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Activation of Strychnine-Sensitive Glycine Receptors by Shilajit on Preoptic Hypothalamic Neurons of Juvenile Mice

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Abstract

Shilajit, a mineral pitch, has been used in Ayurveda and Siddha system of medicine to treat many human ailments, and is reported to contain at least 85 minerals in ionic form. This study examined the possible mechanism of Shilajit action on preoptic hypothalamic neurons using juvenile mice. The hypothalamic neurons are the key regulator of many hormonal systems. In voltage clamp mode at a holding potential of -60 mV, and under a high chloride pipette solution, Shilajit induced dose-dependent inward current. Shilajit-induced inward currents were reproducible and persisted in the presence of 0.5 μM tetrodotoxin (TTX) suggesting a postsynaptic action of Shilajit on hypothalamic neurons. The currents induced by Shilajit were almost completely blocked by 2 μM strychnine (Stry), a glycine receptor antagonist. In addition, Shilajit-induced inward currents were partially blocked by bicuculline. Under a gramicidin-perforated patch clamp mode, Shilajit induced membrane depolarization on juvenile neurons. These results show that Shilajit affects hypothalamic neuronal activities by activating the Stry-sensitive glycine receptor with $\alpha_2/\alpha_2\beta$ subunit. Taken together, these results suggest that Shilajit contains some ingredients with possible glycine mimetic activities and might influence hypothalamic neurophysiology through activation of Stry-sensitive glycine receptor-mediated responses on hypothalamic neurons postsynaptically.

Key Words: glycine, hypothalamic neuron, patch clamp, Shilajit, Strychnine

Introduction

Shilajit (Shilajeet or Salajeet), a mineral pitch, is a blackish-brown organic mass found in the steep rocks of the Himalayan region, and has a prominent history in traditional medicine systems of Nepal and India. Shilajit is obtained as a tar-like sticky substance from steep rocks of different formations found in high-altitude mountain ranges of Nepal, India, China, Bhutan and other Himalayan regions of Asia (13, 19). There are two distinct types of Shilajit, one as a semi-hard, brownish black to dark, greasy resin with a distinct coniferous smell and bitter taste, and is called gomuthira Shilajit. The other type, karpura Shilajit, is a white variety with camphor odor (31). Gomuthira Shilajit is again classified into four subtypes according to the predominance of the metal ore found in the mountains from where shilajit exudates (31). These four different varieties of Shilajit have been described in charka samhita, namely savrana meaning gold Shilajit with red color, rajat meaning silver Shilajit in white color, tamra meaning copper Shilajit in blue color, and lauha meaning iron-containing Shilajit in brownish-black colour (1). Of these, lauha Shilajit is the most

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commonly found and widely used (31).

Charaka Samhita, a great Ayurvedic text of Hindu traditional medicine system, describes Shilajit as a Rasayana (rejuvenator) and panacea for all diseases promising to increase longevity (28), and has been used as part of traditional systems of medicine in many countries since time immemorial (27). A number of studies have demonstrated that Shilajit has multiple biological effects, and is used in the treatment of diabetes, bronchial asthma, genitourinary infections and nerve disorders (9). Shilajit has also been found to: enhance learning acquisition and memory retention (14), help in immunopotentiating (12), have antistress activity (15), be antiallergic (13), reabsorb tumors and pimples (27), and be safe for use in pregnancy (2). Some recent reports also have elucidated that Shilajit has anti-inflammatory activity (16) and anxiolytic effects (19), and eliminates free radicals (5). Apart from these effects, Shilajit has been found to enhance fertility by improving spermatogenesis (8, 24). Despite the wealth of information available for treatment of various ailments by Shilajit, no information is so far available on the Shilajit-mediated current *via* membrane receptors, and its significant role on hypothalamic neurons. Here we provide evidence supporting the presence of Shilajit-mediated responses on postsynaptic neurons of preoptic hypothalamic area.

Materials and Methods

Animals

All experiments were approved by Chonbuk National University Animal Welfare and Ethics Committee. Juvenile male and female mut5 mice (17) were housed under 12-h light, 12-h dark cycles (lights on at 07:00 h) with access to food and water *ad libitum*.

Brain Slice Preparation

Brain slices were prepared as described previously (6). Briefly, male and female juvenile mice (5-20 day old) were decapitated and their brains were removed rapidly and placed in ice-cold bicarbonate-buffered artificial cerebrospinal fluid (ACSF) with the following composition: 126 mM NaCl, 2.5 mM KCl, 2.4 mM $CaCl_2$, 1.2 mM $MgCl_2$, 11 mM D-glucose, 1.4 mM NaH_2PO_4 and 25 mM $NaHCO_3$ (pH 7.3~7.4 when bubbled with 95% O_2 and 5% CO_2). Brains were supported with a 4% agar block and glued with cyanoacrylate to the chilled stage of a vibratome (Microme, Walldrof, Germany), where 150 to 250 μm-thick coronal slices containing the hypothalamus were cut. The slices were allowed to recover in oxygenated ACSF for at least one hour at room temperature.

Electrophysiology and Data Analysis

Slices transferred to the recording chamber were submerged and continuously superfused with carboxygenated ACSF at a rate of 4-5 ml/min. The slices were viewed with an upright microscope. Neurons from the preoptic hypothalamic area were randomly identified at 40X objective magnification and patched under Nomarski differential interference contrast optics. Patch pipettes were pulled from thin-wall borosilicate glass-capillary tubing (PG52151-4, WPI, Sarasota, USA) on a Flaming/Brown puller (P-97; Sutter Instruments Co., Novato, CA, USA). The tip resistance of the electrode was 4-6 MΩ. The pipette solution was passed through a disposable 0.22 μm filter and contained the following: 140 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, 4 mg ATP, 10 mM EGTA, adjusted to pH 7.3 with KOH. Whole-cell patch clamp recordings were performed using an Axopatch 200B (Axon Instruments, Union City, CA, USA). The cells were clamped at -60 mV after nullifying the junction potential between the patch pipette and bath solution. Membrane current changes were sampled online using a Digidata 1322A interface (Axon Instruments) connected to an IBM personal computer. For the current clamp recordings, the holding current was not adjusted during the experiment and was 0 pA. For the gramicidin-perforated mode, gramicidin (Sigma, St. Louis, MO, USA) was first dissolved in dimethylsulfoxide (Sigma) to a concentration of 2.5-5 mg/ml, and then diluted in the pipette solution, just before use, to a final concentration of 2.5-5 μg/ml, and sonicated for 10 min. Before backfilling the electrode with the gramicidin-containing solution, the tip of the electrode was loaded with a small volume of gramicidin-free pipette solution. The gramicidin-perforated patch recordings were performed using an Axopatch 200B amplifier (Axon Instruments). The tip resistance of the electrode was 4-6 Mohm. The junction potential between the patch pipette and bath solution was nulled before giga-seal formation. Access resistance was monitored and experiments begun when resistance stabilized at 50-90 Mohm. This typically took 15-20 min after giga-seal formation and always corresponded to the resting membrane potential (RMP) of the cell reaching a stable level below 45 mV. In all subsequent cells, drug application was started when the RMP reached a stable level below 45 mV. Spontaneous rupture of the seal was evident by a sudden overshooting of action potentials above 0 mV. Acquisition and subsequent analysis of the acquired data were performed using the Clampex 9 software (Axon Instruments). For current clamp experiments, any neurons that displayed a shift in membrane potential >2 mV upon Shilajit application were considered as responded (7). In the case of voltage clamp

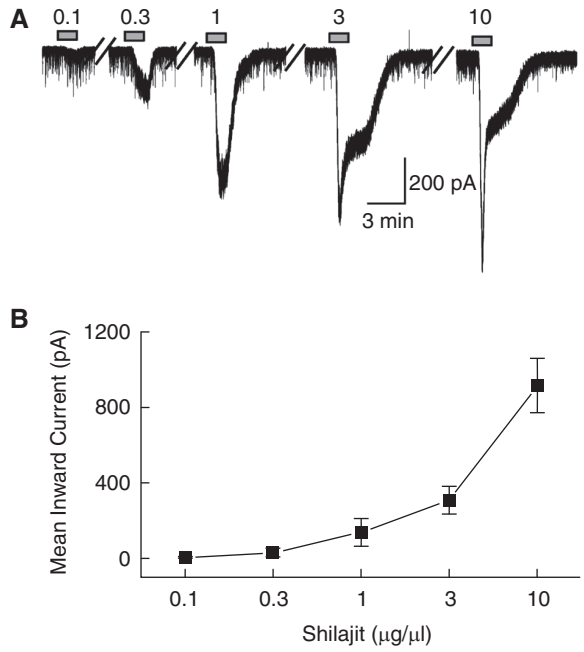

Fig. 1. Shilajit-induced concentration-dependent inward currents on preoptic hypothalamic neurons. (A) A representative trace showing inward currents induced by Shilajit at different concentrations (0.1, 0.3, 1, 3, 10 μg/μl). (B) The mean inward current induced by Shilajit at different concentrations.

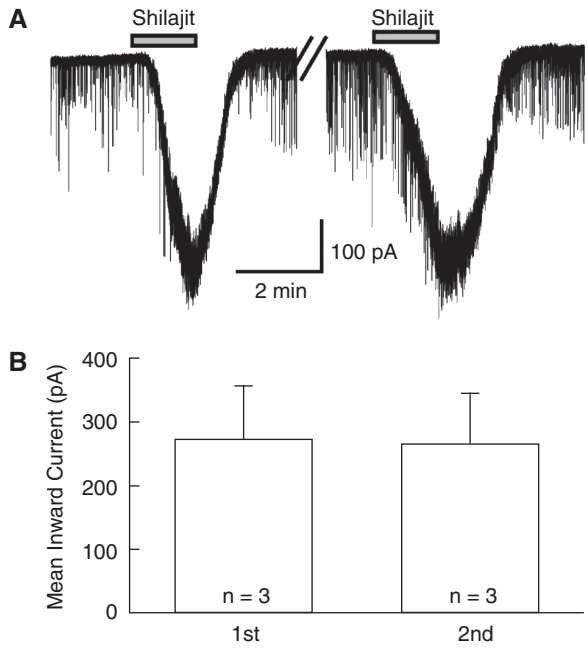

Fig. 2. Shilajit-induced non-desensitizing inward current on the hypothalamic neuron. (A) A representative current trace showing the induction of non-desensitizing repeated inward currents induced by Shilajit. (B) The mean inward current induced by Shilajit in the first and the second applications.

recordings, neurons that displayed a shift in holding current of >5 pA were considered to have responded (6). The traces were plotted using Origin 7 software (MicroCal Software, Northampton, MA, USA). All recordings were made at room temperature.

Chemicals

Shilajit was purchased from Dekha Herbals (Lalitpur, Nepal). Strychnine (Stry), a glycine receptor antagonist, and chemicals for ACSF were purchased from Sigma (USA), and tetrodotoxin citrate (TTX), a Na^+ channel blocker, was purchased from Tocris Bioscience (Bristol, UK). Shilajit was freshly prepared at a stock concentration of 100-300 mg/ml in ACSF and was further diluted in ACSF at a desired concentration just before the application. All the drugs and the ACSF were applied with the gravity flow system at a rate of 4-5 ml/min.

Statistical Analysis

All values are expressed as the mean ± S.E.M. A student *t*-test was used to examine the differences between the two experimental groups. *P* value < 0.05 was considered significant.

Results

Shilajit-Mediated Currents Exists on Preoptic Hypothalamic Neurons

Electrophysiological recordings were obtained from neurons in juvenile male and female mice brain preoptic hypothalamic slices. Under whole-cell voltage clamp (-60 mV), high chloride pipette solution conditions, application of Shilajit induced the dose-dependent inward currents in 7 neurons (Fig. 1A) at varying concentrations (0.1 μg/μl: -4.67 ± 3.6 pA, 0.3 μg/μl: -31.9 ± 21.0 pA, 1 μg/μl: -139 ± 72.2 pA, 3 μg/μl: -310 ± 72.5 pA and 10 μg/μl: -914 ± 142 pA, Fig. 1B). The inward currents induced by Shilajit at 3 μg/μl were reproducible (Fig. 2, A and B) (First application: -273 ± 84.2 pA n = 3; Second application: -265 ± 80.3 pA, n = 3, Fig. 2B) and the response by the second application was similar to that of the first application ($P > 0.05$, paired *t*-test, Fig. 2B).

Shilajit-Induced Currents on Preoptic Hypothalamic Neurons Are Action Potential-Independent

In order to investigate whether the Shilajit acts directly on postsynaptic hypothalamic neurons, Shilajit was applied in the presence of 0.5 μM TTX, a voltage-gated Na^+ channel blocker. TTX completely blocked the action potential-dependent neuro-transmission, but did not inhibit the Shilajit-induced inward currents

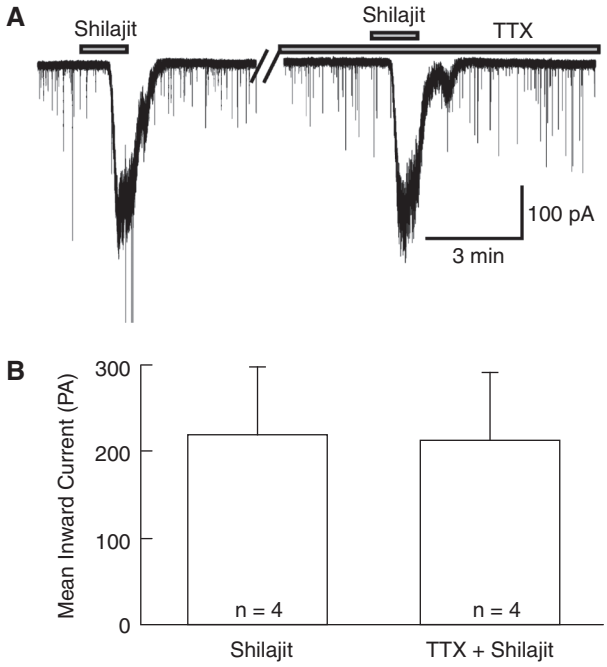

Fig. 3. Shilajit-induced inward currents were purely postsynaptic. (A) A representative trace showing the inward current induced by Shilajit at 3 mg/ml alone and in the presence of 0.5 μM TTX. (B) The mean inward current induced by Shilajit in the absence and presence of Shilajit ($P > 0.05$).

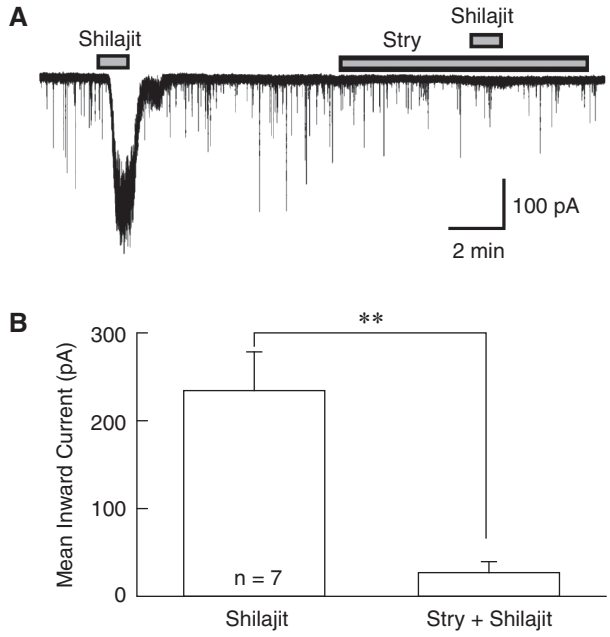

Fig. 4. Shilajit-induced inward currents were mediated by strychnine (Stry)-sensitive glycine receptors. (A) A representative trace showing the inward currents induced by Shilajit at 3 mg/ml alone and in the presence of 2 μM Stry. (B) Mean inward currents induced by Shilajit in the absence and the presence of Stry. (**$P < 0.01$ paired *t*-test)

in all 4 neurons tested (Control: -220 ± 78.9 pA, n = 4; TTX: -213 ± 78.9 pA, n = 4) ($P > 0.05$, paired *t*-test, Fig. 3, A and B).

Glycine Mimetic Action of Shilajit on Preoptic Hypothalamic Neurons

To shed light on Shilajit-induced action on hypothalamic neurons, the action of Shilajit was tested in the presence of 2 μM Stry, a potent glycine receptor blocker (32). Shilajit-induced responses were almost completely inhibited in the presence of 2 μM Stry (Fig. 4A). The Shilajit-mediated responses shown in Fig. 4A (first part) were almost completely blocked by 2 μM Stry (Fig. 4A, second part). The Shilajit-mediated inward currents (Control: -227 ± 51.1 pA; Stry: -14.6 ± 3.81 pA, n = 6, Fig. 4B; $P < 0.01$, paired *t*-test). The mean relative current induced by Shilajit in the presence of Stry was 0.06±0.01 of the control. As Stry completely blocked the Shilajit-mediated response, we used another antagonist, bicuculline, which partially antagonizes glycine receptors with the $\alpha_2/\alpha_2\beta$ subunit configuration (23, 29). Interestingly, the bicuculline partially blocked the Shilajit induced inward current (Fig. 5, A and B, n = 5; $P < 0.05$) suggesting the involvement of glycine receptor with the $\alpha_2/\alpha_2\beta$ subunits in Shilajit mediated inward current. The mean relative current induced by Shilajit in the presence of bicuculline with respect to Shilajit intact was 0.32 ± 0.05.

Physiological Action of Shilajit on Preoptic Hypothalamic Neurons

Using the gramicidin-perforated patch-clamp technique, recordings were made from 4 (postnatal d 9 to 17) immature neurons located at the preoptic area. In nearly all cases, only single-recorded cell was obtained for each mouse. Under the gramicidin-perforated patch-clamp mode, the mean resting membrane potential was -59 ± 2.9 mV. In immature mice, repeated applications of Shilajit at 3 μg/μl induced the membrane depolarization of 6.67 ± 1.96 mV and 6.15 ± 1.72 mV (n = 4) (Fig. 6, A and B, former part). The Shilajit-mediated membrane depolarization persisted in the presence of TTX (4.91 ± 1.86 mV; Fig. 6B; n = 3). These results suggest that the hypothalamic neurons were excited by application of Shilajit.

Discussion

In this study, we examined the effects of Shilajit on juvenile preoptic hypothalamic neurons, and showed its possible action at the hypothalamic level affecting

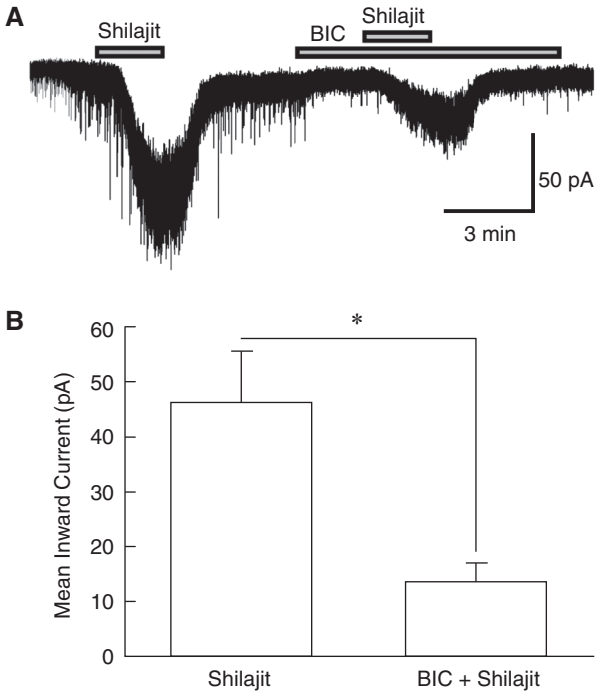

Fig. 5. Shilajit-induced inward currents were mediated by α_2 glycine receptors. (A) A representative trace showing the inward current induced by Shilajit at 3 mg/ml alone and in the presence of 20 μM bicuculline (BIC). (B) Mean inward current induced by Shilajit in the absence and the presence of bicuculline. (*$P < 0.01$ paired *t*-test)

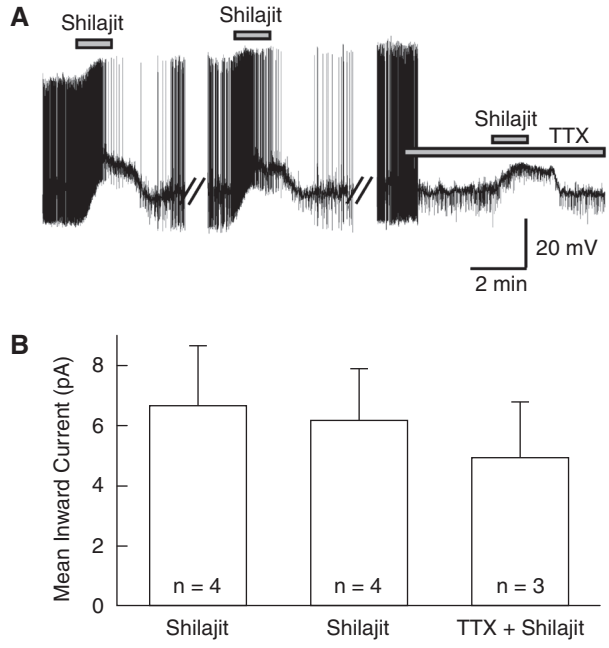

Fig. 6. Shilajit induced membrane depolarization. (A) Gramicidin-perforated recording showing robust membrane depolarization induced by Shilajit in the presence and the absence of 0.5 μM TTX. (B) The mean membrane potential changes induced by Shilajit in intact and in the presence of TTX.

various hypophysiotropic hormones release. In the high Cl^- pipette solution, Shilajit induced concentration-dependent, reproducible, non-desensitizing and short lasting inward currents. Persistence of Shilajit-mediated inward currents in the presence of TTX, a Na^+ channel blocker, further revealed that Shilajit acts directly on the glycine receptors expressed in the neuronal membrane or dendrites rather than on any presynaptic action potential-mediated action mechanism. In the gramicidin-perforated mode in which the intracellular milieu remains intact, Shilajit induced non-desensitizing membrane depolarization which persisted in the presence of TTX, suggesting that one of the constituents of Shilajit acts as a ligand on the glycine receptors expressed in the postsynaptic membranes of preoptic hypothalamic neurons. In addition, our results also demonstrated that Shilajit activated Stry-sensitive glycine receptors with α_2 and/or $\alpha_2\beta$ subunits on hypothalamic neurons. To the best of our knowledge, this is the first report of direct membrane effects of Shilajit at the hypothalamic level using the patch-clamp technique.

Furthermore, apart from metallic characterization, Shilajit was reported to contain more than 85 minerals in ionic form and also humic substances, mainly fulvic and humic acids (4). Ingredients like bioactive dibenzo alpha pyrones, along with humic and fulvic acids present in Shilajit, have been reported to induce physiological actions (12). Shilajit has been found to increase the plasma corticosterone levels and to decrease adrenal gland weight during chronic fatigue syndrome, and to prevent mitochondrial dysfunction by stabilizing the complex mitochondrial enzymes (30), and has been reported to show anxiolytic activity in rodents (19). Clinical research has confirmed that some ingredients of Shilajit are quickly absorbed through the intestinal tract and once in the systemic circulation, it can penetrate the blood-brain barrier (21). Our results suggest that there might be a possibility to inhibit the corticotropin-releasing hormone neurons by ingredients of shilajit *via* the glycine receptors.

Glycine plays an important role as an inhibitory neurotransmitter in the central nervous system (CNS) of various animals, and has been found to serve both inhibitory and excitatory functions in the CNS (11, 18, 20, 26, 33). Stry, a potent and widely used antagonist, reversibly suppresses the glycine current (3). On the other hand, existence of Stry-insensitive glycine receptor has been reported (10). In the present study, blockade of Shilajit-induced inward current by Stry suggests activation of Stry-sensitive glycine receptors on hypothalamic neurons. In mammals, glycine receptors are formed by a combination of five distinct

transmembrane protein subunits, one β subunit and four α subunit (α1-α4) (22, 25). In this study, to pharmacologically characterize the type of glycine receptors activated by Shilajit, Shilajit was applied in the presence of bicuculline, a γ-aminobutyric acid (GABA) receptor antagonist which partially antagonize glycine receptors with the $\alpha_2/\alpha_2\beta$ configuration (23, 29). The results indicated that Shilajit-induced inward currents were partially blocked by bicuculline at 20 μM, suggesting the presence of $\alpha_2/\alpha_2\beta$ glycine receptors. However, this blockade was not complete and the unblocked remainder implies activation of other types of Stry-sensitive glycine receptors. Further studies will be needed to determine the actions of the Shilajit on the hypothalamic pituitary axis.

In conclusion, Shilajit, a well-known Ayurvedic medicine, has postsynaptic response on preoptic hypothalamic neurons, and act *via* the Stry-sensitive glycine receptors. These results suggest that Shilajit can be a potent modulator of hypophysiotropic hormones acting directly on preoptic hypothalamic area. This study is a basic study aimed to elucidate the receptor types activated by Shilajit at the hypothalamic level. However, the effects of Shilajit on membrane receptors of the preoptic hypothalamic neurons in clinical pathogenic conditions are still unknown. Further studies will be needed to isolate and identify the compounds associated with these Shilajit-mediated actions as well as the action of those compounds in pathogenic conditions.

Acknowledgments

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