ORIGINAL RESEARCH ARTICLE EXPERIMENTAL

# Phasic and tonic type A γ-Aminobutryic acid receptor mediated effect of *Withania somnifera* on mice hippocampal CA1 pyramidal Neurons

*Janardhan Prasad Bhattarai, Seong Kyu Han*

*Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, 561-756, Republic of Korea*

## ABSTRACT

**Background:** In Nepali and Indian system of traditional medicine, *Withania somnifera* (WS) is considered as a rejuvenative medicine to maintain physical and mental health and has also been shown to improve memory consolidation. **Objective:** In this study, a methanolic extract of WS (mWS) was applied on mice hippocampal CA1 neurons to identify the receptors activated by the WS. **Materials and Methods:** The whole cell patch clamp recordings were performed on CA1 pyramidal neurons from immature mice (7-20 postnatal days). The cells were voltage clamped at -60 mV. Extract of WS root were applied to identify the effect of mWS. **Results:** The application of mWS (400 ng/μl) induced remarkable inward currents (-158.1 ± 28.08 pA, $n = 26$) on the CA1 pyramidal neurons. These inward currents were not only reproducible but also concentration dependent. mWS-induced inward currents remained persistent in the presence of amino acid receptor blocking cocktail (AARBC) containing blockers for the ionotropic glutamate receptors, glycine receptors and voltage-gated $Na^+$ channel (Control: -200.3 ± 55.42 pA, AARBC: -151.5 ± 40.58 pA, $P > 0.05$) suggesting that most of the responses by mWS are postsynaptic events. Interestingly, these inward currents were almost completely blocked by broad $GABA_A$ receptor antagonist, bicuculline- 20 μM (BIC) (BIC: -1.46 ± 1.4 pA, $P < 0.001$), but only partially by synaptic $GABA_A$ receptor blocker gabazine (1 μM) (GBZ: -18.26 ± 4.70 pA, $P < 0.01$). **Conclusion:** These results suggest that WS acts on synaptic/extrasynaptic $GABA_A$ receptors and may play an important role in the process of memory and neuroprotection via activation of synaptic and extrasynaptic $GABA_A$ receptors.

**Keywords:** $GABA_A$ receptors, hippocampal CA1 neurons, patch clamp technique, *Withania somnifera*

## INTRODUCTION

*Withania somnifera* (WS), which is commonly known as *Ashwagandha*, has been used in autochthonal medicine system i.e., Ayurveda of Nepal and India[1] since time immemorial. WS is also referred as Indian ginseng[2] and is also regarded as *Rasayana* (rejuvenative medicine) in Ayurveda.[3] Animal studies have shown that WS has profound effects on the hematopoietic system, acting as an immunoregulator and a chemoprotective agent.[4] Studies done in mouse have revealed that administration of a powdered root extract from WS enhanced total white blood cell count,[5] showed anti-aging effect,[6] and inhibited breast and colon cancer cell proliferation.[7] In addition, WS also reversed chronic stress-induced immunosuppression.[8]

Studies have shown WS to be effective in cardiovascular protection,[9] and in the treatment of hypothyroidism,[10] osteoarthritis,[11] inflammation,[12,13] stroke[14] and tardive dyskinesia.[15] Studies also reveal Ashwagandha to be a potential antimicrobial agent, with antifungal activity[16,17] and moderate antibacterial activity against *Staphylococcus aureus* and *Pseudomonas aeruginosa*.[18]

*Withania somnifera* also has variety of biological effects, including anti-inflammation,[19] cardioprotection,[20] thyroid hormone release,[21] cardiorespiratory endurance,[22] and

**Address for correspondence:**
Prof. Seong Kyu Han,
Department of Oral Physiology and Institute of Oral Bioscience,
School of Dentistry, Chonbuk National University,
Jeonju, 561-756, Korea.
E-mail: skhan@jbnu.ac.kr

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spermatogenesis.[23] WS also has wide range of effects on the central nervous system (CNS) including antistress,[24] anxiolytic[25] and anticonvulsant activity.[26] In addition, WS has been reported to have a neuroprotective role.[27,28] Kulkarni and Dhir (2008) discussed that WS could be used for the treatment of epilepsy, cognitive and neurological disorders, senile dementia, Alzheimer's and Parkinson's disease.[26]

The hippocampus is a workhouse of memory and is essential for converting short-term memory into long-term memory.[29,30] In particular, the CA1 region of the hippocampus is involved in this process.[30] Recently, our study showed that methanolic extract of *Withania somnifera* potentiate the NMDA-mediated responses on CA1 neurons with a little effect on glycine receptors.[31] WS has a neuroprotective effect in the hippocampal CA1 and CA3 sub-regions[27] and reduces the release of glucocorticoids in chronic stress.[3] Further, Mehta *et al.* (1991) reported that the root extract of WS inhibits the specific binding of $^{3}$[H]γ-amino butyric acid (GABA) in a rat brain mitochondrial and microsomal fraction and increases the influx of $^{36}[Cl^{-}]$ in the absence of GABA. Moreover, the $^{36}[Cl^{-}]$ influxes in mouse cultured spinal cord neurons were antagonized by $GABA_{A}$ receptor antagonists.[32] It was also revealed that WS induces GABA-mimetic action on hypothalamic GnRH neurons affecting reproductive physiology.[1] Recent study suggested that the GABAergic neurons can critically modulate the electrical activity of the cortex, hippocampus, septum and amygdala during the multiple consolidation process of memory storage.[33] Although, there are a number of studies suggesting the neuroprotective effects of WS in the hippocampus, the direct membrane effect of WS and the related ion channels are not completely understood. In this study, whole cell patch clamp technique was used to examine the direct membrane effect of mWS on CA1 pyramidal neurons. In addition, this study also aimed at figuring out the receptor type activated by mWS on CA1 neurons, which may put insight in role of mWS in memory enhancement and neuroprotection.

## MATERIALS AND METHODS

### Animals

All experiments were approved by Chonbuk National University Animal Welfare and Ethics Committee. Immature male mice (5-20 postnatal days) 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 and electrophysiology

Brain slices were prepared as described by earlier study[34] with slight modifications in position of brain used. Immature male mice (5-20 Postnatal days) were sacrificed by cervical dislocation between 10:00 and 12:00 h and their brains rapidly removed and placed in the ice-cold low calcium (0.5 mM), high magnesium (6 mM) bicarbonate-buffered artificial cerebrospinal fluid (ACSF)[1] (ACSF (in mM): 126 NaCl, 2.5 KCl, 0.5 $CaCl_2$, 6 $MgCl_2$, 11 D-glucose, 1.4 $NaH_2PO_4$ and 25 $NaHCO_3$ (pH 7.4 when bubbled with 95% $O_2$ and 5% $CO_2$)) equilibrated with 95%$O_2$/5%$CO_2$. Brains were then cut into 150 to 200 μm-thick coronal slices containing the hippocampal formation and incubated for at least 1 hour at 30°C in oxygenated ACSF of the following composition (in mM): 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 11 D-glucose, 1.4 $NaH_2PO_4$ and 25 $NaHCO_3$ (pH 7.4 when bubbled with 95% $O_2$ and 5% $CO_2$).

Individual brain slices were placed in a recording chamber and continuously perfused with ACSF at a rate of 3-4 mL/min. Individual CA1 pyramidal neuron was visually identified in the pyramidal layer of CA1, and subsequently approached using Nomarski differential interference contrast optics. Patch pipettes (4-6 MΩ) were pulled from thin-wall borosilicate glass-capillary tubing (outer diameter, 1.5 mm; inner diameter, 1.17 mm) (PG52151-4, WPI, Sarasota, USA) on a horizontal Flaming/Brown puller (P-97; Sutter Instruments Co., Novato, CA). The pipette solution was passed through a disposable 0.22 μm filter and contained the following (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 4 MgATP, 10 EGTA (pH 7.3 with KOH). The whole cell patch clamp recordings were performed under voltage clamp using an Axopatch 200B (Axon Instruments, Union City, CA). The cells were voltage clamped at-60 mV after nullifying the junction potential between the patch pipette and bath solution. The changes in membrane current were sampled online using a Digidata 1322A interface (Axon Instruments, USA). Acquisition and subsequent analysis of the acquired data were performed using Clampex 9 software (Axon Instruments, USA). The traces were plotted using Origin7 software (MicroCal Software, Northampton, MA). All recordings were made at room temperature.

### Extraction procedure

Extraction was done as previously described.[1] Briefly, the powder of WS root (Dekha herbals, Hattiban, Dhapakhel-1, Lalitpur, Nepal) was shaken overnight (24 h) in methanol (Sigma, St Louis, MO, USA) at a concentration of 100 mg/ml at room temperature. The supernatant was centrifuged twice at 8,000 rpm at 4°C for 15 min (Rotor NO. 9, Supra 22K, Hanil Science Industrial, Incheon, Korea). The supernatant was then evaporated at 50-55°C using a rotary evaporator (Laborata 4,000-*efficient*, Heidolph, Schwabach, Germany). The concentrated solution was frozen at −70°C overnight and freeze-dried using a

centrifugal vacuum concentrator (Hanil Science Industrial, Incheon, Korea). Finally, DMSO (Sigma, St Louis, MO, USA) was added at a concentration of 200 mg/ml. The extract stock was diluted (0.5-4 μl/ml, as per the doses used) in an ACSF bath solution prior to use.

### Chemicals and statistical analysis

Bicuculline (BIC), a broad $GABA_A$ receptor antagonist, and the chemicals for ACSF were purchased from Sigma (USA). Tetrodotoxin citrate (TTX), a $Na^+$ channel blocker, was supplied by Tocris bioscience (Bristol, UK). All values are expressed as the mean ± S.E.M. One-way ANOVA was performed to analyze more than two experimental groups. All pair-wise comparisons were performed by the *post hoc* Scheffé test using ORIGIN 7.0. Student's *t*-test was used to examine the differences between the two experimental groups. *P* of < 0.05 was considered significant.

## RESULTS

### mWS-induced dose-dependent inward current on CA1 neurons.

Whole cell currents were recorded from the pyramidal neurons in CA1 region of hippocampal slices from male immature mice brain. Under whole cell voltage clamp high chloride pipette solution conditions, mWS was applied in concentration dependent manner. Bath application of 50, 100, 200, 400 and 800 ng/μl of mWS revealed a clear concentration-dependent increase in the mWS-induced inward currents [Figure 1a]. The mean inward currents induced by mWS were 50 ng/μl: -13.6 ± 4.08 pA, 100 ng/μl: -37.1 ± 6.01 pA, 200 ng/μl: -82.4 ± 16.3 pA, 400 ng/μl: -225 ± 51.0 pA and 800 ng/μl: -484 ± 104 pA, respectively. The negative sign indicates inward current. The current induced at different concentration were found to be statically significant ($n$ = 7) ($P$ < 0.05, One-way ANOVA Figure 1b).

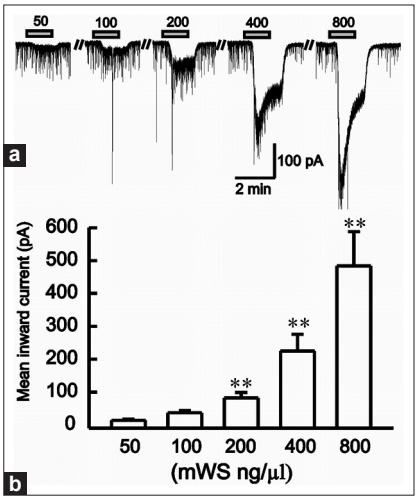


**Figure 1:** The methanol extract of Withania somnifera root induces potent concentration dependent inward currents on CA1 pyramidal neurons. (a), A representative current trace of a juvenile hippocampal CA1 neuron showing concentration-dependent increase in mWS-induced inward currents by 50, 100, 200, 400 and 800 ng/μl concentrations of mWS. (b), Cumulative bar graph shows the mean inward currents by mWS (50, 100, 200, 400 and 800 ng/μl) shown in figure a. (**$P$ < 0.01, One way ANOVA post-hoc Scheffé test)

### mWS-induced repeated inward current on CA1 neurons.

In another set of experiment we used two stimulation protocol to figure out whether mWS-induced inward current get desensitized on CA1 neurons. Inward currents induced by the application of mWS (400 ng/μl) were reproducible [Figure 2a] and the response by second application was similar to that of the first application. The mean membrane inward currents in the first and the second application were -120 ± 52.0 pA and -115 ± 53.9 pA, respectively. The mean relative current induced by second application with respect to first was 0.87 ± 0.05 [Figure 2b].

### Postsynaptic action of mWS

To check whether mWS acts directly on postsynaptic CA1 pyramidal neurons, the effect of mWS on the CA1 pyramidal neurons was investigated in the presence of amino acid receptor blocking cocktail (AARBC), containing the following: AP-5: NMDA receptor antagonist, 20 μM; CNQX: Non-NMDA glutamate receptor antagonist, 10 μM; strychnine: Glycine receptor antagonist, 20 μM; and tetrodotoxin (TTX, 0.5 μM), a voltage-gated $Na^+$ channel blocker. AARBC completely blocked the action potential dependent and independent transmission but did not inhibit the mWS-induced inward currents in all 5 CA1 pyramidal neurons tested. (Control: -200.3 ± 55.42 pA, $n$ = 5; AARBC: -151.5 ± 40.58 pA, $n$ = 5) ($P$ > 0.05, Figure 3a). The mean relative current induced by second

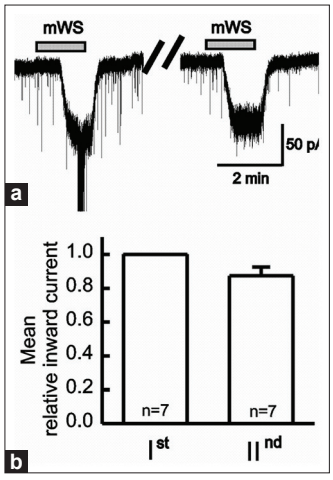


**Figure 2:** (a) Representative current trace showing the reproducible non-desensitizing effect of WS (400 ng/μl) on a juvenile CA1 neuron, (b), Bar graph shows the mean relative inward currents induced by mWS (400 ng/μl) ($P$ > 0.05, paired *t* test)

application in presence of AARBC with respect to first was 0.81 ± 0.09 [Figure 3b].

### $GABA_A$ receptor mediated phasic and tonic receptor activation by mWS

In this study, to determine if the mWS-induced inward currents were mediated by the $GABA_A$ receptor, mWS was applied in the presence of $GABA_A$ receptor antagonists. As shown in Figure 4a, the mWS-induced inward currents were blocked partially by gabazine-1 μM (GBZ) (Control: -85.23 ± 32.48 pA; GBZ: -18.26 ± 4.70 pA, $n$ = 8, Figure 4c), which blocks the synaptic $GABA_A$ receptors but not the extrasynaptic $GABA_A$ receptors.[35,36] The mean relative current induced by second application in presence of gabazine with respect to first intact application was 0.34 ± 0.07 [Figure 4c]. In addition, bicuculline (BIC, 20 μM), an antagonist for all types of $GABA_A$ receptors, was applied.[36] The mWS-mediated responses shown in Figure 4b (first part) were completely blocked by BIC (second part). The mWS-mediated inward currents (Control: -138.4 ± 50.18 pA; BIC: -1.46 ± 1.4 pA, $n$ = 5, Figure 4d) ($p < 0.001$). These results suggest that mWS acts on both synaptic $GABA_A$ and extrasynaptic $GABA_A$ receptors. The mean relative current induced by second application in presence of BIC with respect to first intact application was 0.028 ± 0.02 [Figure 4d]. Further, to evaluate whether tonic receptors remained active after removal of phasic events, CA1 pyramidal neurons were pretreated with GBZ to block the phasic receptors and then exposed to BIC that would additionally suppress tonic $GABA_A$ receptors [Figure 5a]. In the presence of GBZ, the outward shift in holding current was observed following application of BIC (4.58 ± 0.54 pA, $n$ = 5) [Figure 5c]. The magnitude showed a trend to be significantly different to that evoked in the presence of mWS [Figure 5b and c] (BIC, 21.7 ± 3.5 pA, $n$ = 4 $P < 0.01$), suggesting sub-population of CA1 neurons is subject to both phasic and tonic $GABA_A$ receptor activation by mWS, probably through synaptic and extrasynaptic $GABA_A$ receptors, respectively.

## DISCUSSION

In this study, bath application of mWS induced reproducible and short-lasting inward currents, which remain persisted in the presence of AARBC containing TTX, suggesting that mWS acted on the CA1 pyramidal neurons directly rather than through action potential-dependent or/and independent transmission. In addition, the inward currents induced by mWS were partly blocked by gabazine-1 μM a synaptic $GABA_A$ blocker and almost completely by BIC a $GABA_A$ receptor broad antagonist, suggesting that mWS exhibits GABA-mimetic activity via synaptic and extrasynaptic $GABA_A$ receptors.

These results provide evidence that mWS acts directly on the GABA-binding site of CA1 pyramidal neurons of hippocampus. In earlier studies as well, methanolic extract of WS root has been shown to inhibit the specific binding of [$^3$H] GABA and [$^{35}$S] TBPS, and enhanced the binding of [$^3$H] flunitrazepam to their putative receptor sites.[32] Further, WS was shown to increase [$^{36}$Cl]$^-$ influx in the absence of GABA.[17]

However, recently it is becoming clear that, in addition to fast inhibitory synaptic transmission, a second type of GABAergic signaling exists at the $GABA_A$ receptors in many locations within the nervous system,[37] including

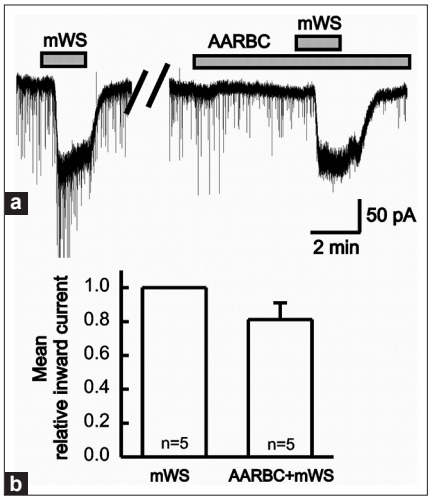


**Figure 3:** (a) The mWS-induced inward currents persisted in the presence of amino acid blocking cocktail containing: AP-5: an NMDA-receptor antagonist, 20 μM; CNQX: A non-NMDA glutamate receptor antagonist, 10 μM; strychnine: A glycine receptor antagonist, 20 μM; and tetrodotoxin (TTX, 0.5 μM), a voltage-gated $Na^+$ channel blocker. (b) Bar graph shows the mean relative inward currents by mWS (400 ng/μl) alone and mWS in the presence of AARBC ($P > 0.05$, paired $t$ test)

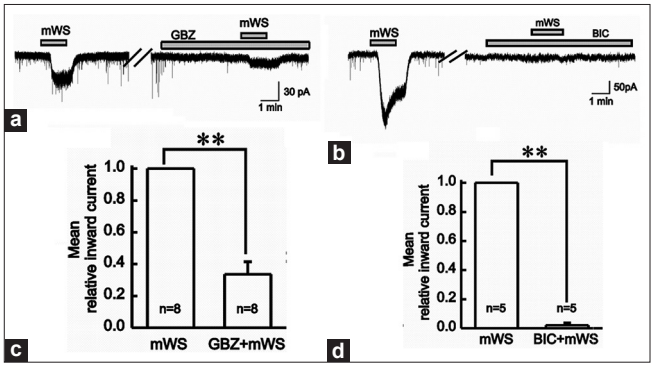


**Figure 4:** The mWS-mediated inward currents were through the GABAA receptors. A current trace of a juvenile CA1 neuron held at -60 mV, showing that the mWS (400 ng/μl)-mediated inward current was partially blocked by gabazine (GBZ, 1 μM), (a) and almost completely blocked by bicuculline (BIC, 20 μM) (b) a GABAA receptor antagonist. c,d. Bar graphs show the mean relative in current changes by mWS alone and mWS in the presence of BIC, (c) and GBZ, (d) (**$P < 0.01$, paired $t$ test)

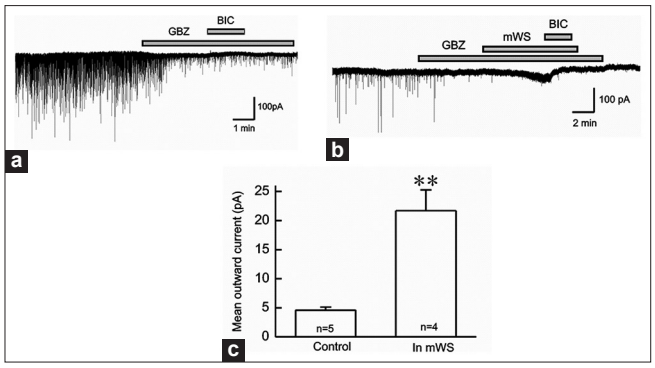


**Figure 5:** The mWS-mediated inward currents occurred partly through the extrasynaptic GABAA receptors. A current trace of a juvenile CA1 neuron held at -60 mV, showing presence of slight outward shift in the presence of gabazine (1 μM) and bicuculline (20 μM), (a) which got intensified in the presence of mWS, (b) Cumulative bar graphs show the mean outward current by BIC alone and BIC in the presence of mWS, (c) (**$P < 0.01$, *t* test)

the hypothalamus.[35,38] This activation, so-called tonic, results from the spillover of GABA from the synaptic cleft to activate extrasynaptic $GABA_A$ receptors and is a product of vesicular GABA spillover caused by the increased firing of GABAergic interneurons,[39] release of GABA from glial sources[40] or an impairment of GABA transporter (GAT).[41] These currents, exist in the form of a persistent inhibitory current that affects the resting membrane potential.[37] Hippocampal pyramidal cells also express a range of structurally diverse $GABA_A$ receptors in a domain-specific manner. Although the $\alpha_1$ and $\alpha_2$ $GABA_A$ receptors are largely synaptic, the $\alpha_5$ $GABA_A$ receptors are located extrasynaptically at the base of the spine and on the adjacent shaft of the pyramidal cell dendrites.[42] Activation of the $GABA_A$ receptors on CA1 by mWS may affect these $GABA_A$ receptor subtypes in the synaptic and extrasynaptic sites.

The mWS of root contains major biochemical constituents as withanolide A; lyceum substance B; withacoagin; Withanolide D; Withaferin A; withanolide glycosides called withanosides I, II, III, IV, V, VI, and VII, coagulin Q and physagulin D.[43] Further, phytochemical compounds screened in same *WS* root powder used in this study by GC-MS analysis shows the presence of alkaloid, sugar compound mainly glucose, fructose and sucrose followed by some sugar alcohols and plant fatty acids like palmitic acid, lenoleic acid and oleic acid.[44] In contest of neuroprotection, it has been illustrated that the oleic acid helps in neuronal proliferation. Medina and Tabernero reported that the oleic acid promotes axonal outgrowth, neuronal clustering and the expression of the axonal growth associated protein, GAP-43.[45] Further insight is needed to clarify the role of plant fatty acid on memory function. Furthermore, *WS* also has a remarkable property of neuroprotection reducing the stress-induced changes in the neuronal soma[28] and its derivatives facilitate neurite outgrowth.[46,47] Following this further, it has been known that $GABA_A$ receptor agonist can improve the survival of hippocampal CA1 neurons after global ischemia.[48,49] Overall, this study provides clear evidence that in addition to other actions of its derivatives, the mWS has a $GABA_A$ receptor activation effect and may play an important role in neuroprotection and neurite outgrowth via regulation and activation of the $GABA_A$ receptors. Though the $GABA_A$ has been shown to have neuroprotective effect, further studies in this direction are required. Although, the mechanism of neuroregeneration and cytoprotective effects of WS can be explicated by the action of $GABA_A$ receptor activation, it is possible that an increase in the corticosteroid level during stress might be associated with cell degeneration. Many authors have reported the neurotoxic effects of corticosterone on hippocampal cells[50] and WS has been found to down-regulate the stress-induced increase in the corticosterone level.[51] Furthermore, GABAergic transmission in the brain has been suppressed by stress and the stress-induced increase in corticosterone.[52] In reference to above studies and the effect of mWS suggest that there may be a relationship between the neuroprotection and $GABA_A$ signaling. Therefore, further studies will be needed to determine the actions of the mWS on intact CA1 pyramidal neurons as well as the biochemical constituents involved.

## CONCLUSION

This study demonstrates that mWS affects the CA1 neuronal activities by mediating all types of $GABA_A$ receptors i.e., synaptic and extrasynaptic $GABA_A$ on hippocampal CA1 pyramidal neurons. In addition, GABA being inhibitory neurotransmitter, it inhibits neuronal excitation by maintaining the synaptic and tonic inhibition and may facilitate in neuroprotection.

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