Neurotropic and Trophic Action of Lion's Mane Mushroom *Hericium erinaceus* (Bull.: Fr.) Pers. (Aphyllophoromycetidae) Extracts on Nerve Cells *in Vitro*

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ABSTRACT: The neurotropic and trophic effect of extract obtained from the edible and medicinal mushroom *Hericium erinaceus* on nerve cells was studied. Spike reactions of hippocampal neurons during application of *H. erinaceus* fruiting bodies extract were studied on rat brain slices *in vitro*, using whole-cell patch clamp recording. Spike activity was inhibited in a concentration-dependent, reversible manner in 30%–90% of studied neurons by extracts obtained with ethanol, ether, or broth. Extract suppressed the excitation of neurons caused by L-glutamic acid application. The assumption is made that the *H. erinaceus* extract contains substances that may activate receptors that cause an inhibition of spike activity. The inhibitory effect of extract was not induced by GABA and serotonin receptor activation or activation of M- and N-cholinoreceptors. Inhibition of spike activity was caused by hyperpolarization of the neuronal membrane during extract application. The hyperpolarization was accompanied by an increase of apamin-sensitive Ca-activated K+ current (I_{Ca,AP}) and apamin-insensitive, slow Ca-activated K+ current (I_{Ca,SK}), but it was not caused by an increase of inward rectifier K+ current (I_{K1}) or by changes of hyperpolarization-activated cationic currents (I_{H}). The effect of the extract was observed in the presence of tetrodotoxin, suggesting that the extract acts postsynaptically. Extract application did not suppress biochemical processes in cell respiratory circuit. The extract did not affect the regenerating abilities of the neurons and glial cells of the cerebellum and hippocampus. It was demonstrated that the *H. erinaceus* extract concentratively exerted neurotropic action and improved the myelination process in the mature myelinating fibers, did not affect nerve cell growth *in vitro*, and did not evoke a toxic effect on nerve cell damage.

KEY WORDS: *Hericium erinaceus*, edible and medicinal mushrooms, extracts, neurons, brain slices, spike activity, cell cultures

INTRODUCTION

Recently, much attention has been given to the mechanisms of neurotropic action of medicinal mushroom components (Moldavan et al., 1999a–c, 2000a–c). One of the most promising mushroom species for use in medicine is *Hericium erinaceus* (Bull.: Fr.) Pers. It is a good edible cultivated mushroom and is widely used in Eastern traditional medicine (Dudka and Wasser, 1987). In Japan it is known by the name
ABBREVIATIONS

ACSF: artificial cerebrospinal fluid; AFSA: average frequency spike activity of neurons; BR: extract prepared on the basis of *H. erinaceus* mushroom broth; DMSO: dimethyl sulfoxide; ETIA: ethanol extract of *H. erinaceus*; 
ETHE: ether extract of *H. erinaceus*; I_{APP}: amphoteric Ca-activated K⁺ current; 
I_{AHP}: amphoteric-insensitive, slow Ca-activated K⁺ current; I(h): hyperpolarization-activated cationic currents; 
I(ir): inward rectifier K⁺ current; GABA: γ-aminobutyric acid; GABA_A receptors: A-type receptors sensitive 
to GABA; M-cholinoreceptors: muscarine-sensitive receptors; 
N-cholinoreceptors: nicotine-sensitive receptors; NMDA receptor: N-methyl-D-aspartate receptor; NGF: nerve growth factor; 
5-HT: 5-hydroxytryptamine (serotonin); 5-HT₁A, 5-HT₁D, 5-HT₆: the various types of serotonin receptors.

"Yamabushitake" and forms the basis for many medicinal preparations. Polysaccharides (1→3)-β-D-glucans (T-4-N and T-5-N), which are included in its structure, promote antitumor treatment as a result of their regulating influence on the immune system (Mizuno et al., 1992; Wasser and Weis, 1999). The mushroom primarily contains saccharides and proteins, some micronutrients, and a small quantity of lipids (Mizuno, 1995, 1998). It also includes not less than 18 amino acids and several vitamins (Mizuno, 1999). Hericenones C, D, E and Y-A-8-c, the substances that are extracted from the *H. erinaceus* mushroom, show nerve growth factor (NGF) synthesis-induction-promoting activity that opens new prospects in the treatment of Alzheimer’s disease (Kawagishi et al., 1990, 1991; Furukawa and Kawagishi, 1991; Mizuno, 1999). At the same time, the study of the neurotropic action of this mushroom has just begun (Moldavan et al., 1999c). In this respect, the purpose of the present work was to study the mode of action of an extract on spike activity, receptor activation, and ion currents of brain neurons, as well as on the growth and development of nerve cell cultures that have been cultivated on nutrition medium enriched with an extract.

MATERIALS AND METHODS

Cultivation of *Hericium erinaceus* and Preparation of the Dried Powder of Fruiting Bodies

Fruiting bodies of *H. erinaceus* used in these experiments were received from Weser-Champignon Ltd. (Germany). As a substrate, a mixture of beech sawdust (80%) and corn flour (20%) was used. Moistened to 65%, a substrate mix equal to 2 kg was packaged in polypropylene bags and autoclaved at 121°C for 1.5 hours. After cooling, substrate blocks were inoculated with stick spawn of *H. erinaceus* mushroom at 2 weeks’ growth. The substrate colonization period lasted 4 months at 25°C in darkness. After colonization, the substrate blocks were transferred to the fruiting room and incubated at 18°C, 95% relative humidity, under artificial illumination at an intensity of 800 lux.

Mature fruiting bodies were used for powder preparation. Fruiting bodies of the first flush were picked after beginning sporulation, dried at 50°C for 4-5 days, and ground in a petty-dispersion fraction (powder).

Preparation of *Hericium erinaceus* Extracts

The extracts were prepared by the three following methods:

1. **Ethanol Extraction Method.** The mushroom fruiting body powder was added to 95% ethanol in the ratio 1:10 to achieve a 10% tincture. At 10 days, the ethanol extract was separated from the insoluble part of the mushroom and evaporated at a temperature no higher than 40°C. Distilled water was added to reproduce the original volume, creating the water extract that was determined as an extract with 100% concentration. Extracts from 1-16% concentration were produced by adding artificial cerebrospinal fluid (ACSF) to the 100% extract.
The ethanol content in the extracts did not exceed 0.01% and did not cause changes in the spike activity of the hippocampal neurons. For application, only fresh water extract was used.

2. *Ether Extraction Method.* Mushroom powder was added to ether in a ratio of 1:10. The procedure was the same as in method 1.

3. *Distilled Water Extraction Method.* Mushroom fruiting body powder was added to distilled water in the ratio of 1:10 and left for 20 hours at room temperature. The infusion was heated to boiling and boiled for 5–7 minutes. The broth was covered for 30 minutes, then filtered cooled. The procedure continued as presented in 1.

The prepared extracts and broth during all procedures was kept at 4°C.

**Brain Slice Preparation and Technique of Neuronal Spike Activity Recording**

All experimental procedures with animals were carried out according to the Ethical Code of the International Scientific Societies on conducting experiments with use of animals, and all efforts were made to minimize pain and the number of animals used (Gigaty et al., 1988).

Wistar rats (weight 150 g each) were anaesthetized with ether. Rats were decapitated and the brain was quickly extracted and placed into ice-cold ACSF (0–3°C). The 400 μm-thick brain slices were prepared using a vibrating-blade microtome. At first, prepared slices were put into a vessel filled with ACSF that contained (in mM): NaCl, 124.0; KCl, 3.0; MgSO₄, 2.0; KH₂PO₄, 1.25; CaCl₂, 2.0; NaHCO₃, 26.0; glucose, 25.0; pH 7.4; and saturated with carbogen (95% O₂, 5% CO₂). Slices were transferred to a recording chamber following 4–6 hours preincubation in the above solution at 30°C. The neurons of hippocampus (paleocortex) present in the forebrain limbic system responsible for vegetative function regulation, motivational/emotional reactions, and memory were the object of these studies (Vinogradova, 1975). These cells' sensitivity to changes in extracellular liquid composition were extreme and much higher than in neurons in the neocortex and cerebellum (Arienshoek and Oerasing, 1983). The spike activity of individual neurons from the hippocampal stratum pyramidale (CA1 region) in brain slices was recorded in ACSF (control) and in *H. erinaceus* mushroom extract made on the ACSF basis followed by wash at 30°C at a flow rate of 2 ml/min in the chamber. Spike activity was recorded extracellularly by glass micropipettes with tip resistance of 5–12 MΩ filled with 2M NaCl. Recorded action potentials were converted by trigger into standard pulses, and a pulse flow was processed on a PC to present data as an average frequency spike activity (AFSA) histogram. The background spike activity was recorded for 3–4 minutes. Mushroom extract was applied for 2–4 minutes and washed for 5–20 minutes. The reactions of the neurons were ignored if their spike activity was reduced and was not restored during washout. When one neuron spike activity was recorded, the extract application was repeated a few times. The spike activity of individual neurons was recorded for 1–3 hours. Neuronal response was regarded as significant when the variation of its discharge frequency exceeded an average background activity of ±28.

To discover the receptor types of nerve cells activated during extract application, specific blockers of synaptic transmission were used. Atropine (100 μM, M-cholinoreceptor antagonist), bicuculline methiodide (10 μM, GABA₅ receptor antagonist), L-glutamic acid (100 μM), and GABA (100–300 μM) were purchased from Sigma (St. Louis, Missouri). Benzoxoxonium (hexamethonium benzoxoxalones, 1.7 mM, N-cholinoreceptor antagonist), serotonin (5-HT creatinine sulfate, 100 μM), and ritanserin (100 μM, serotonin 5-HT₆ receptors antagonist) were used. Ritanserin was dissolved in DMSO and then in ACSF. All substances were dissolved in ACSF to a final concentration before experiments.

**Method of Ion Current Registration on Single Neurons**

Wistar rats 18–28 days old were decapitated under halothane anaesthesia, and coronal hippocampal slices
(300–400 μm) were cut either with a tissue chopper (Mickle Laboratory, England) or a vibrating-blade microtome (Leica, Germany) and subsequently incubated in a humidified interface chamber at room temperature for ≥1 hour.

Tight-seal whole-cell voltage-clamp recordings were obtained using the “blind method” (Blanton et al., 1989). Patch electrodes (4–6 MΩ) were pulled from borosilicate glass (Hilgenberg, Germany) with a two-stage vertical puller (Narishige PP-830, Japan) and filled with standard intracellular solution containing (in mM): 140 potassium glutamate, 10 HEPES, 2 Na₂ATP, 0.4 Na₃GTP, 5 MgCl₂ (osmolality 280–300 mOsm, pH 7.3 with KOH). Drugs were either included in the intracellular solution or applied in the bath solution. Recordings were performed in a submerged recording chamber with a constant flow of ACSF, 2 ml/min, at room temperature. In all recordings, tetrodotoxin (TTX; 0.5 μM) and either bicuculline (10 μM) or picrotoxin (5 μM) were added to the superfusing ACSF. Neurons were电压-clamped at -60 mV, and 100–200 ms-long depolarizing pulses to 0–30 mV were delivered every 30 seconds. These pulses led to unclamped Ca²⁺ action currents sufficient to activate SlAM. Series resistance was estimated from 100 ms-long hyperpolarizing test pulses (-10 mV) and was monitored at regular intervals throughout the recording. Only recordings with stable series resistance ≤23 MΩ were included in this study. No series resistance compensation and no corrections for liquid junction potentials were made. Data were acquired using a patch-clamp EPC9 amplifier (HEKA, Germany), filtered with a -3 dB cutoff frequency at 250 Hz, sampled at 1 kHz, and stored on a Macintosh Power PC. Analysis was made using the programs Pulse and Pulseefer (HEKA), Igor Pro 3.01 (Wave Metrics, USA), and Excel (Microsoft, USA). We analyzed the amplitude, charge transfer (area enclosed by SlAM), and time course of SlAM decay. No discrepancies in the variations of these parameters upon pharmacological manipulations were observed. Therefore, we reported only the values concerning SlAM amplitude in the results. The amplitude of SlAM was determined at a point 700–900 ms after the end of the command pulse, where possible contamination by the partially overlapping apamin-sensitive I₅H₅ was negligible.

Technique of Hippocampal and Cerebellum Cell Culture Cultivation in Vitro. Analysis of Growth and Development of Neurons and Glial Cells

A 12-day dissociated monolayer culture of hippocampal and cerebellum cells was used to study the influence of H. erinaceus extract on nerve process growth and nerve cell development. To determine the effect of H. erinaceus extract on myelination process, a 26-day cerebellum cell culture was used. Cultivation was performed as described previously (Skibo and Koval, 1992; Fedoroff and Richardson, 1997). To get a cell suspension, newborn Wistar rats were anesthetized with ether, then the hippocampus and cerebellum were removed and dissected. The tissue was washed in Ca-Mg-free solution and put into Dulbecco’s minimal essential medium (Gibco, USA), supplemented with 10% fetal bovine serum (Intergen, USA), 10% horse serum (Gibco), and glucose (Sigma) (6 g/L), pH 7.2–7.4. H. erinaceus extract was added into culture medium (10%) on plating day as well as on the second and fourth day of cultivation (groups 1, 2, and 3, respectively). Cell suspension was dissociated thoroughly and plated on cover glasses (12 × 12 mm), previously coated with poly-L-lysine. Cells were placed into a CO₂ incubator, where they were incubated in an atmosphere of 5% CO₂ and 95% air at 37°C for 12 and 26 days. Nutrient medium was changed every 5 days. Experimental and control cultures were photographed every day to perform comparative morphological analysis.

To reveal myelinated fibers, 26-day cultures were stained by Sudan black dye. Cells were fixed with 4% paraformaldehyde for 12 hours and 0.1% OsO₄ for 1 hour; they were then dehydrated, stained with 0.5% Sudan black dye for 1 hour, rehydrated, and placed into a mixture of glycerine and gelatine. Stained tissue was observed under a phase-contrast microscope and photographed.

Mathematical and Statistical Analysis

Values are presented as mean ± standard error of the mean (SEM). For statistical analysis, the un-
paired, two-tailed Student's *t* test was used, and differences were considered statistically significant if *p* ≤ 0.05.

**RESULTS**

The spike reactions of 81 hippocampal neurons were studied during application of *H. erinaceus* extracts, which were prepared using ethanol (ETHA) or ether (ETHE) extractions and by preparation of broth from dried mushroom fruiting body powder (BR).

**Extract Action on Neuronal Spike Activity**

1. **ETHA Effect.** Reactions of 63 neurons were studied during application of ETHA. A total of 40 (63%) showed inhibitory reactions, 6 neurons (10%) displayed excitatory responses, and 17 cells (27%) did not respond (Fig. 1A). The parameters of inhibition were: latency, 53 ± 6 seconds; duration, 241 ± 25 seconds; and parameters of excitation, 35 ± 10 seconds and 253 ± 92 seconds, respectively (Table 1).

   The changes in spike activity during extract application were reversible. The spike activity was restored completely at washout and sometimes could exceed background level. The extract inhibited neuronal spike activity in a concentration-dependent, reversible manner. An increase of extract concentration from 1% to 16% led to an increase of duration and magnitude of inhibition of the tested neurons. An example of the reaction of such neurons is given in Figure 2A. The curve showing the concentration dependence of AFSA of neurons from extract concentration is given in Figure 2B. This curve repre-

![Graph A](image)

![Graph B](image)

![Graph C](image)

**FIGURE 1.** Ratio (in %) of neurons, reacted by inhibition and excitation on application of different kinds of extracts prepared from *Hericium erinaceus* fruiting bodies. N = quantity of CA1 hippocampal neurons in pyramidal layer studied during application of extracts prepared on the basis of broth (BR) and by extraction with ethanol (ETHA) and ether (ETHE). (A) ETHA application; (B) ETHE application; (C) BR application. Abscissa: total amount of neurons, in %; number of neurons reacting on extract application by inhibition (black), excitation (grey), unresponding (white), excitation followed by inhibition (striped), specified in %. Total quantity of neurons studied during application of each kind of extracts was taken as 100%.
ments the mean data of spike activity of 12 neurons that showed inhibitory reactions. The concentration-dependence curve has an exponential character, with IC\(_{50}\) = 3% extract concentration (IC\(_{50}\); concentration of tested substance at which the frequency of spike activity is reduced twice). Changes of AFSA correlated significantly with changes of extract concentration (p < 0.01; R = 0.86; N = 10). Changes of duration and delay of inhibitory reactions of studied neurons were tested using a wide range of extract concentrations. In a range of concentration from 2% to 4%, the duration of inhibition correlated with the duration of extract application. The duration of inhibition considerably exceeded the duration of extract application when concentrations increased to 8%–16%. The correlation was observed between duration of inhibition and extract concentration (p < 0.01; coefficient of correlation R = 0.85; N = 10).

2. ETHE Effect and Comparison of Intensity of Neuronal Spike Reactions During ETHE and ETHE Application. The reactions of 26 neurons were recorded during application of ETHE. A total of 9 neurons (34%) reacted by inhibition, 2 neurons (8%) by excitation, and 15 cells (58%) did not respond to ETHE application (Fig. 1B). The parameters of inhibition were: delay, 48 ± 13 seconds; duration, 227 ± 36 seconds; and parameters of excitation: 78 ± 41 seconds and 259 ± 46 seconds, respectively (Table 1).

Spike reactions of the same neurons (17 cells) were compared during application of ETHE and ETHE (Fig. 3). Usually, the effect of ETHE was stronger than the effect of ETHE (Fig. 3A and B). In some cases, ETHE inhibited a neuron, whereas ETHE did not show any effect (Fig. 3A). To estimate the force of ETHE and ETHE actions objectively, the value of extract action was estimated as a ratio (in %) of AFSA during extract application to a level of background spike activity. A background (control) level was taken as 100%. The data were averaged separately for cells showing inhibitory reactions and for neurons reacting by excitation. The mean AFSA for a group of neurons showing inhibitory reactions during ETHE and ETHE application was 48 ± 9% and 74 ± 7% of control, respectively. For the group of neurons responding by excitation during ETHE and ETHE application, the mean AFSA was 242 ± 32% and 135 ± 19% of control, respectively.

It is well known that neurons are very sensitive to a change in pH of the washing solution in the recording chamber. It was necessary to learn whether the distinctions in the neuronal responses at ETHE and ETHE action were explained by pH differences of these extracts. It was established that initial water extract undiluted in ACSF with substances extracted by ethanol or ether had a pH of 5.5. ETHE and ETHE diluted in ACSF up to 1–16% and pH 7.4 were used. Thus, the distinctions in ETHE and ETHE action could be explained by the effect of those substances, which were better extracted by ethanol or ether.

3. BR Effect and Comparison of Spike Reaction Intensity of the Same Neurons at Action ETHE and BR. The reactions of 21 neurons were studied during BR application. A total of 12 (57%) reacted only by inhibition, 7 (33%) showed complex reaction (excitation with subsequent inhibition), 1 (5%) reacted only by excitation, and 1 (5%) did not respond.

Table 1. Parameters of Neuronal Reactions on Applications of *Hericium erinaceus* Extracts with Ethanol (ETHE), Ether (ETHE), and Prepared as Mushroom Broth (BR)

<table>
<thead>
<tr>
<th>Type of extract</th>
<th>No. of neurons studied</th>
<th>Duration of extract application (in sec)</th>
<th>Delay of reaction (in sec)</th>
<th>Duration of reaction (in sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ETHE</td>
<td>63</td>
<td>185 ± 7</td>
<td>53 ± 6</td>
<td>241 ± 25</td>
</tr>
<tr>
<td>ETHE</td>
<td>75</td>
<td>240 ± 27</td>
<td>48 ± 13</td>
<td>277 ± 36</td>
</tr>
<tr>
<td>BR</td>
<td>21</td>
<td>172 ± 3</td>
<td>87 ± 19</td>
<td>339 ± 68</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>No. of neurons application (in sec)</th>
<th>Delay of reaction (in sec)</th>
<th>Duration of reaction (in sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>211 ± 32</td>
<td>35 ± 10</td>
<td>253 ± 92</td>
</tr>
<tr>
<td>238 ± 63</td>
<td>78 ± 41</td>
<td>259 ± 46</td>
</tr>
<tr>
<td>174 ± 4</td>
<td>26 ± 12</td>
<td>64 ± 22</td>
</tr>
</tbody>
</table>

Note: Data were averaged for all samples of neurons showing the respective reactions.
FIGURE 2. Concentration dependence of spike activity frequency of hippocampal neurons at various concentrations of *Hericium erinaceus* extract. A: histogram of average frequency spike activity (AFSA) changes of a neuron reacting by inhibition on application of extracts with 1%-15% concentration. Abscissa: time, minute; ordinate: amount of spikes per second. Lines with numbers showing extract concentration = interval of extract application. B: concentration dependence of neuronal AFSA from extract concentration. The means of AFSA for 12 neurons reacted by inhibition during extract application. Abscissa: concentration, %; ordinate: AFSA (spikes/s), the background spike activity was given as 100%. IC_{50} = 3% (IC_{50} = concentration of tested extract at which the frequency of spike activity was reduced twice).

Thus, the inhibitory component of the reaction was observed in 19 neurons (90%), and the excitatory component in 8 neurons (38%) (Fig. 1C). The mean parameters of inhibition were: delay, 87 ± 19 seconds; duration, 339 ± 68 seconds; and mean parameters of excitation, 26 ± 12 and 64 ± 22 seconds, respectively (Table 1). The reactions of 2 neurons are shown in Figure 3: 1 reacted by inhibition to BR application (Fig. 3C) and 1 reacted by initial excitation with the subsequent inhibition (Fig. 3D).

FIGURE 3. Comparison of neuronal reactions on application of *Hericium erinaceus* extracts, extracted with ethanol (ETH-A) and ether (ETH-E). Histograms of changes of spike activity average frequency of single neurons during extracts application. A, B: effect of ETH-A and ETH-E on the same neuron; C, D: inhibitory reaction and complex reaction consisting of initial excitation with the subsequent inhibition on BR application, respectively; E: effect of BR and ETH-A on the same neuron. Lines under an abscissa axis: represent the time of application of extract and used substances. Extract concentration, 8%. Other signs are the same as in Figure 2.
The force of ETHA and BR action on the same neurons (13 cells) was compared as in No. 2 (above). Data were averaged separately for groups of cells responding by inhibition and for neurons reacting by excitation. The mean AFSA for a group of neurons with inhibitory responses on ETHA and BR application was 39 ± 7% and 36 ± 7% of control, respectively. Only one neuron reacted by excitation on ETHA and BR application; thus, the AFSA produced 170% and 259% of control, respectively. Figure 3E shows that the same neuron reacted by excitation with the subsequent inhibition on BR application and by inhibition on ETHA application. Broth freezing up to −20 °C kept its activity and revealed the neurotropic action at defreezing.

Application of BR suppressed the neuronal excitation caused by l-glutamic acid application (Fig. 4). The effect of ETHA and ETHE on excitation evoked by l-glutamic acid was not studied.

4. Absence of Correlation Between Effects of Extract and Cell Respiration. The temporary termination of ACSF flow caused spike activity inhibition in 6 studied neurons, caused by reduction of entry to cell of carbogen (95% O₂ and 5% CO₂) and glucose dissolved in ACSF (Fig. 5). Such a phenomenon can occur at ischemic conditions and hypoxia. Four neurons from this group did not react on extract application. The tolerance of these neurons to the extract on the one hand, and the sensitivity to reduction of oxygen quantity in a washing solution on the other, indicate that the extract components did not suppress biochemical processes in circuits of cell respiration.

5. Physiological Mechanism of Extract Effect. Antagonists and agonists of different types of receptors were used to study the physiological mechanism of extract effect. Their effect was tested during 8% ETHA application. The action of bicuculline (GABA<sub>-</sub> receptor antagonist) on spike reactions caused by extract application was investigated in 8 neurons. Earlier it was shown that all these neurons reacted by inhibition on extract application. Bicuculline did not block reaction in 7 neurons (Fig. 6A). In 1 neuron, inhibitory reaction caused by extract application did not occur on the background of tonic activation evoked by bicuculline. However, after bicuculline action cessation, the inhibitory reaction caused by the extract appeared at maximum. Apparently, bicuculline, by blocking the GABA<sub>-</sub> receptors, caused excitation of the neurons and prevented inhibitory reaction development.

![Figure 4](image_url) **Figure 4.** Suppression of excitation caused by l-glutamic acid application by broth extract of *Hericium erinaceus*. BR, extract on the basis of broth from *H. erinaceus* mushrooms; Glu, l-glutamic acid (100 mM); Glu+BR, application of BR on a background of l-glutamic acid application. Lines under an abscissa axis: the application time of used substances. Other signs the same as in Figure 3.
FIGURE 5. Neuronal spike activity during Hericium erinaceus extract application and after reduction of oxygen in an artificial cerebrospinal fluid washing brain slice. ETHA, H. erinaceus extract. Extract concentration, 8%. Arrows designate time of termination and restoration of solution flow containing oxygen. Other signs are the same as in Figures 1 and 3.

The action of ritanserin (serotonin 5-HT2A/5-HT2C receptors antagonist) on reactions caused by extract application was investigated in 3 neurons. In 1 neuron the reaction did not change, in another the reaction was blocked partially (Fig. 6B); and in the remaining cell the reaction was blocked completely.

The group of neurons (10 cells) did not react (or reacted by excitation) during application of ETHA or ETHE. The responses of this group of neurons to application of 1 or 2 synaptic transmitters, such as serotonin and GABA, were studied (Fig. 6C). All 9 cells showed inhibition when affected by serotonin. A total of 4 neurons out of 7 showed inhibition, and others did not react during GABA action. Thus, neurons of the given sample reacted by inhibition on serotonin and GABA application but did not respond or showed excitation on extract application. Such neuronal responses testify to the different mechanisms of action of the investigated extracts and specified transmitters.

The application of benzogexonium (N-cholinergic receptor antagonist) (Fig. 7A) and atropine (M-cholinergic receptor antagonist) (Fig. 7B) did not block inhibitory reactions caused by extract application. In some cases, the blocking of M- and N-cholinoreceptors (as well as GABA_a receptors) not only did not block the response on extract application, but, on the contrary, extended its inhibitory action.

Extract Action on Nerve Cell Ion Currents

In the first set of experiments, the effect of H. erinaceus extracts on the resting membrane potential and firing properties of the neurons was studied using the current-clamp technique. A hyperpolarization of the membrane potential (–5–7 mV) and a decrease in the number of action potentials generated by prolonged depolarizing current injections in the 6 studied cells were always observed. In the second set of experiments, four different currents were studied using the voltage-clamp technique: (1) inward rectifier K⁺ current (I_ir); (2) I(i); (3) outward-sensitive Ca-activated K⁺ current (I_AHP); and (4) outward-insensitive, slow Ca-activated K⁺ current (I_AHP). Also in voltage clamp, an outward shift in the holding current, corresponding to a hyperpolarization of the membrane potential, was always observed. I(i) and I(i) were not apparently affected, whereas both I_AHP and I_AHP increased (n = 5). Both effects on the membrane potential and on the AHP currents were reversible upon washout of the extracts. The increase in AHP
current amplitude could explain the reduction in the number of spikes observed in current clamp. All these effects apparently did not have a synaptic origin, because they still were observed in the presence of tetrodotoxin.

**Action of Extract on Growth and Development of Nerve and Glial Cells**

According to obtained data, following mechanical dissociation, the resulting cell suspensions of control and treated cultures appeared under phase contrast microscope as rounded cell bodies without processes during the first day *in vitro*, although some cells had initial segments of processes. Cell adherence to the surface took place within 3-10 hours of cell plating. During the next 48 hours, the processes renewed or formation *de novo* was observed. By day 4 *in vitro*, neurons (usually of pyramidal or round bodies) and glial cells with well-developed processes made a dense interlacing network and were easily identified by different refraction under phase contrast microscope. Nerve processes grew and increased during culture maturation.

Comparative morphological analysis of groups 1, 2, and 3 (see Materials and Methods) and control cells in both cerebellum and hippocampus 12-day culture did not reveal any significant difference in nerve process growth and nerve cell development (Figs. 8A and 8B). *In vitro* maturation of all cells had approximately equal time and rate. A 10% *H. erinaceus* extract did not speed up or slow down culture development, nor did it show a toxic effect on the cells. The 26-day cerebellum culture was observed to analyze the myelination process in culture after the influence of *H. erinaceus* extract. Light-microscopic analysis of these cultures revealed mature myelinating fibers in stained tissue and showed that myelin sheaths in *H. erinaceus*-treated cultures were thicker than those in control samples (Koboshkin et al., 2003). Thus, it is possible to conclude that 10% *H. erinaceus* extract significantly influenced neuronal spike activity and improved the myelination process in the mature myelinating fibers, but it did not evoke a toxic effect or nerve cell damage and did not affect the nerve cell growth *in vitro*.

**FIGURE 6.** Neuronal reactions on application of *Histrichium erinaceus* extract, antagonists and agonists of synaptic transmission. A-C: reaction of three neurons on application of He, *H. erinaceus* extract; B, bicuculline (bicuculline, 10 μM, antagonist of GABA<sub>A</sub> receptors); R, ritanserin (ritanserin, 100 μM, antagonist of 5-HT/5-HT<sub>1c</sub> serotonin receptors); GABA (GABA, 300 μM); Ser, serotonin (serotonin (5-HT), 100 μM); B+He, extract application on a background of bicuculline; R+He, extract application on the background of ritanserin. Other signs are the same as in Figure 3.

**DISCUSSION**

Neurotropic components of *H. erinaceus* extract were well dissolved in water (pH 5.5) and kept their own effectiveness even 1 month after preparation (at T = 4°C). The study of the neurotropic effect of extract showed that neurotropic components of extract are extracted better by boiling mushrooms (during broth preparation) and by ethanol, but worse by ether. The mushroom broth showed neurotropic,
action at short-term boiling and at freezing up to -20°C. The force and character of the neurotropic action of *H. erinaceus* extract depend on the extraction method of its components. The ratio of neurons reacting to broth, ETHA, and ETHE application was 9:7:4. Upon the specified extract’s application, the inhibitory responses prevailed. The ratio of cells reacting by inhibition to the specified extracts was 9:6:3, and those reacting by excitation was 4:1:0.8. Thus, broth and ETHA caused stronger inhibitory and excitatory reactions in neurons than did ETHE. Some distinction in delay and duration of neuronal reactions during these kinds of extract applications apparently reflected their different chemical composition. Thus, the most effective was the action of mushroom broth, followed by the extract obtained with ethanol, then by the extract obtained with ether. The pH of applied extracts was the same (pH 7.4 at concentration 1%-15%), which excluded the possibility of an effect of pH changes on the pattern of neuronal spike activity. The extract inhibited neuronal spike activity in a concentration-dependent, reversible manner. The duration of inhibitory reaction significantly increased with an increase in extract concentration.

*H. erinaceus* contains glutamic acid (42.2 mg %) (Mizuno, 1999) that also should be found in its broth, because glutamic acid dissolves in water and is not destroyed by boiling; thus, the broth of fungi contains a significant amount of glutamate (the anionic form of glutamic acid) (Kinoshita et al., 2004). It should be expected that glutamic acid contained in broth (extracts) will evoke excitation of the studied neurons. However, the received experimental data did not reveal this effect of glutamic acid caused by extract; rather, on application of different kinds of extracts, 34%-90% of neurons reacted by inhibition, and only 5%-10% by excitation. We cannot explain this inhibitory effect of broth (extracts) by activation of metabotropic glutamate receptors by glutamic acid contained in the broth, because the bath application of L-glutamate evoked a strong excitation of tested neurons in the same experiments. Furthermore, the broth suppressed the neuronal excitation caused by L-glutamic acid applied in the bath. The assumption is that the *H. erinaceus* extract contains substances

**FIGURE 7.** Atropine and benzogoxenonium action on inhibitory reactions, caused by *Hericium erinaceus* extract application in the same hippocampal neuron. A, B: responses of 2 neurons on application: He, *H. erinaceus* extract; Benz+He, extract application on a background of benzogoxenonium (Benz) (benzogoxenonium, 1.7 mM, antagonist of N-cholinoreceptors); At+He, extract application on a background of atropine (At) (atropine, 100 µM, antagonist of M-cholinoreceptors). Other signs are the same as in Figure 3.
that may activate receptors that cause an inhibitory effect. The most common substances that usually evoke inhibitory reactions in the central nervous system are GABA, serotonin, and M- and N-cholinoreceptor agonists. To check the effect of extract on receptors activated by the substances indicated above, blockers of these receptors and extract were applied together. In addition, the effect of extract was compared with the effect of agonists of these receptors. It appears that inhibiting substances in *H. erinaceus* extract do not belong to GABA, serotonin receptors or to choline family substances. Some neurons that were not inhibited by *H. erinaceus* extract were inhibited by GABA and serotonin.

These data also showed that inhibition evoked by *H. erinaceus* extract could not be explained by its effect on GABA, or GABA receptors or on serotonin receptors. GABA was not found in *H. erinaceus* mushroom fruiting bodies (Mizuno, 1999), which explains an inefficiency of extract action on different types of GABA receptors. The extract components did not suppress biochemical processes in cell respiration circuits, because neurons that showed inhibition during hypoxia did not react to the extract application. Additional studies are needed to check the effect of neurotropic substances (especially amino acids) contained in extract to find the source of the inhibitory effect of extract on nerve cells.

Whole-cell current- and voltage-clamp recordings determined that inhibition of spike activity during extract application was caused by hyperpolarization of the neuronal membrane. This hyperpolarization was not caused by an increase of inward rectifier K+ current (I*(in))), which was not changed. Also, the hyperpolarization was not accompanied by changes of hyperpolarization-activated cationic currents I*(h), but aminergic-sensitive Ca-activated K+ current (I*Ca,K) and aminergic-insensitive slow Ca-activated K+ current (I*Ca,K1) increased. This study shows an important role of some K+ currents in the mechanism of extract effect on nerve cells. All the effects observed with this technique did not seem to have a synaptic origin and revealed the effect of extract on a cell membrane.

Two different cultivation terms were used to study the influence of *H. erinaceus* extract on nerve tissue. A 12-day culture was used to observe the nerve processes' development or renewal, and a 26-day culture was used to determine whether this extract affects myelination process *in vitro*. Selection of the 12-day term is explained by the fact that cells *in vitro* mature from process renewal to formation of the intercellular contacts' network and synapses over 10–12 days (Skibo and Koval, 1992). A longer cultivation term is necessary for myelogenesis *in vitro*, because myelin proteins in culture were produced only after the 19th day *in vitro* (Notterpek et al., 1993), and mature myelinated fibers were observed in 26-day cultures (Kotoluska et al., 2000). Selected cultivation terms allow testing the *H. erinaceus* extract's effect on different stages of cell development *in vitro*. The fact that there was no

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**FIGURE 8.** Neurons (arrowheads) and glial cells in 12-day-old cultures of hippocampus (A) and cerebellum (B) in the presence of *H. erinaceus* extract (phase contrast microscopy, magnification x20).
considerable difference in morphology among the control and test groups of cell cultures grown on nutrient medium containing 10% H. erinaceus extract may suggest that this extract does not affect the regenerating abilities of neurons and glial cells of the cerebellum and hippocampus during the first days in vitro, nor does it show any promoting or destructive influence on the cell maturation process during cultivation. The destructive influence of H. erinaceus extract on the myelination process was not observed either. On the contrary, the extract promotes faster myelogenesis in vitro in cerebellum cell culture, and these results correspond with our previous data (Kolotushkina et al., 2000, 2003). Electron microscopic analysis showed that the process of the myelin sheath formation in the presence of H. erinaceus extract proceeded faster and was complete by day 26 in vitro (in controls, it was finished by day 31). Only one extract concentration was used in our experiments (10%), so any influence in greater concentrations should not be ignored.

Thus, it was shown that the H. erinaceus extract exerts neurotropic action and improves the myelination process in mature myelinating fibers in that concentration, did not affect nerve cell growth in vitro, did not evoke toxic effect, and did not cause nerve cell damage. These data allow a better understanding of the mechanisms of the neurotropic and neurotrophic action of H. erinaceus extract and help to design additional approaches for their study. However, it is very important to learn: (1) What kinds of substances contained in the extract evoke inhibition of neuronal spike activity and hyperpolarization of cell membrane? (2) What kinds of currents (other than K⁺ currents studied) are affected by the extract in a cell membrane? (3) Could the extract show presynaptic effect and does it influence Ca²⁺ influx in axon terminals? (4) Could the extract application alter glutamatergic and GABAergic synaptic transmission? (5) Could the H. erinaceus extract influence the regenerating abilities of neurons and glial cells and show any promoting or destructive influence on the cell maturation process during cultivation in the nutrient medium containing a large concentration of extract?

ACKNOWLEDGMENT

The authors express gratitude to Dr. F. Dohme (Wester-Champignon Ltd., Hessisch-Oldendorf, Germany) for assistance in Hericium erinaceus cultivation, for the fruiting bodies, and also for rendering technical support.

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