Study on electrophysiological properties of neurons from a rat cortex model under the influence of the phytoalkaloid compound Berberine

Final Master Thesis

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1. SUMMARY

The research “Study on electrophysiological properties of neurons from a rat cortex model under the effects of phytoalkaloid compound Berberine” was carried out by Miguel Solis Gonzalez. This research was aimed to analyse and summarize the information related to berberine and its effect in cortical neurons.

To reach the aim of the thesis, 5 major objectives were stated: (1) Prepare viable neuronal culture from newborn rats. (2) To analyse published data on Alzheimer’s disease and on Berberine compound. (3) To prepare and evaluate electrophysiological properties of the 2-week-old mixed neuron-glia in vitro cultures before and after berberine injection. (4) To analyse the electrophysiological response in the presence and absence of berberine and to compare data with that available from the literature. (5) To evaluate the effects of berberine derivatives exposed in previous studies and to compare these findings with the effects of the berberine compound extracted directly from the plant.

The method that was used to analyse and record this activity was the voltage clamp technique commonly known as patch-clamp recording. The electrodes were clamped on the cell membrane that were previously dissociated from newborn rat pups and cultivated for two weeks in in vitro cell culture conditions in order to obtain mature neuronal network. Electric current was injected into the neuron of interest through an amplifier and spontaneous network activity was recorded regarding threshold potential characterised by the network properties.

Some other characteristics were evaluated before and after introduction of BBR during the study, including spike amplitude and relative refractory period.

BBR did not interrupt the signal transmitted by the neurons and it did not enhance neuronal network activity during evaluation of the activity. However, it produced an interference in current injection, where neurons needed higher amount of current to achieve threshold potential. Yet results are not conclusive and it was not possible to evaluate if the function of BBR in the treatment of neurodegenerative diseases is related to only the anticholinergic inhibition and anti-inflammatory process or also there is enhancement on the conductivity itself.

A good next step to be considered would be to record the electrical current in cortical neurons impregnated with Aβ amyloid plaques, and its comparison with affected cells under BBR influence.
2. CONFLICTS OF INTEREST

The author reports no conflicts of interest.

3. ETHICS COMMITTEE CLEARANCE

This research has state food and veterinary permission to work with laboratory animals. Permission issued on 2012-09-12, number 0239. LUHS neuroscience institute veterinary permission number is LT 61-19-001.

4. ABBREVIATIONS LIST

ACh: Acetylcholine
AChE: Acetylcholinesterase
AD: Alzheimer’s disease
APP: Amyloid precursor protein
Aβ: Amyloid beta
BACE: Beta-site amyloid precursor protein cleaving enzyme
BBR: Berberine
ChEI: Cholinesterase inhibitor
COX-2: Cyclooxygenase 2
CRP: C reactive protein
EDTA: Ethylenediaminetetraacetic acid
GBSS: Gey’s balanced salt solution.
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IL: Interleukin
5. INTRODUCTION

Alzheimer's disease (AD) is one of the most studied neurodegenerative disorders nowadays. Although treatment yet remains palliative and temporary some new treatment therapies evolve; therefore, further studies of the disease and its treatment strategies remain actual and important.

Though there is no effective treatment available till date, inhibition of acetylcholine (ACh) breakdown by blocking the acetylcholinesterase (AChE), has been proved to be helpful in slowing down the disease progression. Berberine (BBR) is a compound that acts as an anticholinergic drug having similar properties as Galanthamine (a FDA approved cholinesterase inhibitor (ChEI) used for the sustainment of AD). [1]

BBR is an isoquinoline alkaloid produced by a variety of plants, such as *Hydrastis canadensis*, *Berberis aristata*, *Coptis chinensis*, *Coptis rhizome*, *Coptis japonica*, *Phellodendron amurense* and *Phellodendron* chinese schneid [2]. It’s been used in Chinese traditional medicine for centuries in the treatment of many different pathologies including diabetes and inflammatory processes. Recently, many studies have been performed to understand the mechanism of action and scientific properties of this compound. One of these characteristics is the cholinergic effect in neurodegenerative diseases such as Alzheimer's disease, by inhibiting the AChE enzyme.

It has also been proven that BBR alters conductivity of cells from different tissues. Therefore, the electrophysiological properties of the neurons should be evaluated to understand the effect of BBR to the neural network and to determine how the conductivity of these cells is modified.
The aim of this research is to analyse and compare data obtained during the laboratory research and to collect and summarize all the data that has been found in previous studies about BBR effect in electrophysiology of different cells, focusing on the neuronal network.

6. AIM AND OBJECTIVES OF THE THESIS

It is important to recognize the effect of the BBR isochloride in healthy neuron population to be able to understand its effect on AD type of dementias for the possible treatment approach. Therefore, experimental data that was collected was also compared to that of other researchers collected from both scientific papers as well as monograph published. Rat cortical neuron mixed glia-neuron population have been grown in the laboratory and studies by voltage clamp technique were performed for this purpose. Spontaneous activity generated by the cells under the influence of BBR was measured during this study.

The aim of the study was to analyse current treatment strategies for Alzheimer’s type dementia and to evaluate possible modes of action of the phytoalkaloid BBR in healthy rat neuron-glia cultures in regard to observe the electrophysiological properties of the neurons. To reach the aim 4 major objectives were stated:

1. Prepare viable neuronal culture with papain from newborn rats and assess quality of the media during its growth in laboratory.

2. To analyse published data on the current hypothesis of AD development, progression and treatment and on BBR compound as a treatment of several diseases, focusing on its effects in the nervous system.

3. To prepare and evaluate electrophysiological properties of the 2-week-old mixed neuron-glia in vitro cultures prepared from newborn rat pups in control conditions as well as after introduction to BBR.

4. To analyse the electrophysiological response in presence and absence of BBR and to compare data with that available from the literature.

5. To evaluate the effects of BBR derivatives exposed in previous studies and to compare these findings with the effects of the BBR compound extracted directly from the plant.
7. LITERATURE REVIEW

7.1. Background

BBR, an isoquinoline alkaloid existing in *Berberis Vulgaris*, *Cortex phellodendri* (Huangbai) and *Rhizoma coptidis* (Huanglian), has a long history in China as a non-prescription drug. In recent years, many studies have indicated that BBR has multiple pharmacological effects [3], including the inhibition of AChE in AD. Alzheimer’s is the most common cause of dementia; affecting approximately 35.6 million people worldwide in 2012 [4]. It occurs in elderly, and diagnosis is performed clinically. From a histological viewpoint, the progression of AD is associated with 3 neuropathological hallmarks: the accumulation of extracellular senile amyloid-beta (Aβ) plaques, intracellular neurofibrillary tangles (NFT) and synaptic degeneration [5]. It is important to take into consideration the pathogenesis in AD in order to understand how BBR might be beneficial.

Until recently, two major hypotheses have been proposed regarding the molecular mechanism of pathogenesis in AD: the cholinergic hypothesis and the amyloid cascade hypothesis. [6]

Amyloid cascade hypothesis posits that the deposition of the amyloid-β peptide in the brain is a central event in AD pathology. [7] Aβ assemblies can be seen as Aβ-derived diffusible ligands, which are small oligomers and their molecular weights range from 1 to 42 kDa. The aggregation and accumulation of extracellular and intracellular Aβ40/42 in brain parenchyma impairs synaptic plasticity and memory. Aβ40/42 is generated by BACE-mediated (beta-site amyloid precursor protein cleaving enzyme) and γ-secretase-mediated sequential cleavages of amyloid precursor protein (APP). Inhibition of the production of Aβ40/42 can be expected to delay the development of AD. [3]

Aβ plaques are frequently associated with both reactive astrocytes and activated microglia, plus neuroinflammatory markers such as interleukins (IL), tumor necrosis factor-α (TNF-α) and C-reactive protein (CRP). [8] Therefore, neuroinflammation plays a fundamental role in the progression and appearance of these plaques during the degenerative process in AD.

On the other hand, the pathogenesis of AD is linked to a deficiency in the brain of ACh. Cholinergic-deficiency theory claims that due to non-regenerable nature of neurons, the levels ACh neurotransmitter produced by neuronal network declines. This is known as cholinergic-deficit hypothesis for the AD (*Fig. 1*). [1] [9]
Figure 1 Schematic diagram of a neuron representing (A) alterations in neurotransmission in Alzheimer’s disease and (B) the hypothetical mode of action of AChE inhibitors. Key to figure (A): (1) reduced cortical cholinergic innervation; (2) reduced corticocortical glutamatergic neurotransmission due to neuron or synapse loss; (3) reduced coupling of muscarinic M1 receptors to second messenger system?; (4) shift of tau to the hyperphosphorylated state—precursor of neurofibrillary tangles; (5) reduced secretion of soluble APP; (6) increased production of amyloid protein; (7) decreased glutamate production. *It is hypothesised that these changes give rise to the clinical symptoms of Alzheimer’s disease and contribute to the spread of pathology.12 49 54 Key to figure B: (1) AChE inhibitors reduce the breakdown of endogenously released ACh, resulting in greater activation of postsynaptic ACh receptors; hypothesised consequences: (2) reduced phosphorylation of tau; (3) secretion of sAPP returned towards normal; (4) reduced â-amyloid production; (5) glutamatergic neurotransmission returns towards normal, possibly due to activation of muscarinic and nicotinic receptors. ACh=acetylcholine; mAChR=ACh muscarinic receptor; APP=amyloid precursor protein; AChE=acetylcholinesterase; nAChR=ACh nicotinic receptor; Glu=glutamate [9]

7.2. Acetylcholinesterase therapy

AChE is mainly present in the central nervous system and its principle role is to catalyze the hydrolysis of the ACh to choline. This process is important in recovering activated cholinergic neuron back to its resting state, thus AChE is an important pathogenic factor of AD [10]. Nowadays, inhibition of ACh breakdown is the only known palliative treatment in AD; there are no other known methods in slowing down the disease till date.

The first ChEI available and effective against AD was approved by the FDA in 1993 by the name of Tacrine. Currently only few other ChEIs such as donepezil, galanthamine or rivastigmine are generally used for treating cognitive symptoms in persons with mild to moderate AD. [1]
The risk of developing newer ChEIs is that they will need to be more effective than donepezil and rivastigmine. [11] Future research in this class will need to focus on whether ChEIs directly affect the pathophysiology of AD.

Recently, numerous studies have been performed supporting the correlation between AD, neuroinflammation, and oxidative stress (reactive oxygen species (ROS) such as H₂O₂, -OH or reactive nitrogen species (RNS), e.g. -ONOO) [6] Therefore, several activities relevant to anti-AD approach have been proposed: anti-inflammatory, nicotinic receptor-stimulating, and antioxidant effects.

### 7.3. The isoquinoline alkaloid berberine as anti-Alzheimer drug

BBR has proven its effect in AD in many different pathways. First, due to its chemical structure (Fig. 2) BBR can pass through the blood-brain barrier and reach the brain parenchyma in a dose- and time-dependent manner, [3] which makes BBR more suitable to treat neurodegenerative disorders. Furthermore, BBR is an acetylcholinesterase inhibitor similar to Galanthamine, and might be a low-molecular-weight neurotrophic drug to neurodegenerative disorders by potentiating the nerve growth factor (NGF)-induced differentiation in neural cells. [12]

It has been demonstrated that BBR exerts anti-inflammatory effects by inhibiting arachidonic acid metabolism and the production of some inflammatory factors including cyclooxygenase-2 (COX-2), IL-1β, TNF-α, IL-6 and inductible nitric oxide synthase (iNOS). [12] [13]

Moreover, BBR has a very powerful antioxidant properties against ROS and RNS species leading to reduction of these factors. [14]

Possible mechanisms by which BBR modifies metabolism of APP are described in Fig. 3. It has also been reported a restraining effect in the hyperphosphorylation of tau protein, which is responsible for the formation of NFT. [13]

Nevertheless, it has been postulated that potassium current play a very important role in memory processing [15]. In a study performed by the Tongji Medical College of Huazhong, BBR 30μmol/L blocked transient outward potassium current and delayed rectifier potassium current in a concentration-dependent manner in rat hepatocytes [16] Some other studies focusing on CA1 pyramidal cells under the influence of BBR [17] [18] agreed that the alkaloid produces a blockade in potassium current. The same reaction can be seen in rabbit sinoatrial node cells [19] [20]. Therefore, we can assume that BBR interrupts potassium current through the cell membrane.
7.4. Other effects of berberine in human body

Apart from the role that berberine plays in Alzheimer's disease, some other effects in nervous system have been studied. In rats with temporal lobe epilepsy, BBR exhibited a significant decrease in seizure severity (by Racine score) and in rate of incidence of seizure. [21] In peripheral nervous system, it seems like BBR facilitated neurite extension and promoted axonal regeneration. [22] In multiple sclerosis and in experimental autoimmune encephalomyelitis, some studies suggest that BBR could provide a neuroprotective effect against neuronal damage in the brain by inhibiting enzyme activity and reducing laminin degradation. [23] Furthermore, it has been described a broad spectrum of pharmacological actions for BBR. It is significantly effective, for example, in the treatment of type 2 Diabetes Mellitus and dyslipidemia [24]. BBR may also prevent liver fibrosis. [25] Clinical investigations showed that BBR supplement may reduce alanine and aspartate transaminase levels in patients with type 2 diabetes, indicating the restoration of liver function. Nonetheless BBR showed antiarrhythmic [26] and antihypertensive [27] properties in several studies. These studies suggest that BBR can be used for treatment of atrial fibrillation and heart failure. Moreover, many BBR-derived molecules have provided more information about its effects in different tissues. Stepholidine, which is a protoberberine alkaloid, has shown D1 agonist and D2 antagonist activity in nucleus accumbens [28], [29]. The 8-oxoberberine compound inhibits potassium current in atrial tissue similar to the effect by some class III and class Ia antiarrhythmic drugs. [30] The different BBR derivatives shown in Fig. 4 were studied to determine their effect in colon cancer cells, and it was
proven that all derivatives, (being BBR the less potent drug) reduced the viability of two different colon carcinoma cell lines (HCT116 and SW613-B3) and produced different morphological changes (vacuolization of the cells). [31].

![Molecular structure of BBR, NAX053, NAX056, NAX057, NAX080, and NAX081](image)

Figure 4 Molecular structure of BBR, NAX053, NAX056, NAX057, NAX080, and NAX081 Different chemical modifications at positions 7, 8, 9, and 13 in the BBR molecule are shown in red; the added groups at position 13 are drawn in blue [31]

## 8. RESEARCH METHODOLOGY AND METHODS

For the purpose of this experiment, 5 day old postnatal rat pups were sacrificed by decapitation and their brains were removed from cranium in an ice cold Gey's Balanced Salt Solution (GBSS) solution. Animals were maintained and bred at Lithuanian University of Health Science Veterinary Academy small laboratory animal breeding facility. Cortex was separated by removing any adherent tissue including cerebellum.

GBSS solution with poli L-lysine and laminin on pretreated glass coverslips as an adhesion surface for axonal growth mimicking intracellular interactions through laminin-like molecules was prepared. In order to obtain the neuron cultures, cell culture suspension technique was used. For dissociation, serine cleaving enzyme papain was added to the cell dissociation media as explained below. As a chelating agent, we used an ethylenediaminetetraacetic acid (EDTA) solution.

### 8.1. Cell dissociation media with Versene solution and papain

Coverslips were degreased with xylene. they were cleansed with acetone, 100% ethanol and heat sterilization was performed by boiling degreased cover-slips in ethanol solution. Coverslips were then covered with poli L-lysine (10 µL) and laminin (10 µL) solution diluted in GBSS (980 µL) to provide a suitable substrate for axonal growth.
EDTA solution (Versene) was prepared for dissociation of neurons. Neuron culture dissociation was carried out with versene solution and papain, a cysteine protease found in papaya fruit that was used to dissociate cells in the first step of cell culture preparation. Dissociation media contained (in 10mL) 4µL 1M sodium bicarbonate; 15µL 1M calcium chloride; 2.5µL 1M potassium chloride and 40µL 0.5M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), diluted in 9938.5 µL Versene solution. Papain was added in a proportion of 10U/mL.

8.2. Neuron preparation and cell suspension technique

Brain was taken from 5 days old rat model. All surrounding structures were removed (vascular, debris, cerebellum…) and neuronal tissue remained. Brain slices (2-3 mm) were placed into 3mL 37ºC versene solution (EDTA solution; chelating agent that binds to calcium and prevents joining of cadherins between cells, preventing clumping of cells grown in liquid suspension). Posteriorly, 6mL EDTA solution were added and coverslips were placed in CO₂ incubator. After five minutes, the tissue should be treated with a protease inhibitor solution to stop the protease action. Cystein crystals were used with this purpose.

After incubation preparation was made by triturating with 1000µL pipette 5-10 times and repeating this process with a Pasteur pipette. 3 mL of supernatant were then moved into a tube with 6 mL culture media. Further preparation was carried out by cell suspension culture technique. Once the cells were resuspended in the correct amount of culture media we poured some cell suspension into a petri dish ready for plating. Resulting dissociated cells then attached to coverslips with GBSS solution.

After 2-4 days, incubated cells were supplemented with Ara C (cytosine arabinoside) causing cell proliferation arrest. Subsequently neurons were grown for not less than 14 days before electrophysiological recordings changing half of the neurobasal growth media with preheated fresh media every 3-4 days.

8.3. Measurements by voltage-clamp technique

Electrodes were implanted taking into consideration the compensatory resistance. Electrode used about 7 mΩ electrode solution warmed until 37ºC. Its ingredients were 117.5 mM gluconate, 8.5 mM potassium chloride, 8.5 mM HEPES buffer, 3.4 mM magnessium chloride and 15 % destilated water
We linked the electrodes to the cell using very weak positive pressure releasing it in a proximity of the cell. Filled in cells were verified by intermediate (sealed) resistance (-0.02 nA). Voltage clamp technique was performed in order to record and study electrophysiology of the neurons (Fig. 5).

![Voltage clamp technique diagram](image)

**Figure 5** Voltage clamp technique [32] The membrane voltage (Vm) was measured by the amplifier using a recording internal electrode inserted into the cytoplasm of the cell and an external reference electrode. The command voltage (Vc) is the desired membrane voltage. The difference between the Vm and the Vc was determined by a comparator. If Vm is not equal to Vc, this comparator generates a different signal. This difference signal is used by the voltage clamp amplifier to generate a current that is injected into the cell via the current-passing electrode, in order to make Vm equal to Vc. This keeps the Vm voltage as close to the Vc as possible, as it operates virtually and instantaneously. Finally, the amount of current required to keep Vm equal to Vc, the injected current, can be measured and recorded. This means that its voltage-gated ion channels open and close over time in response to changes in Vm imposed by the voltage clamp. The current generated by these channels can be recorded and analyzed.

Vm was represented as the holding potential of the membrane when the cells were not stimulated by the voltage clamp amplifier. The threshold was seen when enough current is injected into the cell to produce action potential and to depolarize it.

### 8.4. Data analysis

Once data was obtained, all the different voltages were analysed and studied.

Analysis in holding potential, threshold potential (and electric current injected to achieve this voltage), spontaneous activity of the neurons, relative refractory period, spike amplitude, number of spikes when voltage is high enough to produce continuous response of the cell and comparison between control and experimental groups were performed.

The data reported were analyzed measuring the frequency of different parameters through histograms and by the unpaired two-tail Student's *t* test, assuming unequal variances. Analysis was completed via software Microsoft Excel 2016. P<0.05 was considered to represent a significant difference.
9. ANALYSIS OF THE RESULTS

9.1. Initial Recorded Data

Registration of cells was performed using patch-clamp electrophysiological technique. Holding potential and threshold were measured. Spontaneous activity was monitored as described in table 1. Graphs are showing the data obtained during the experiment.

Number of recordings per cell: 10 times; variable: Injection current; Interperiod: 30000 ms (30 seconds); Intraperiod: 2000 ms from which Delay: 200ms; Duration of the current injection: 700 ms; Initial amplitude: ~ -0.02 (*20mV | /5nA); Amplitude increases ~ 0.1 nA each repetition. The clamp electrode resistance was evaluated beforehand of performing experiments and was determined to be close to 7mΩ in every subsequent measurement.

During the experiments 13 cells were recorded out of which 8 (2; 3; 4; 6; 8; 11; 12; 13) were selected for the evaluation. Only cells with resting potential ranging from -52 mV to -77 mV not expressing any electrophysiological or recording abnormalities were selected as suitable for further analysis. For these conditions 4 cells (cells #8, #11, #12, #13) were recorded as experimental group - the group that was introduced to BBR, same cells before the experiment were recorded as an internal control of the research. Also 4 separate cells (cells #2, #3, #4, #6) during subsequent experiments were recorded as negative control group. In case if spontaneous activity was present during the experiment delays in between the events were recorded. All the initial data of the experiments is summarised in the table 1.

Negative control group: Cells #2, #3, #4, #6

Internal control group: Cells #8, #11, #12, #13 before BBR injection

Experimental data: Cells #8, #11, #12, #13 after experiment
<table>
<thead>
<tr>
<th>CELL</th>
<th>HOLDING POTENTIAL</th>
<th>THRESHOL POTENTIAL</th>
<th>SPONTANEOUS ACTIVITY (S)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CELL #1</td>
<td>−40 mV</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CELL #2</td>
<td>−65 mV</td>
<td>−45 mV</td>
<td>54, 15, 45, 40, 42</td>
</tr>
<tr>
<td>CELL #3</td>
<td>−65 mV</td>
<td>−42 mV</td>
<td>10, 45, 30, 35, 25, 28, 20, 14, 18</td>
</tr>
<tr>
<td>CELL #4</td>
<td>−77 mV</td>
<td>−55 mV</td>
<td>18, 15, 19, 30, 19, 27, 28</td>
</tr>
<tr>
<td>CELL #5</td>
<td>−80 mV</td>
<td>−61 mV</td>
<td>31, 11, 29, 19, 75</td>
</tr>
<tr>
<td>CELL #6</td>
<td>−60 mV</td>
<td>−41 mV</td>
<td>14, 30, 20, 14, 15</td>
</tr>
<tr>
<td>CELL #7</td>
<td>−70 mV</td>
<td>−41 mV</td>
<td></td>
</tr>
<tr>
<td>CELL #8</td>
<td>−65 mV</td>
<td>−52 mV</td>
<td>23, 7, 13, 31, 12</td>
</tr>
<tr>
<td>CELL #8 (BBR)</td>
<td>−67 mV</td>
<td>−50 mV</td>
<td></td>
</tr>
<tr>
<td>CELL #9</td>
<td>−45 mV</td>
<td>−27 mV</td>
<td>10, 4, 6, 7, 5, 4, 4, 11, 4, 6</td>
</tr>
<tr>
<td>CELL #9 (BBR)</td>
<td>−45 mV</td>
<td>−30 mV</td>
<td></td>
</tr>
<tr>
<td>CELL #10</td>
<td>−45 mV</td>
<td>−36 mV</td>
<td>Not measured</td>
</tr>
<tr>
<td>CELL #10 (BBR)</td>
<td>−43 mV</td>
<td>−38 mV</td>
<td></td>
</tr>
<tr>
<td>CELL #11</td>
<td>−65 mV</td>
<td>−43 mV</td>
<td>9, 10, 10, 11, 12, 9, 6, 6</td>
</tr>
<tr>
<td>CELL #11 (BBR)</td>
<td>−68 mV</td>
<td>−41 mV</td>
<td></td>
</tr>
<tr>
<td>CELL #12</td>
<td>−75 mV</td>
<td>−52 mV</td>
<td>32, 268, 112, 138, 70, 30</td>
</tr>
<tr>
<td>CELL #12 (BBR)</td>
<td>−70/−75 mV</td>
<td>−54 mV</td>
<td>46, 99, 186, 131, 8, 98</td>
</tr>
<tr>
<td>CELL #13</td>
<td>−55 mV</td>
<td>−39 mV</td>
<td>66, 34, 90, 33, 23, 39, 62</td>
</tr>
<tr>
<td>CELL #13 (BBR)</td>
<td>−72 mV</td>
<td>−49 mV</td>
<td>13, 92, 40, 18, 66, 60</td>
</tr>
</tbody>
</table>

Table 1: Initial cell registration data (holding potential, threshold potential and spontaneous activity) obtained during laboratory analysis. Holding potential ranges from −40 mV to −80 mV depending on the different type of cells that were clamped (neurons, glia). Threshold potential ranges from −27 to −61 mV. BBR - With berberine injection. S -Seconds

The analysis was carried out with negative control group, internal control group and experimental group. Spike numbers and time to repolarize was excluded in negative control group due to its lack of relevance. Results are shown in table 2.
<table>
<thead>
<tr>
<th></th>
<th>HP (mV)</th>
<th>T (mV)</th>
<th>SA (s)</th>
<th>CInj (nA)</th>
<th>SpkH (mV)</th>
<th>SpkA (mV)</th>
<th>SpkN (nr (mV))</th>
<th>RRP (ms)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell#2</td>
<td>−65</td>
<td>−45</td>
<td>54, 15, 45, 40, 42</td>
<td>0.63</td>
<td>17.8</td>
<td>62.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell#3</td>
<td>−65</td>
<td>−42</td>
<td>10, 45, 30, 35, 25, 28, 20, 14, 18</td>
<td>0.7</td>
<td>13.1</td>
<td>55.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell#4</td>
<td>−77</td>
<td>−55</td>
<td>18, 15, 19, 30, 19, 27, 28</td>
<td>0.56</td>
<td>16</td>
<td>61</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell#6</td>
<td>−60</td>
<td>−41</td>
<td>14, 30, 20, 14, 15</td>
<td>0.47</td>
<td>37.7</td>
<td>78.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cell#8</td>
<td>−65</td>
<td>−52</td>
<td>23, 07, 13, 31, 12</td>
<td>0.17</td>
<td>21.4</td>
<td>73.4</td>
<td>4 (−40)</td>
<td>152, 231, 236</td>
</tr>
<tr>
<td>Cell#11</td>
<td>−65</td>
<td>−43</td>
<td>9, 10, 10, 11, 12, 9, 6, 6</td>
<td>0.16</td>
<td>24</td>
<td>67</td>
<td>11 (−40)</td>
<td>44, 51, 34, 54, 57, 66, 61, 63, 81, 67</td>
</tr>
<tr>
<td>Cell#12</td>
<td>−75</td>
<td>−52</td>
<td>32, 268, 112, 138, 70, 30</td>
<td>0.47</td>
<td>35.4</td>
<td>87.4</td>
<td>10 (−45)</td>
<td>50, 53, 57, 54, 60, 60, 63, 63, 75</td>
</tr>
<tr>
<td>Cell#13</td>
<td>−55</td>
<td>−39</td>
<td>66, 34, 90, 33, 23, 39, 62</td>
<td>0.16</td>
<td>25</td>
<td>64</td>
<td>5 (−34)</td>
<td>96, 132, 134, 164</td>
</tr>
<tr>
<td>Cell#8(B)</td>
<td>−67</td>
<td>−50</td>
<td></td>
<td>0.16</td>
<td>13.6</td>
<td>63.6</td>
<td>5 (−40)</td>
<td>98, 123, 159, 154</td>
</tr>
<tr>
<td>Cell#11(B)</td>
<td>−68</td>
<td>−41</td>
<td></td>
<td>0.36</td>
<td>12.5</td>
<td>53.5</td>
<td>11 (−40)</td>
<td>5, 71, 73, 45, 64, 65, 61, 89, 72, 102</td>
</tr>
<tr>
<td>Cell#12(B)</td>
<td>−75</td>
<td>−54</td>
<td>46, 99, 186, 131, 8, 98</td>
<td>0.47</td>
<td>32.8</td>
<td>86.8</td>
<td>10 (−45)</td>
<td>45, 46, 56, 64, 67, 71, 78, 75, 82</td>
</tr>
<tr>
<td>Cell#13(B)</td>
<td>−72</td>
<td>−49</td>
<td>13, 92, 40, 18, 66, 60</td>
<td>0.56</td>
<td>41.2</td>
<td>90.2</td>
<td>4 (−45)</td>
<td>146, 173, 205</td>
</tr>
</tbody>
</table>

Table 2 Cells #2, #3, #4 and #6 were selected as negative control group. Cells #8, #11, #12, #13 with and without BBR are the internal control group and the experimental group respectively. B- with berberine; HP-holding potential; T-threshold; SA-spontaneous activity; CInj-current injection; RRP-relative refractory period at a mean voltage of -40mV, SpkH-spike highest point; SpkA-spike amplitude; SpkN-number of spikes at a determined voltage. nr-number of spikes; mV-miliVolts; nA-nanoAmperes; s-seconds; ms-miliseconds.

9.2. Voltage and spike amplitude do not vary under the influence of berberine

BBR effect on holding potential and threshold potential does not show any significant variance to establish a direct effect of the phytoalkaloid in the cell membrane conductivity. By establishing a null hypothesis $H_0$ determining that BBR injection has no effect on holding potential, we compared the
holding potential between cells with and without BBR using the two-tailed Student’s T test (assuming unequal variances).

<table>
<thead>
<tr>
<th>A</th>
<th>Without BBR</th>
<th>With BBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>-65.875</td>
<td>-70.5</td>
</tr>
<tr>
<td>Variance</td>
<td>51.83928571</td>
<td>13.66666667</td>
</tr>
<tr>
<td>Observations</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Hypothesized mean difference</td>
<td>4.625</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B</th>
<th>Without BBR</th>
<th>With BBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>-46.125</td>
<td>-48.5</td>
</tr>
<tr>
<td>Observations</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>Variance</td>
<td>36.125</td>
<td>29.66666667</td>
</tr>
<tr>
<td>Hypothesized mean difference</td>
<td>2.375</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

Table 3 Student’s t test for Holding potential (mV) (A) and threshold potential (B). Mean voltages in both holding and threshold potentials are (-65)to(-70) mV and (-45)to(-50) mV respectively, which are the common values for cortical neurons. \( \alpha = 0.05 \). As student’s t test was carried out assuming unequal variances, hypothesized mean difference must be adjusted. Observations indicate the number of studied cells: 8 without berberine- 4 negative control group and 4 internal control group) and 4 with berberine.

\( P(A) \) and \( P(B) \) are 1 (> 0.05) (shown in table 3). Therefore, we assumed there is no significant relevance between the two groups leading us to the hypothesis that BBR does not cause any repercussions in holding or threshold potential. Threshold potential was evaluated assuming similar null hypothesis \( H_0 \) (BBR injection has no effect on threshold potential).

Spike amplitude was measured and analysed as well. Results are shown in table 4.

<table>
<thead>
<tr>
<th>Without BBR</th>
<th>With BBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>68.675</td>
</tr>
<tr>
<td>Variance</td>
<td>110.8307143</td>
</tr>
<tr>
<td>Observations</td>
<td>8</td>
</tr>
<tr>
<td>Hypothesized mean difference</td>
<td>4.85</td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>0.372174125</td>
</tr>
</tbody>
</table>

Table 4 Student’s t test for Spike Amplitude. \( \alpha = 0.05 \). Parameters are explained in table 3. Values correspond to spike amplitude formed during the absolute refractory period. Mean spikes amplitudes are calculated in mV. Values were calculated by measuring the distance from the threshold potential to the point where inactivation of Na\(^+\) channels occurs (highest point of the spike).

We concluded that there is no significant relevance. (P > 0.05) The fact that spike amplitude does not vary when this drug is administered means that Na\(^+\) channels inactivate at the same point before and after BBR injection. Therefore, we assumed that the was no relationship between BBR and Na\(^+\) gated channels.
9.3. Spontaneous activity after BBR injection shows no difference

Spontaneous activities of the neuronal networks were analyzed and compared by the Student’s t test. The following histograms (Fig. 6) represent the frequency of the interval between each spontaneous activity recording.

![Figure 6 Interval histograms of the spontaneous activity of four different cells. Interspike intervals represent the time between spontaneous activity where cells were not stimulated by the neuronal network.](image)

**Figure 6** Interval histograms of the spontaneous activity of four different cells. Interspike intervals represent the time between spontaneous activity where cells were not stimulated by the neuronal network. Graph A (Cells #12 and #13) and graph B (#12(B) and #13(B)) are compared in table 5. The cells depicted in the graphs were evaluated over cumulative periods of 500 sec, illustrating the results over a 2000 sec period.

<table>
<thead>
<tr>
<th></th>
<th>Without BBR</th>
<th>With BBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>76.69230769</td>
<td>71.41666667</td>
</tr>
<tr>
<td>Variance</td>
<td>4530.730769</td>
<td>2786.44697</td>
</tr>
<tr>
<td>Observations</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>0.999815337</td>
<td></td>
</tr>
</tbody>
</table>

Table 5 Student’s t test for the evaluation of spontaneous activity. Observations indicate the number of interspike intervals taken for the analysis. α=0.05

There is no relation between BBR injection and spontaneous activity of the cell (P>0.05), as it is shown in table 5.

9.4. Berberine blocks electric current injection

Subsequent analysis was performed on berberine effect while electric current was being injected into the cells.

![Figure 7 Interval histograms representing current injection frequencies.](image)

**Figure 7** Interval histograms representing current injection frequencies. Graph A (without BBR) and Graph B (with BBR) represents the current injection intervals necessary to undergo a threshold potential. Current injection in cells without BBR needed a low current injection (0.15–0.17 nA) in 3 out of 4 cells to achieve threshold, while 3 out of 4 cells under BBR stimulus needed a higher current injection (>0.35 nA)
When current injection is compared between the internal group (Fig. 7a) and the experimental group (Fig. 7b), we found that null hypothesis $H_0$ (BBR does not affect current injection) was questioned by the results. As shown in table 6, current injection needed to reach the threshold in those cells from internal group is lower than the current injection needed to reach threshold in cells from the experimental group. There was an increased need of current injection to reach the threshold in this second group.

<table>
<thead>
<tr>
<th></th>
<th>Without BBR</th>
<th>With BBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>0.24</td>
<td>0.3875</td>
</tr>
<tr>
<td>Variance</td>
<td>0.023533333</td>
<td>0.029691667</td>
</tr>
<tr>
<td>Observations</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>Hypothesized mean difference</td>
<td>0.1475</td>
<td></td>
</tr>
<tr>
<td>$P(T&lt;=t)$ two-tail</td>
<td>0.043060721</td>
<td></td>
</tr>
</tbody>
</table>

Table 6 Student’s t test for Current injection (nA). $\alpha = 0.05$. $P < 0.05$, therefore we find significant relevance during the analysis. Current was injected while voltage was recorded. It was found that to achieve the same voltage, a different amount of current was injected. The compared difference is seen in this table.

P is 0.043 ($< 0.05$). Therefore, there is a considerable influence of BBR in cell, causing a need of higher amount of current injection to start depolarization. We can assume that BBR at some level interrupt or slow down passage of ions through the membrane, without any pathological acute changes for the cell (cell did not die after recordings).

**9.5. Relative refractory period and number of spikes are not influenced by berberine**

![Figure 8 Relative refractory period frequencies after each spike in cells A (without the influence of BBR) and B (under the effect of BBR). Frequencies show that RRP most of the times last between 51 and 75 milliseconds, and when BBR is injected, RRP duration is not altered.](image-url)
<table>
<thead>
<tr>
<th></th>
<th>Without BBR</th>
<th>With BBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>86.84615385</td>
<td>88.03846154</td>
</tr>
<tr>
<td>Observations</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Hypothetized mean difference</td>
<td>1.192308</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>0.865521358</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Without BBR</th>
<th>With BBR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>105.8333333</td>
<td>100</td>
</tr>
<tr>
<td>Observations</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Hypothetized mean difference</td>
<td>5.833</td>
<td></td>
</tr>
<tr>
<td>P(T&lt;=t) two-tail</td>
<td>0.999990333</td>
<td></td>
</tr>
</tbody>
</table>

Table 7 Student's t test for relative refractory period after every recorded spike (A) and after the last three recorded spikes on each cell (B). α= 0.05 P(T<=t) two-tail represents the value of significance at which we consider the null hypothesis rejected. As error α is 0.05, any value below 0.05 is considered with significant relevance to be evaluated. In this case P(A) is 0.86 and P(B) is 0.99

Number of spikes was entirely dependant on the relative refractory periods recorded in the laboratory. Mean voltage of the studied cells was -40mV. While measuring the relative refractory period, there is a delay from interval to interval, due to neuronal fatigue to continuous current injection. Accordingly, we analyzed relative refractory period intervals. None of them showed significant relevance, thus there is no relation between BBR presence and the relative refractory period produced by the cells (shown in table 7). As table 2 indicates, there are no changes in the number of spikes during the experiment.

### 10. DISCUSSION OF THE RESULTS

According to the results obtained during this experiment, BBR delays current injection without any further effect in the action potential, at least in an acute state. This means that the cell membrane has enough plasticity to recover from a blocked current. Moreover, it is clear that berberine does not delay the sodium inward current or the function of the membrane protein channels that intervene during depolarization and repolarization periods because this would cause a shortened relative refractory period with less number of spikes.

The goal of this investigation was to examine the acute electrophysiologic effects of BBR administration in cultured cortical neurons. Our results indicate that BBR did not evoke significant effects in these neurons, at the current injection rate of 0.1 nA per repetition. BBR effects in neurons are evident and its potential as anti-inflammatory and acetylcholinesterase inhibitor has been proven many times. Regarding its effect in neurons, it seems that berberine affects neurons through many different mechanisms. Previous researches had already demonstrated that this beneficial effect is associated with antioxidant action, scavenging oxygen-free radicals and anti-inflammation, which may explain, in part,
the neuroprotective mechanism of berberine. The simultaneous studies on both cholinesterases and BACE inhibitory effects, as well as antioxidant effects, including ONOO scavenging and ROS inhibitory effects of Coptidis Rhizoma alkaloids is worthy of development. [6].

Moreover, it has been studied BBR inhibition of N-methyl-D-aspartate (NMDA) receptor activity [33] and reduction of glutamate release from nerve terminals [34] with very positive results.

However, from an electrophysiological point of view there is no strong evidence that BBR affects spontaneous activity of the neuronal network or the membrane potential of the cell. Although there is no evidence that berberine affects holding potential, threshold potential or spontaneous activity of the neuronal network, it has been demonstrated that berberine blocks outward potassium current in many types of cells [25] [26] [27] [28] [30] [16] [35] [20], which matches the results obtained in this investigation regarding the increased current injection needed to reach the threshold potential.

As berberine does not alter the action potential in normal healthy neurons, we can assume that the effect that berberine exerts in neurodegenerative disorders is merely chemical. Other effects from berberine derivatives such as vacuolization of colon cancer cells [31] or D1 agonist and D2 antagonist activities [29] demonstrate the importance of berberine as a potent modulator of cell membrane properties.

Taking into consideration the berberine acetylcholinesterase inhibition activity, the different changes in the electrophysiology of the neuronal network and the different effects of berberine derivatives, it is very likely that BBR can alter the stage of Alzheimer’s disease. Still it is necessary further investigations and innovative approaches to determine if BBR or any of its different synthetic derivatives can improve, paliate or slow down the neuronal damage that is caused in diseases such as Alzheimer’s.

Regarding the electrophysiological properties of neurons, some recent studies reported that potassium channels are regarded to play a key role in neurodegeneration including AD, [35] [36] which is a very important fact in order to investigate a new treatment line. One of these studies demonstrates the increased potassium channel expression in microglia from cryopreserved brains with AD. [36] However, although BBR alters the potassium current in the neuronal network, it has not been proven that potassium loss has any consequences in AD. As all the other electrophysiological properties remained unchanged in the studied healthy neurons, we can suppose that BBR has no effect in the electrophysiological properties of any affected neurons by AD.
11. CONCLUSIONS

1. It was possible to obtain a total of 13 neuron cultures from newborn rat pups by achieving a stable culture through the cell suspension technique in a well-prepared dissociation media with versene solution and papain. The 13 neuron cultures were cultivated for 2 weeks before performing the electrophysiological recording by voltage-clamp technique. Quality of the dissociated samples was good and there were not unexpected incidences.

2. According to the literature review we went through, AD clinical picture is thought to be caused due to the amyloid plaques formation or due to the action of AChE causing ACh deficiency. Therefore, available treatment consists of ChEI which has demonstrated a reduction on symptoms and in the progression of the disease. BBR plays a fundamental role as a treatment approach in AD. It does not only act as a ChEI, but also it reduces neuroinflammation by inhibiting expression of inflammatory markers and through antioxidation, reducing the formation of ROS and RNS. Other BBR functions have been observed in human body, such as antidiabetic, antidyslipidemic or antiarrhythmic effects. It has been reported that BBR promotes axonal regeneration and although further studies are necessary, there is some evidence of improvement in neurodegenerative disorders such as multiple sclerosis or epilepsy.

3. 2-weeks-old mixed neuron-glia cultures were studied with voltage clamp technique and out of the 13 cells obtained during the recording, 8 neurons were taken for the study. 4 of these neurons served as negative control group, and the other 4 neurons were selected for the experimental analysis. We compared the holding potential, threshold potential, spontaneous activity of the cells, spike density, relative refractory period and current injection before and after BBR injection with the Student’s t test and a value of significance P < 0.05.

4. Results showed that electrophysiological properties of neurons have significantly changed after introduction of berberine only in sense of current injection to reach threshold and not having significant effect on any other recorded and monitored properties. These results match with the data, that berberine is important in blocking outward potassium current and this effect in turn can be important in regulating not only neuronal activity but also metabolic properties that should be further evaluated using molecular biology techniques. Yet, results are not conclusive regarding the neuroprotective effect in AD, and it was not possible to evaluate if the function of BBR in the treatment of neurodegenerative diseases is related to only the anticholinergic inhibition and anti inflammatory process or also there is enhancement on the conductivity itself.
5. Data also reported an increased effect of different BBR derivatives compared to those in the primitive BBR compound, as a higher outward potassium blockade, reducing viability of cancer colon cells or as dopamine modulator. Further analysis should be carried out regarding these findings and electrophysiological properties of BBR derivatives.

12. PRACTICAL RECOMMENDATIONS

The investigation requires more cortical cells to get a better statistical results. A good next step to be considered would be to record the electrical current in cortical neurons impregnated with Aβ amyloid plaques, and its comparison with affected cells under BBR influence as it is known that BBR affects inflammation and it has been hypothetised its benefit in dementia treatment. It is important to understand what effects this alkaloid can generate for the neuronal network activity.

BBR derivatives should be analysed from a electrophysiological viewpoint in order to establish which kind of synthetic compound can achieve better results in the treatment of different diseases such as AD.

Combination of BBR compound and derivatives with other treatment approaches can determine an innovative combined therapy, not only for neurodegenerative diseases, but also for diabetes, cancer, dyslipidemia, heart arrhythmias and other pathologies where BBR is known to have beneficial effects.
13. REFERENCES


[12] Z. Feiqi and Q. Caiyun, "BioMed Central: Berberine chloride can ameliorate the spatial memory impairment and increase the expression of interleukin-1beta and inducible nitric oxide synthase in the


