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Novel mechanisms for botanical effect

St. John's Wort enhances the synaptic activity of the nucleus of the solitary tract

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ABSTRACT

Objective: St. John's Wort (SJW) extract, which is commonly used to treat depression, inhibits the reuptake of several neurotransmitters, including glutamate, serotonin, norepinephrine, and dopamine. Glutamatergic visceral vagal afferents synapse upon neurons of the solitary tract (NST); thus, the aim of this study was to evaluate whether SJW extract modulates glutamatergic neurotransmission within the NST.
Methods: We used live cell calcium imaging to evaluate whether SJW and its isolated components hypericin and hyperforin increase the excitability of prelabeled vagal afferent terminals synapsing upon the NST. We used voltage-clamp recordings of spontaneous miniature excitatory postsynaptic currents (mEPSCs) to evaluate whether SJW alters glutamate release from vagal afferents onto NST neurons.
Results: Our imaging data show that SJW (50 μg/mL) increased the intracellular calcium levels of stimulated vagal afferent terminals compared with the bath control. This increase in presynaptic vagal afferent calcium by the extract coincides with an increase in neurotransmitter release within the nucleus of the solitary tract, as the frequency of mEPSCs is significantly higher in the presence of the extract compared with the control. Finally, our imaging data show that hyperforin, a known component of SJW extract, also significantly increases terminal calcium levels.
Conclusion: These data suggest that SJW extract can significantly increase the probability of glutamate release from vagal afferents onto the NST by increasing presynaptic calcium. The *in vitro* vagal afferent synapse with NST neurons is an ideal model system to examine the mechanism of action of botanical agents on glutamatergic neurotransmission.

Introduction

The nucleus of the solitary tract (NST) is located in the dorsal medulla and controls a number of homeostatic and behavioral functions including gastric motility, feeding, blood pressure, heart rate, and respiration. The NST controls these functions through axonal connections with parasympathetic premotor neurons in the medulla, direct and indirect connections with preganglionic sympathetic neurons, projections to oral motor segments of the reticular formation, and projections to the ventral forebrain and hypothalamus as well as the spinal cord [1]. Glutamatergic visceral vagal afferent terminals synapse upon second-order NST neurons [2–4], thereby controlling homeostasis through the connections made by the NST [5,6].

Over the past few decades, there has been increasing interest in the use of herbal remedies to treat disease. A 2007 National Health Interview Survey indicated that nearly 18% of American adults used a nonvitamin or nonmineral natural product in the previous year [7]. St. John's Wort (SJW; *Hypericin perforatum*) extract has been used for centuries to treat a number of disorders and is now available as an over-the-counter compound widely used to treat mild to moderate depression [8,9]. Although the cellular and molecular mechanisms of action of SJW in treating depression are not clear, it has been proposed that a number of

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KMV, GEH, and RCR designed the study and conducted the experiments. D. Ribnicky purified the St. John's Wort extract. All authors participated in writing the manuscript.

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different neurochemical pathways are influenced by the extract. SJW extract has been postulated to inhibit the neuronal uptake of neurotransmitters such as serotonin, norepinephrine, dopamine, γ-aminobutyric acid (GABA), and L-glutamate [10–13], alter noradrenergic and serotonergic receptor expression [14,15], and inhibit monoamine oxidase enzymatic activity [14].

St. John's Wort extract is composed of many biologically active compounds, including naphthodianthrones, flavonoids, prenylated phloroglucinols, tannins, phenols, and volatile oils [9,16,17]. The phloroglucinol derivative hyperforin is thought to be the major component of SJW extract that acts as an antidepressant, although the naphthodianthrone hypericin may also act as an antidepressant [9,10,16,18–20]. It is thought that hyperforin activates the nonselective cation transient receptor potential (TRP) channel TRPC6 to increase intracellular sodium and calcium content, therefore reducing neurotransmitter reuptake [21–24]. Indeed, previous studies on hyperforin suggest that the compound enhances miniature synaptic transmission in hippocampal CA1 and CA3 pyramidal neurons and alters dendritic spine morphology [21].

Because of SJW's potential effects on neurotransmitter release and synaptic transmission, we evaluated whether the extract influenced the excitability of glutamatergic vagal afferents synapsing upon the NST using live cell calcium imaging. We also evaluated whether SJW extract altered the probability of glutamate release from vagal afferents onto the NST neurons by recording miniature excitatory postsynaptic currents (mEPSCs). Finally, we evaluated whether hyperforin or hypericin could mediate the effects of SJW on glutamatergic neurotransmission.

Methods

Twelve male and female Long-Evans rats (weight 130–250 g) were used for these studies. Animals were obtained from the breeding colony at Pennington Biomedical Research Center, were maintained in a room with a 12-h light–dark cycle with constant temperature and humidity, and had access to food and water ad libitum. All experimental protocols were approved by the Institutional Animal Care and Use Committees of Pennington Biomedical Research Center and were performed according to the guidelines determined by the National Institutes of Health.

Vagal afferent labeling for calcium imaging

Visceral vagal afferents were labeled as previously described [25]. Briefly, a glass microinjection pipette pulled from 1.8 mm OD starbore capillary tubing (Radnoti, LLC, Monrovia, CA, USA) using a Narishige Model 1 D puller was filled with 20% CalciumGreen 1-dextran 3000 molecular weight conjugate (CG; Life Technologies, Carlsbad, CA, USA) reconstituted in 1% Triton X-100 and distilled water. Rats were anesthetized using 2.5% to 5% isoflurane. Using sterile technique, the right nodose ganglion was accessed through a ventral incision in the neck. Approximately 500 nL of the calcium reporter dye (CG) was microinjected through the sheath of the exposed ganglion using the filled micropipette connected to a Picospritzer (General Valve, Cleveland, OH, USA). The cervical wound was then closed, and the animal was housed in its home cage for 4 to 5 d to allow for anterograde transport of the dye to the vagal varicosities in the NST.

Brainstem slice preparation

The prelabeled animals were deeply anaesthetized using ethyl carbamate (urethane; 3 g/kg; Sigma, St. Louis, MO, USA). The brainstem was rapidly removed and glued to the stage of a vibrating microtome (Leica VT1200); the chamber was filled with cold (4°C) carbogenated (95% O_2/5% CO_2) cutting solution [26]. The brainstem was cut into coronal sections (300 μm thick), which were incubated at 32°C to 34°C in the cutting solution for 10 to 15 min. Brainstem sections subsequently were incubated at room temperature (22°C–24°C) for 1 to 5 h in carbogenated Krebs recording solution supplemented with 5 mM sodium ascorbate, 3 mM sodium pyruvate, and 2 mM thiourea and titrated to pH 7.4 with HCl [26].

Live cell calcium imaging

Live cell calcium imaging of CG-labeled vagal varicosities was performed as previously described [27]. Briefly, slices are placed in the recording chamber of a Nikon F1 fixed-stage upright microscope and perfused with carbogenated Krebs recording solution at 33°C with a 2.5 mL/min flow rate. A Prairie Technologies (Middleton, WI, USA) confocal head with a Photometrics CoolSNAP HQ camera (Tucson, AZ, USA) was used to perform time-lapse laser confocal calcium imaging. The CG-labeled varicosities were visualized using a 488 nm excitation/509 nm long-pass emission filter, and images were collected a rate of three frames per second. Adenosine triphosphate (ATP; 100 μM) was applied in the bath for 60 sec to activate $P2X_3$ ligand-gated cation channels on vagal afferent varicosities and test for the ability of the terminals to produce calcium signals [25]. Following a 10-min bath application of Krebs alone or SJW prepared in Krebs solution, ATP was reapplied for 60 sec; thus, each varicosity acted as its own control.

Patch-clamp recording from NST neurons

During the whole cell voltage-clamp recordings ($V_{HOLD} = -60$ mV), the slices were placed in the recording chamber of an upright microscope and were perfused with normal Krebs solution at 33°C with a 2.5 mL/min flow rate. Thin-walled borosilicate glass (Warner Instruments, Hamden, CT, USA) was used to form recording electrodes, which were filled with (in mM) 120 Cs-methanesulfonate, 15 CsCl, 10 tetraethylammonim chloride, 10 HEPES, 8 NaCl, 3 Mg-ATP, 1.5 $MgCl_2$, 0.3 Na-GTP, and 0.2 EGTA at pH 7.3 [28]. Recordings were made using a Multiclamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), filtered at 8 kHz, and were digitized at 20 kHz using Axon pClamp10 software.

In vitro solutions and drugs

The cutting solution contained (in mM) 92 *N*-methyl-D-glucamine, 30 $NaHCO_3$, 25 glucose, 20 HEPES, 10 $MgSO_4$-7 H_2O, 5 sodium ascorbate, 3 sodium pyruvate, 2.5 KCl, 2 thiourea, 1.25 NaH_2PO_4, and 0.5 $CaCl_2$, titrated to pH 7.4 with HCl [26]. The Krebs recording solution contained (in mM) 124 NaCl, 25 $NaHCO_3$, 10 glucose, 3 KCl, 2 $CaCl_2$, 1.5 NaH_2PO_4, and 1 $MgSO_4$-7 H_2O. The recording solution was supplemented with 10 μM bicuculline and 0.5 μM TTX, and the $CaCl_2$ was increased to 3 mM during the patch-clamp recordings.

The SJW extracts were prepared at the Rutgers University Botanical Research Center. The flowering herb *Hypericum perforatum* L. was greenhouse grown from seed and harvested at the flowering stage, freeze-dried, and stored at −20°C. The dried herb was extracted in 80% ethanol (1:20 w/v) at 50°C with sonication for 1 h followed by shaking at room temperature for 24 h. The solid material was removed by centrifugation at 3000*g*, and the solvent was subsequently removed by evaporation. The extracts were resuspended in 1000X (50 mg/mL) stocks in DMSO [29].

Data analysis

Nikon Elements AR software was used to analyze the confocal live cell fluorescent signal as previously described [25]. The relative changes in cytoplasmic calcium were expressed as changes in fluorescence [($\Delta F/F$)%] of the CG reporter dye, where F is the intensity of the baseline fluorescence signal before stimulation, and ΔF is the difference between the peak fluorescence intensity and the baseline signal. Briefly, the afferent fiber and varicosity regions of interest were outlined, and the background fluorescence was subtracted from the fluorescence signals before the relative changes in cytoplasmic calcium are calculated. Each varicosity acted as its own control, and data were evaluated for statistical significance using the paired t test; significance was set at $P < 0.05$. Data are reported as mean ± SEM.

Spontaneous mEPSCs from the whole cell voltage-clamp recordings from NST neurons were analyzed as previously described using the Mini Analysis Program (Synaptosoft, Inc., Decatur, GA, USA) [30]. The mEPSCs were collected over 2-min periods and were detected automatically. Only events with amplitudes >2.5 times the root mean square noise and rise times more rapid than 10 ms were included in the analysis. Selected mEPSCs from each recording were scaled and averaged, and the deactivation time constants were calculated by fitting the following single exponential equation to the data:

$$\text{Response} = \text{Amp} \exp(-\text{time}/\tau) \quad (1)$$

where τ is the deactivation time constant, and Amp is the current amplitude of the deactivation component.

Statistical significance of the distribution of mEPSC inter-event intervals was determined using the Kolmogorov-Smirnov nonparametric analysis, while the paired t test was used to evaluate amplitudes and deactivation time constants. Significance was set at $P < 0.05$. Data are reported as mean ± SEM.