence of SS on α2 and β3 subunits [10,11], suggesting that the internalization of GABA$_A$Rs might be increased. According to the results of western blotting and electrophysiology experiments, after inactivating D1Rs, the suppression of surface GABA$_A$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$_A$Rs.

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

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

There have been several studies revealing the time course of GABA$_A$R endocytosis. For example, brain-derived neurotrophic factor (BDNF) [28], Mg^{2+}-free condition [29], and diazepam [30] can trigger GABA$_A$R internalization at between 15 and 60 min. Our present data from western blotting revealed that GABA$_A$Rs on the surface of SGNs were significantly internalized after

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treatment with SS or D1R agonist for 1 h, and the time course observed was consistent with other findings.

D1Rs-Mediated GABA$_A$R Internalization is Modulated by PKC

GABA$_A$R 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$_A$R internalization [32] and inhibit GABA$_A$R function by reducing GABA currents [33,34]. Therefore, we observed the changes in GABA$_A$Rγ2 internalization after inhibiting PKC. Our data from western blotting shown that the reduction of surface GABA$_A$Rγ2 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$_A$Rs. Inhibition of PKC also attenuated SS-induced GABA$_A$R internalization, suggesting that the mechanism by which D1Rs mediate the effect of SS on SGNs involves the PKC signaling pathway. A previous study reported that inhibition of PKCγ partially blocked the ethanol-induced GABA$_A$R internalization in cultured cerebral cortical neurons [35]. In hippocampus neurons, BDNF-induced α1-GABA$_A$R internalization was completely disrupted by PKC inhibition [36]. Moreover, in our study, inhibition of PKC only partially blocked GABA$_A$R 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$_A$R internalization in SGNs. For example, D1 receptors in the striatum decreased the GABA$_A$R response through PKA-dependent regulation [14]. SS might increase the GABA$_A$R internalization via CaMKII [10]. Therefore, the failure of PKC inhibition to completely block the D1R-mediated internalization of GABA$_A$Rs 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$_A$R internalization, and inhibition of PKC enhanced the GABA$_A$R function. Brandon et al found that inhibition of PKC markedly increased GABA currents in A293 cells and *Xenopus* oocytes transfected with GABA$_A$Rs [33]. In prefrontal cortical neurons, PKC inhibitor blocked the inhibitory effect of (-)-2,5-dimethoxy-4-iodoam-phetamine 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$_A$R function.

The Subject of SS Regulation Mediated by D1Rs is γ2 S327

PKC phosphorylation sites on GABA$_A$Rs include β3 S408/409 and γ2 S327 [16]. We observed that the D1R agonist markedly

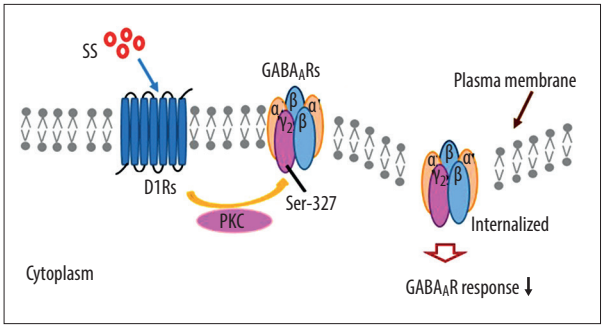

Figure 6. One pathway by which dopamine D1-like receptors mediated the salicylate-induced GABA$_A$ receptor internalization was through the effect of PKC on spiral ganglion neurons. After SS increased the PKC-dependent phosphorylation at γ2 S327 by D1Rs, the internalization of GABA$_A$Rs was increased through a clathrin/dynamin-dependent pathway, then the surface level of GABA$_A$Rs was reduced, resulting in a weakened GABA$_A$R-mediated response. PowerPoint 2020 (Microsoft, USA) was used for the creation of the figure.

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$_A$Rs, thus increasing the phosphorylation of 327 by the PKC pathway, thereby promoting the internalization of GABA$_A$Rs. As observed in other research, inhibition of CaMKII blocked the phosphorylation of β3 S383 and decreased the SS-induced GABA$_A$Rs 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$_A$Rs was positively correlated with the degree of GABA$_A$R internalization and negatively correlated with cell surface GABA$_A$R 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$_A$Rs 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$_A$R 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 calcineurin in rat cortical neurons, which led to increased GABA$_A$R

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internalization [30]. These results show that the regulation of $GABA_ARs$ by PKC was not completely the same, and the phosphorylation at S327 also produced a different effect on $GABA_AR$ surface expression and function. The reasons for this may include the following: (1) $GABA_ARs$ 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 γ2 S327 through D1Rs, thus triggering $GABA_AR$ internalization through a clathrin/dynamin-dependent endocytosis pathway and resulting in suppressed $GABA_AR$ surface levels, eventually leading to reduced $GABA_AR$-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 $GABA_AR$ 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, GABARγ2 protein expression in the absence or presence of dynasore.

Groups	Density (mean±SD)				Relative expression as % of control (mean±SD)	
	Surface GABARγ2	Surface Flotillin-1	Total GABARγ2	Total βIII-tubulin	Surface GABARγ2	Total GABARγ2
Ctrl	138.47±29.11	196.57±11.34	100.95±13.51	197.86±4.28	100±0.00	100±0.00
SKF	72.09±19.13	209.35±10.51	101.34±5.24	200.21±20.37	48.57±7.12	100.53±28.99
SKF+SCH	141.36±21.36	194.75±5.37	102.69±9.03	206.96±10.07	103.79±5.69	98.58±28.59
Dynasore	139.43±15.05	199.91±7.64	107.51±9.91	206.27±15.85	99.77±13.03	103.21±31.09
SKF+Dynasore	123.07±22.19	186.04±14.32	116.57±6.47	199.87±14.63	94.44±7.24	115.66±36.49
SKF+SCH+Dynasore	125.56±7.11	188.29±17.55	107.77±8.66	208.61±11.38	94.57±13.83	102.90±35.77
Ctrl	103.04±17.51	112.93±11.65	119.81±21.26	159.16±33.16	100±0.00	100±0.00
SS	52.76±20.82	121.77±12.96	126.28±10.11	131.89±34.01	47.15±11.11	127.97±56.15
SS+SKF	54.89±19.24	135.92±18.99	119.76±7.12	136.72±36.37	44.76±7.18	117.06±41.60
SS+SKF+SCH	101.31±16.04	146.16±22.45	120.72±24.93	134.21±36.61	76.56±1.43	120.25±50.28
SS+Dynasore	127.41±24.80	122.01±21.27	106.53±16.60	130.01±30.71	115.19±22.57	109.43±35.46
SS+SKF+Dynasore	127.53±21.77	123.06±32.65	117.21±7.79	140.48±39.40	114.30±7.02	112.58±35.96
SS+SKF+SCH+Dynasore	136.35±14.02	130.11±28.63	105.01±11.70	131.24±37.08	115.52±9.36	107.66±7.16

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Supplementary Table 2. Data of Figure 3, GABARγ2 protein expression in the absence or presence of Bis I.

Groups	Density (mean±SD)				Relative expression as % of control (mean±SD)	
	Surface GABARγ2	Surface Flotillin-1	Total GABARγ2	Total βIII-tubulin	Surface GABARγ2	Total GABARγ2
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, GABARγ2 p-S327 protein expression in the absence or presence of Bis I.

Groups	Density (mean±SD)		Relative expression as% of control (mean±SD)
	GABARγ2 p-S327	Total βIII-tubulin	GABARγ2 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	169.62±11.63	198.74±5.85	142.12±5.49
SKF+SCH+Bis I	145.26±20.81	201.31±19.52	120.14±16.92
Ctrl	83.82±19.37	186.92±14.93	100±0.00
SS	160.27±23.26	191.88±3.13	185.48±27.05
SS+SKF	162.13±26.45	186.42±12.18	193.28±30.23
SS+SKF+SCH	115.98±23.08	181.09±10.15	141.82±41.26
SS+Bis I	113.96±22.41	186.47±1.82	135.70±31.09
SS+SKF+Bis I	109.24±18.63	182.13±24.46	134.02±32.55
SS+SKF+SCH+Bis I	112.55±14.60	178.71±16.77	139.59±51.96

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