Gymnopilins, a product of a hallucinogenic mushroom, inhibit the nicotinic
acetylcholine receptor

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1 ABSTRACT

2 Gymnopilins are substances produced in fruiting bodies of the hallucinogenic
3 mushroom, *Gymnopilus junonius*. Although, only a few biological effects of gymnopilins
4 on animal tissues have been reported, it is believed that gymnopilins are a key factor of
5 the *G. junonius* poisoning. In the present study, we found that gymnopilins inhibited
6 ACh-evoked responses in neuronal cell line, PC12 cell, and determine the underlying
7 mechanism. Gymnopilins were purified from wild fruiting bodies of *G. junonius*
8 collected in Japan. Ca^{2+} -imaging revealed that gymnopilins reduced the amplitude of
9 ACh-evoked $[\text{Ca}^{2+}]_i$ rises by about 50 % and abolished the ACh responses remaining in
10 the presence of atropine. Gymnopilins greatly reduced the amplitude of $[\text{Ca}^{2+}]_i$ rises
11 evoked by nicotinic ACh receptor agonists, 1,1-Dimethyl-4-phenylpiperazinium iodide
12 (DMPP) and nicotine. In the whole-cell voltage clamp recording, gymnopilins inhibited
13 the DMPP-evoked currents, but did not affect the voltage-gated Ca^{2+} channel currents.
14 These results indicate that gymnopilins directly act on nicotinic ACh receptors and inhibit
15 their activity. This biological action of gymnopilins may be one of the causes of the *G.*
16 *junonius* poisoning.

1 Abbreviations

2 ACh, acetylcholine; DMPP, 1,1-Dimethyl-4-phenylpiperazinium iodide; NGF, nerve
3 growth factor; nAChR, nicotinic acetylcholine receptor; VGCC, voltage-gated Ca^{2+}
4 channel; cAMP, cyclic adenosine monophosphate

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6 Keywords

7 Gymnophilin, Mushroom poisoning, Nicotinic acetylcholine receptor, PC12 cell

1. Introduction

Gymnopilus junonius (“Ohwaraitake” in Japanese) is a hallucinogenic poisonous mushroom grown in Japan. Gymnopilins were isolated and purified from the fruiting bodies of *G. junonius* (Aoyagi et al., 1983) and identified as one of the polyisoprene polyol compounds consisting of 9 to 12 isoprene units (Fig. 1; Nozoe et al., 1983). In similar kinds of mushrooms (*Gymnopilus* species) grown in America and Europe, a serotonin receptor agonist, psilocybin was detected (Hatfield and Valdes, 1978). On the other hand, *G. junonius* in Japan was reported not to produce psilocybin (Kusano et al., 1986), suggesting that the hallucinogenic effect is due to other substances. It has been reported that gymnopilins give these mushrooms their bitter taste (Lee et al., 2008) and activate spinal cord neurons of the rat (Tanaka et al., 1993). Although a mechanism of action of gymnopilins on the nervous system is unclear, gymnopilins are believed to exert hallucinogenic effects in the poisoning by *G. junonius*.

We have recently reported that gymnopilins activate phospholipase C and increase intracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_i$) in non-neuronal cells isolated from dorsal root ganglia of the rat (Miyazaki et al., 2012), and that gymnopilins act directly on vascular smooth muscles and activated the cAMP-dependent cascade to cause the vasodilation (Nishio et al., 2012). However, direct evidence that gymnopilins act on neurons is still lacking. Therefore, we examined effects of gymnopilins on Ca^{2+} signaling in PC12 cells

1 with the aim of exploring actions of gymnopilins on neurons. PC12, a clonal cell line
2 derived from rat adrenal chromaffin cells, has been used extensively as a model system to
3 study properties of endocrine and neuronal cells (Greene and Tischler, 1976).

4 In our PC12 cell preparation, we found two actions of gymnopilins. One is that
5 gymnopilins induce $[Ca^{2+}]_i$ rises, and the other is that gymnopilins inhibit acetylcholine
6 (ACh)-evoked $[Ca^{2+}]_i$ rises. Here, we focus on the latter action of gymnopilins, and
7 clarify the underlying mechanisms of the inhibitory effect of gymnopilins on ACh
8 responses through the measurement of the $[Ca^{2+}]_i$ in fura-2-loaded PC12 cells and the
9 ionic current across cell membranes using the whole-cell voltage clamp technique.

2. Materials and methods

2.1. Isolation and purification of gymnopilins

Gymnopilins were purified from the wild fruiting body of *G. junonius* collected in Japan according to the procedure reported previously (Aoyagi et al., 1983; Miyazaki et al., 2012). In brief, wild fruiting bodies of *G. junonius* were lyophilized, crunched and stored at -20°C . Six hundred ml of methanol was added to 20 g of the *G. junonius* powder and stirred for 1 h at room temperature. After subsequent filtration of the supernatant, the precipitate was washed with methanol. The combined supernatant was concentrated by an evaporator to give 7.45 g of methanol extract. The residue was dissolved in H_2O and washed with ethyl acetate to remove polar compounds (mono- and oligosaccharides, amino acids and so on). The pH of the aqueous phase was adjusted to 3 with HCl and extracted with ethyl acetate three times. The combined organic layer was dried over anhydrous sodium sulfate (Na_2SO_4), filtered, and concentrated *in vacuo* to give 558 mg of crude gymnopilins. They were further purified on a C18 cartridge with 5 %, 25 %, 50 %, 75 % and 100 % methanol as an eluent. Gymnopilins were eluted in 75 % methanol fractions. They were combined and concentrated to give 127 mg of gymnopilins. The ^1H NMR and ^{13}C NMR spectra of the product were compared with that reported by Aoyagi (Aoyagi et al., 1983), and the purified products were assigned to gymnopilins.

2.2. PC12 cell culture

The PC12 cell line was provided by RIKEN cell bank (Tsukuba, Japan). Cells were plated in 25-cm² culture flasks (Nunc, Roskilde, Denmark) and cultured in Dulbecco's modified Eagle medium (DMEM; Invitrogen, Carlsbad, CA, USA) containing 4.5 g/l of glucose. The medium was supplemented with 10 % fetal bovine serum (MP Biochemicals, Irvine, CA, USA), 100 U/ml of penicillin (Sigma, St Louis, MO, USA) and 100 ng/ml of streptomycin (Sigma). Cells were maintained at 37 °C in a humidified atmosphere of 95 % air and 5 % CO₂. The subculturing was done once a week using 0.25 % trypsin dissolved in phosphate-buffered saline. Cells used for the measurement of [Ca²⁺]_i and the patch clamp recording were transferred to round coverslips (11 mm in diameter) coated with poly-D-lysine (Sigma), and were cultured for 5-7 days before use. During this period of culture, cells were treated with NGF-7S (50 ng/ml, Sigma) for longer than 3 days.

2.3. Measurement of the intracellular Ca²⁺ concentration

The [Ca²⁺]_i in PC12 cells was measured with the fluorescent Ca²⁺ indicator, fura-2, according to the procedure reported previously (Ozaki et al., 2009). The PC12 cells were

incubated in the HEPES-buffered solution (HBS) containing (mM); 154 NaCl, 6 KCl, 2 CaCl₂, 1.2 MgCl₂, 10 glucose and 10 HEPES (pH = 7.4 adjusted with tris) supplemented with 3- μ M fura-2/AM (Merck, Whitehouse Station, NJ, USA) and 0.01% Pluronic F-127 (Invitrogen) for 60 min at room temperature (22–24 °C). After the incubation, a coverslip, on which cells adhered, was mounted in a chamber fixed on the stage of an inverted fluorescence microscope (IX71; Olympus, Tokyo, Japan). Cells were continuously perfused with the HBS through polyethylene tubes connected to a peristaltic pump (Minipulse 3; Gilson, Middleton, WI, USA) at a flow rate of 3.2 ml/min. In this system, the solution around the cells was changed within a few seconds. A solution containing high concentration of K⁺ (60 mM) was made by the isotonic replacement of Na⁺ with K⁺. Changes in [Ca²⁺]_i were measured by dual excitation microfluorometry using a digital image analyzer (Aqua Cosmos/Ratio; Hamamatsu Photonics, Hamamatsu, Japan). The fluorescent signal was detected using a UV objective lens (UApo 20 $\times$ 3/340, Olympus), and the emission, passing through a band-pass filter (500 $\pm$ 10 nm), was detected by a cooled CCD camera (ORCA-ER, Hamamatsu Photonics). All experiments were performed at room temperature (22 – 24 °C).

Data acquisition was performed using a procedure reported previously (Kayano et al., 2010; Komori et al., 2010). Data were obtained at a sampling frequency of 0.2 Hz using Aqua Cosmos Software (Hamamatsu Photonics). The fluorescence intensity at the excitation wavelengths of 340 nm (F340) and 380 nm (F380), and fluorescence ratio

(F340/F380) in each pixel was determined by the analytical software (Aqua Cosmos, Hamamatsu Photonics). To calculate $[Ca^{2+}]_i$ in individual cells, regions of interest (ROIs) were chosen to include the whole cell, and the average values of $[Ca^{2+}]_i$ in all pixels contained in each cell were calculated. Changes in the calculated $[Ca^{2+}]_i$ were analyzed by IGOR Pro (Wavemetrics, Lake Oswego, OR, USA) and Excel (Microsoft, Redmond, WA, USA).

2.4. Measurement of the membrane current

The method used for electrophysiological experiments has been described elsewhere (Fukumoto et al., 2012). Recording pipettes were pulled from micro glass capillaries (GD-1.5; Narishige, Japan) by a framed puller (P-97; Sutter, USA), and electrodes with 2.5- to 4-M Ω tip resistance were used. In the experiments to record 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP)-evoked currents, pipettes were filled with the K⁺-rich pipette solution containing (mM); 130 K⁺-gluconate, 10 Na⁺-gluconate, 10 EGTA-2K, 4.5 MgCl₂, 0.74 CaCl₂, 10 HEPES (pH = 7.3 adjusted with tris). In the experiments to record voltage-gated Ca²⁺ channel (VGCC) currents, pipettes were filled with the Cs⁺-rich pipette solution containing (mM); 140 Cs⁺-gluconate, 10 EGTA-2Cs, 4.5 MgCl₂, 0.74 CaCl₂, 10 HEPES (pH = 7.3 adjusted with tris). To reduce contamination of VGCC currents by voltage-gated Na⁺ and K⁺ currents, the HBS was

supplemented with TTX (1 μ M; Tocris Bioscience, UK), TEA (3 mM, Sigma) and 4-AP (2 mM, Sigma). Ca^{2+} in the HBS was replaced with Ba^{2+} at 10 mM and VGCC currents carried by Ba^{2+} were recorded. Cells were continuously perfused with the HBS at a flow rate of 1.5 ml/min by gravity and overflow solution was removed with an electronic pump. An agar bridge containing 2 % agar and 154-mM NaCl in conjunction with an Ag-AgCl wire was used as a reference electrode. The liquid junction potential between the pipette and bath solutions (10 mV in the whole-cell recording) was corrected after the data acquisition. The patch-clamp amplifier used was EPC-10 (HEKA, Lambrecht/Pfalz, Germany). Currents were controlled and measured by Patch Master Software (HEKA) running on Macintosh (Apple, Cupertino, CA, USA). All electrophysiological experiments were performed at room temperature (22 – 24 °C). Stored data were analyzed off-line by IGOR Pro Software (Wavemetrics).

2.5. Statistics

Data are presented as the mean $\pm$ SEM. The statistical significance was assessed by Student's *t* test or the analysis of variance (ANOVA) followed by Tukey-Kramer test. Differences were considered statistically significant if $P < 0.05$.

2.6. Drugs

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2 A concentrated stock of NGF-7S (Sigma) at 10 µg/ml was made in DMEM and
3 stored at –30 °C. The stock solutions of ACh (0.1 M, Sigma),
4 1,1-Dimethyl-4-phenylpiperazinium iodide (DMPP, 10 mM, Sigma) were prepared in
5 distilled water and stored at –30 °C. Nicotine (0.1 M, Sigma) was dissolved in dimethyl
6 sulfoxide and stored at –30 °C. All these drugs were diluted to desired concentrations by
7 the experimental solution on the day of use. Other drugs and chemicals used were of an
8 analytical grade and were purchased from Sigma or Wako pure chemical (Osaka, Japan).

3. Results

3.1. Effects of gymnopilins on $[Ca^{2+}]_i$ rises induced by ACh

In the present study, an effect of gymnopilins on ACh-induced $[Ca^{2+}]_i$ rises in PC12 cells was examined. Excitable cells, such as muscles and neurons, express VGCCs, which are activated by a membrane depolarization. Increasing the concentration of extracellular K^+ depolarizes the membrane potential and activates VGCCs. Since it is well known that damaged cells show a high basal $[Ca^{2+}]_i$ and no (or small) response to high K^+ , cells showing a low basal $[Ca^{2+}]_i$ and a large (>100 nM) $[Ca^{2+}]_i$ rise in response to high K^+ were considered to be in a healthy condition and used for further analyses in the present study. In our preparation, among 314 cells that showed $[Ca^{2+}]_i$ increase larger than 100 nM in response to 60 mM K^+ stimulation, 46 cells responded to stimulation with gymnopilins at 100 μ g/ml and showed $[Ca^{2+}]_i$ rises. In subsequent experiments, we assessed $[Ca^{2+}]_i$ changes in the PC12 cells that did not respond to gymnopilins. An application of ACh at 100 μ M induced a rapid $[Ca^{2+}]_i$ rise. The cells showing $[Ca^{2+}]_i$ increases larger than 100 nM in response to the stimulation with ACh at 100 μ M were analyzed in this study. As shown in Fig. 2A, PC12 cells showed a $[Ca^{2+}]_i$ increase in response to repetitive stimulation with ACh applied for 20 s three times with intervals of 15 min. Amplitudes of the responses to ACh decreased slightly during the repetition of the stimulation. The

1 amplitude of the 3rd response was significantly smaller than that of the 2nd response;
2 there was no significant difference between the amplitude of the 1st response and that of
3 the 2nd response (Fig. 2B, n = 59). To examine effects of gymnopilins on ACh-induced
4 $[Ca^{2+}]_i$ rises, gymnopilins were applied 120 s before the 2nd application of ACh. In the
5 presence of gymnopilins at 100 μ g/ml, the amplitude of the ACh response was
6 significantly smaller than that in the absence of gymnopilins (Fig. 2C, D). Gymnopilins
7 suppressed the amplitude of the ACh-induced $[Ca^{2+}]_i$ rise to 54 ± 3 % of that of the 1st
8 responses (Fig. 2D, n = 33). ACh was applied again 15 min after washing out of
9 gymnopilins, and the amplitude of the $[Ca^{2+}]_i$ rise did not recover.

11 3.2. Effects of gymnopilins on $[Ca^{2+}]_i$ rises induced by DMPP and nicotine

13 It is reported that PC12 cells express nicotinic ACh receptors (nAChRs) and
14 respond to ACh stimulation (Avila et al., 2003; Henderson et al., 1994; Rogers et al.,
15 1992). To determine whether nAChRs are involved in the gymnopilin-induced
16 inhibition of ACh responses, we examined effects of gymnopilins on $[Ca^{2+}]_i$ rises
17 evoked by the nAChR agonists, DMPP and nicotine. In the following experiments, cells
18 showing a $[Ca^{2+}]_i$ increase larger than 100 nM in response to the 1st stimulation with
19 100- μ M DMPP or 100- μ M nicotine were analyzed. DMPP and nicotine were applied
20 repetitively at an interval of 15 min. Applications of 100- μ M DMPP also induced rapid

[Ca²⁺]_i rises (Fig. 3A). The amplitude of the DMPP response slightly but significantly increased during the repetitive stimulation (Fig. 3C). The DMPP-induced [Ca²⁺]_i rise was inhibited by pretreatment of cells with gymnopilins at 100 µg/ml. The amplitude of the DMPP response was reduced to 11 ± 1.3 % of the control response by gymnopilins and recovered to 90 ± 3.5 % after the removal (Fig. 3B, D; n = 28). Likewise, an effect of gymnopilins on the [Ca²⁺]_i rise evoked by nicotine was examined. The amplitude of the nicotine-induced [Ca²⁺]_i rise was also reduced by pretreatment with gymnopilins. Gymnopilins reduced the amplitude of the nicotine response to 12 ± 0.7 % of the control response, and the amplitude of the nicotine response also recovered to 61 ± 1.5 % after the removal of gymnopilins (n = 70).

The remaining fraction of the [Ca²⁺]_i response to ACh in the presence of gymnopilins was larger than that to DMPP and nicotine (Fig. 3E). The remaining [Ca²⁺]_i responses to ACh in the presence of gymnopilins may be due to activation of muscarinic ACh receptors expressed in PC12 cells. To examine the contribution of muscarinic ACh receptors to the [Ca²⁺]_i rise evoked by ACh, an effect of the muscarinic receptor antagonist, atropine, was evaluated. Atropine at 10 µM reduced the amplitude of [Ca²⁺]_i rise evoked by ACh to 67 ± 3.5 % (n = 60). Furthermore, gymnopilins at 100 µg/ml abolished the ACh-evoked [Ca²⁺]_i rise in the presence of atropine (Fig. 3F, n = 47).

To examine concentration dependence of the inhibitory effect of gymnopilins on the [Ca²⁺]_i responses caused by the nAChR activation, DMPP was applied in the

presence of various concentrations of gymnopilins. The higher the concentrations of applied gymnopilins were, the larger reduction of the DMPP responses was recorded as shown in Fig. 4. The half maximal inhibitory concentration (IC_{50}) of gymnopilins on DMPP-induced $[Ca^{2+}]_i$ rises was estimated to be 15 μ g/ml.

3.3. Effects of gymnopilins on DMPP-induced currents

To further investigate underlying mechanisms of the inhibition of the ACh-evoked $[Ca^{2+}]_i$ rises by gymnopilins, we next examined effects of gymnopilins on DMPP-induced current responses in the whole-cell voltage clamp configuration. Cells were voltage clamped at a holding potential of -70 mV. DMPP at 100 μ M was applied for 20 s three times with an interval of 2.5 min. DMPP induced a transient inward current (Fig. 5A). Although the amplitude of the inward current evoked by DMPP decreased gradually during repetitive stimulations, they were not significantly different (Fig. 5C, $n = 9$). Gymnopilins at 100 μ g/ml, that were applied 1 min before the 2nd DMPP stimulation, significantly reduced the amplitude of the DMPP-induced current by 75 ± 6.8 % (Fig. 5B, D; $n = 9$). After washing out of gymnopilins, the DMPP current responses partially recovered.

3.4. Effects of gymnopilins on VGCCs

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2 Since the activation of nAChRs causes a depolarization of the cell membrane, it is
3 possible that inhibition of VGCCs by gymnopilins causes the reduction of the amplitude
4 of $[Ca^{2+}]_i$ rises evoked by the nAChR activation. To assess this possibility, an effect of
5 gymnopilins on the VGCC current was examined. The VGCC conducts Ba^{2+} more
6 efficiently than Ca^{2+} . Moreover, Ca^{2+} flowing into the cell through VGCCs causes
7 inactivation of the VGCC and activation of some other types of ion channels, but Ba^{2+}
8 does not. These are the reasons to use Ba^{2+} as a charge carrier instead of Ca^{2+} in VGCC
9 current measurements. In this study, we also recorded Ba^{2+} current (I_{Ba}) to assess the
10 activity of VGCCs. Cells were voltage clamped at -90 mV in the whole-cell
11 configuration. To evoke currents through the high voltage-activated VGCCs, cells were
12 depolarized by a 50-ms voltage pulse to -10 mV every 20 s and I_{Ba} was repetitively
13 recorded. After recording current responses to a train of the voltage pulses, Cd^{2+} at 1
14 mM was applied to block currents through VGCCs in all the cells tested. In the presence
15 of Cd^{2+} , inward currents were abolished, indicating that the majority of the inward
16 current was I_{Ba} through high voltage-activated VGCCs. In this study, the net current,
17 which were calculated by subtracting the remaining current in the presence of Cd^{2+} from
18 the current in the absence of Cd^{2+} , are considered to be the net current through VGCCs.
19 It is well known that the amplitudes of the VGCC currents recorded in the whole cell
20 configuration tend to decrease gradually depending on washout of intracellular

1 substances. This property is called “rundown”. PC12 cells used in the present study also
2 showed rundown of the I_{Ba} . Therefore, we assessed effects of gymnopilins on the I_{Ba}
3 considering the effect of I_{Ba} rundown. Gymnopilins at 100 $\mu\text{g/ml}$ were applied to the
4 extracellular solution. Although the amplitude of I_{Ba} continued to decrease, the rate of
5 rundown of I_{Ba} in the presence of gymnopilins was not statistically different from that in
6 the absence of gymnopilins, indicating that gymnopilins did not affect amplitudes of I_{Ba}
7 (Fig. 6).

4. Discussion

Here, we describe an action of gymnopilins on neurons using PC12 cells as a model system. It is reported that PC12 cells normally express both neuronal nAChRs (Mousavi et al., 2001; Sala et al., 2008) and muscarinic receptors (Cross et al., 1984; Jumblatt and Tischler, 1982). It is also reported that the activation of either type of receptor induces $[Ca^{2+}]_i$ rises (Bennett et al., 1998; Rathouz et al., 1996; Robinson and McGee, 1985; Takashima and Kenimer, 1993; Takashima and Koike, 1985). In this study, we also showed that an application of ACh induced $[Ca^{2+}]_i$ rises in PC12 cells. Gymnopilins reduced the amplitude of $[Ca^{2+}]_i$ rises evoked by ACh and nAChR agonists. The inhibitory effect of gymnopilins was much greater on the $[Ca^{2+}]_i$ rises evoked by nAChR agonists than on that by ACh. Moreover, in the presence of atropine, a muscarinic receptor antagonist, gymnopilins abolished the ACh-evoked $[Ca^{2+}]_i$ rise. These results strongly suggest that gymnopilins mainly inhibited the $[Ca^{2+}]_i$ response to the nAChR activation, but not to the muscarinic ACh receptor activation.

It is widely accepted that when nAChRs are activated, the membrane potential is depolarized. The depolarization, in turn, activates VGCCs and allows Ca^{2+} influx (Catterall, 1998; Dickinson et al., 2007; Harkins and Fox, 1998; Taylor and Peers, 2000; Zhou and Neher, 1993). Ca^{2+} can also enter into cells through pores of nAChRs themselves (Harkins and Fox, 1998; Taylor and Peers, 2000; Zhou and Neher, 1993).

We hypothesized two mechanisms of the inhibitory action of gymnopilins on the nAChR-dependent $[Ca^{2+}]_i$ rise: 1) gymnopilins inhibit nAChRs, 2) gymnopilins inhibit VGCCs. In the whole-cell voltage clamp recording, gymnopilins inhibited DMPP-induced currents, but did not affect I_{Ba} . In addition to these results, we confirmed that an application of DMPP stimulation induced an increase $[Ca^{2+}]_i$ even in the presence of Cd^{2+} and gymnopilins inhibited these responses (data not shown). These results strongly suggest that the hypothesis 1 is likely: gymnopilins inhibit the nAChR itself, but not the VGCC, thereby reducing the amplitude of $[Ca^{2+}]_i$ rises by ACh. The IC_{50} of gymnopilins for the inhibition of the DMPP current was 15 μ g/ml. It is not possible to calculate the exact concentration of gymnopilins on a molar basis, because the molecular weight of gymnopilins ranges between 985.41 and 1261.81 depending on their structure. Assuming that the average molecular weight of gymnopilins is 1000, the IC_{50} is estimated to be 15 μ M. The effective concentration range of hexamethonium, one of the major inhibitors for neuronal type nAChRs, is reported to be 1–100 μ M (Eaton et al., 2003; Grady et al., 2001), suggesting gymnopilins have a similar potency in the inhibitory effect on nAChR to hexamethonium.

It is known that nAChRs are widely expressed in the central and the peripheral nervous systems (Itier and Bertrand, 2001; Jones et al., 1999). The activation of this receptor leads to multiple effects depending on location in the body. The nAChR can be found at both perisynaptic and postsynaptic sites (Kalamida et al., 2007). The nAChRs

located in presynaptic and perisynaptic sites are responsible for regulating release of various neurotransmitters (Alkondon et al., 1997; Kalamida et al., 2007; Role and Berg, 1996; Wonnacott, 1997). It is also reported that the nAChR located in the postsynaptic site activates cellular functions such as excitation, gene expression, differentiation, and survival (Zouridakis et al., 2009). The actual distribution of gymnopilins in the body is not known. After gymnopilins are absorbed in the gastrointestinal tract, they would be delivered into the peripheral tissues by the circulation. Gymnopilins could then suppress the nAChR functions in the peripheral tissues. If gymnopilins were delivered into the brain passing across the blood-brain barrier, the inhibition of nAChRs might be a cause of some of the symptoms of the mushroom poisoning of patients who ingest *G. junonius*.

We have recently reported that gymnopilins activate two intracellular signal cascades including the phospholipase C-dependent one (Miyazaki et al., 2012) and the cAMP-dependent one (Nishio et al., 2012). In addition, here we report that gymnopilins act on the nAChR. This series of studies reveals that gymnopilins have various biological actions. One of the possible causes is the extract purified as gymnopilins in these studies consists of some different types of gymnopilins with different molecular structures (Aoyagi et al., 1983; Nozoe et al., 1983). It is also an important subject to identify what kinds of gymnopilins are included in the purified extract. We are focusing

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- 1 on this point and developing methods to separate different molecules. We would like to
- 2 clarify the mechanisms underlying various effects of gymnopilins in future studies.

5. Conclusion

The present results demonstrate that gymnopilins directly act on and inhibit the nAChR in PC12 cells. This inhibition by gymnopilins may be one of the mechanisms responsible for *G. junonius* poisoning. Although the full characterization of the toxicity of gymnopilins is incomplete, future studies with mammalian models would clarify the mechanisms of *G. junonius* poisoning. This report may provide helpful clues to understand the mechanism of *G. junonius* poisoning and may contribute to the development of new therapies for the poisoning.

Acknowledgments

We are grateful to Dr. Sathapana Kongsamut for critical reading of the manuscript, the valuable comments and correction of the language. This work was partly supported by Global COE Program (Advanced Utilization of Fungus / Mushroom Resources for Sustainable Society in Harmony with Nature), MEXT, Japan; the Grant from Hokuto Foundation for Bioscience; Grand –in–Aid for JSPS Research Fellow and JST, A-STEP feasibility study program (#AS242Z02021N). We would like to thank Ms. Yoriko Kaneta and Ms. Maki Taniguchi for the technical assistance to extract and purify gymnopilins.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Figure legends

Fig. 1. The structure of gymnopilins. A basic structure of gymnopilins is shown. Three different types of gymnopilins are reported: Gymnopilin 1, $m = 1$ and $n = 7-9$; gymnopilin 2, $m = 2$ and $n = 5-7$; gymnopilin 3, $m = 3$, $n = 5-6$. The calculated molecular weights are 1089.55 ($m = 1$, $n = 7$), 1175.68 ($m = 1$, $n = 8$), 1261.81 ($m = 1$, $n = 9$), 985.41 ($m = 2$, $n = 5$), 1071.54 ($m = 2$, $n = 6$), 1157.67 ($m = 2$, $n = 7$), 1053.53 ($m = 3$, $n = 5$) and 1139.66 ($m = 3$, $n = 6$).

Fig. 2. Effects of gymnopilins on $[Ca^{2+}]_i$ rises evoked by ACh in PC12 cells. A: A typical trace of the $[Ca^{2+}]_i$ responses to the repetitive stimulation with ACh (100 μ M). B: Summarized data of amplitudes of $[Ca^{2+}]_i$ responses to three sequential stimulation with ACh (100 μ M, $n = 59$). C: A typical trace of the $[Ca^{2+}]_i$ responses to ACh (100 μ M) in the absence and presence of gymnopilins (100 μ g/ml). Gymnopilins were applied 120 s before the 2nd application of ACh. D: Summarized data of amplitudes of $[Ca^{2+}]_i$ responses to ACh (100 μ M) in the absence and presence of gymnopilins ($n = 33$). The columns and vertical bars indicate the mean $\pm$ SEM. *, $P < 0.05$; ***, $P < 0.001$ by t test.

Fig. 3. Effects of gymnopilins on $[Ca^{2+}]_i$ rises evoked by DMPP in PC12 cells. A: A

1 typical trace of the $[Ca^{2+}]_i$ responses to the repetitive stimulation with DMPP (100 μ M).
 2 B: A typical trace of the $[Ca^{2+}]_i$ responses to DMPP (100 μ M) in the absence and
 3 presence of gymnopilins (100 μ g/ml). Gymnopilins were applied 120 s before the 2nd
 4 stimulation with DMPP. C: Summarized data of amplitudes of $[Ca^{2+}]_i$ responses to three
 5 sequential stimulation with DMPP (100 μ M, $n = 82$). D: Summarized data of amplitudes
 6 of $[Ca^{2+}]_i$ responses to DMPP in the absence (Ctrl) and presence of gymnopilins (Gym).
 7 The right column (W.O.) shows the amplitude of responses after washing out of
 8 gymnopilins ($n = 28$). E: Relative amplitudes of responses to the 2nd stimulation with
 9 ACh, nicotine (Nic), and DMPP in the presence of gymnopilins (100 μ g/ml) to those
 10 against the 1st stimulation. F: Relative amplitudes of responses to the 2nd stimulation
 11 with ACh in the presence of atropine are shown. The left and right columns show
 12 amplitudes of the ACh responses in the absence and presence of gymnopilins (100
 13 μ g/ml), respectively. The columns and vertical bars indicate the mean $\pm$ SEM. **, $P <$
 14 0.01; ***, $P < 0.001$ by t test. ††, $P < 0.01$ by ANOVA followed by Turkey-Kramer test.

15
 16 Fig. 4. The concentration-inhibition curve for gymnopilins on the amplitude of the
 17 $[Ca^{2+}]_i$ rise evoked by DMPP (100 μ M). Symbols indicate relative amplitudes of the
 18 DMPP responses in the presence of gymnopilins to that of the preceding responses in
 19 the absence of gymnopilins. The amplitudes are plotted against the concentrations of
 20 applied gymnopilins. Symbols and vertical bars indicate the mean $\pm$ SEM. The numbers

of the observations are 45, 70, 64, 24, 28 and 28 in the presence of presence of
gymnopilins at 0, 1, 3, 10, 30 and 100 $\mu\text{g/ml}$, respectively.

Fig. 5. The effect of gymnopilins on the DMPP-induced inward current recorded from
PC12 cells in the whole-cell configuration. The membrane potential was
voltage-clamped at -70 mV . A, B: Typical traces of the current responses to the
repetitive stimulation with DMPP ($100\text{ }\mu\text{M}$). In the cell shown in B, the 2nd stimulation
with DMPP was applied in the presence of gymnopilins ($100\text{ }\mu\text{g/ml}$). Gymnopilins were
applied 30 s before the stimulation with DMPP. C, D: Summarized data of amplitudes
of DMPP-induced currents in the absence (C, $n = 9$) and presence of gymnopilins (D, n
 $= 9$). The columns and vertical bars indicate the mean $\pm$ SEM. *, $P < 0.05$ by t test.

Fig. 6. The effect of gymnopilins on I_{Ba} in PC12 cells. I_{Ba} was evoked by a 50-ms pulse
from -90 to -10 mV every 20 s. The amplitudes of I_{Ba} are normalized by those just
before the application of gymnopilins, and the averages of relative amplitudes of I_{Ba} are
plotted against time ($n = 9$). Gymnopilins ($100\text{ }\mu\text{g/ml}$) were applied as indicated by the
horizontal bar (Gym). The inset indicates raw traces of I_{Ba} in the absence (black traces,
Ctrl) and presence (gray traces, Gym) of gymnopilins. The current remaining in the
presence of Cd^{2+} (1 mM) is also shown. Symbols and vertical bars indicate the mean $\pm$
SEM.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6