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Influence of lithium in neuron-glia interaction in hippocampal neurons: preliminary study

Influência do lítio na interação neurônioglia em neurônios hipocampais: resultados preliminares

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ABSTRACT: Recently, special attention has been given to the possible neuroprotective effects of lithium, especially by the discovery of its regulatory effects on pro and anti-apoptotic proteins. Lithium substantially increases the cytoprotective proteins expression in the central nervous system, both in rat cortex and in human cells of neuronal origin. In addition to neuroprotective actions, it aids in the regeneration of axons in the central nervous system of mammals. Lithium negatively regulates the expression and activity of enzymes that exert important functions in cerebral homeostasis: synaptic plasticity, neurogenesis, and phosphorylation of tau protein. Microglia is known for its importance in neuropathology. However, under physiological conditions, such immune cells interact actively with neurons, being able to modulate the fate and functions of the synapses. Such ability of microglial cells suggests the consequences of changes in microglial phenotype under pathological conditions, which makes it relevant to understand the interaction between microglial and other developing brain cells and their influence on the formation of neuronal and synaptic networks. The current work aims to identify the main pathway of neuronal-glia integration activated by chronic treatment with lithium in neurons, exploring the use of bioinformatics tools in microarray data. Treatment of primary hippocampal neurons with lithium changed the genes related to different neuroprotection pathways at the highest therapeutic dose. There was dissociation between the therapeutic and sub-therapeutic dose of lithium in neuroprotection. Therefore, treatment at therapeutic doses (2mM) modified different signaling pathways when compared to the sub-therapeutic dose (0.02 and 0.2mM).

Keywords: Neuroprotection; Hippocampus; Lithium; Neuroglia; Neurons.

RESUMO: Recentemente, especial atenção foi dada aos possíveis efeitos neuroprotetores do lítio, especialmente pela descoberta de seus efeitos regulatórios sobre proteínas pró e anti-apoptóticas. O lítio aumenta substancialmente a expressão de proteínas citoprotetoras no sistema nervoso central, tanto no córtex de ratos quanto em células humanas de origem neuronal. Além de ações neuroprotetoras, auxilia na regeneração de axônios no sistema nervoso central de mamíferos. O lítio regula negativamente a expressão e a atividade de enzimas que exercem funções importantes na homeostase cerebral: plasticidade sináptica, neurogênese e fosforilação da proteína tau. Microglia é conhecida por sua importância na neuropatologia. No entanto, sob condições fisiológicas, tais células imunes interagem ativamente com os neurônios, sendo capazes de modular o destino e as funções das sinapses. Essa capacidade das células microgliais sugere as conseqüências de mudanças no fenótipo microglial sob condições patológicas, o que torna relevante o entendimento da interação entre micróglia e outras células cerebrais em desenvolvimento e sua influência na formação de redes neuronais e sinápticas. O presente trabalho tem como objetivo identificar a principal via de integração neurônio-glia ativada pelo tratamento crônico com lítio em neurônios, explorando o uso de ferramentas de bioinformática em dados de microarray. O tratamento de neurônios hipocampais com lítio alterou os genes relacionados a diferentes vias de neuroproteção na dose terapêutica mais alta. Houve dissociação entre a dose terapêutica e sub-terapêutica de lítio na neuroproteção. Portanto, o tratamento em doses terapêuticas (2mM) modificou diferentes vias de sinalização quando comparado com as doses sub-terapêuticas (0,02 e 0,2mM).

Descritores: Neuroproteção; Hipocampo; Lítio; Neuroglia; Neurônios.

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INTRODUCTION

Alzheimer's disease

*A*lzheimer's disease (AD) is the most frequent progressive neurodegenerative disease associated with age, whose cognitive and neuropsychiatric manifestations result in a deficiency progression and eventual incapacitation[1]. The disease affects more than 30 million individuals throughout the world[2].

In general, the first clinical aspect is the recent memory deficiency, which can affect the processes of learning, reasoning and attention. With the evolution of the disease, there is a progressive loss of judgment, language, orientation and other cognitive abilities. The individual with AD may not be able to report symptoms of other diseases, follow prescribed treatment and report side effects of medicines. In the final stages of the disease, brain changes begin to affect physical functions, such as swallowing, balancing, and controlling urination, which increases vulnerability to other health problems, namely: aspiration, pneumonia, falls, fractures, malnutrition and dehydration.

The pathogenic process of AD involves different mechanisms of neurodegeneration in different brain regions: initially in the medial portions of the temporal lobe - hippocampus and cortex entorhinal[3] - later in other brain regions responsible for cognitive functions as limbic structures and associative cortices[4]. Parallel to neuronal loss, occur extracellular deposit - which main component is amyloid beta peptide (AB) - and intracellular accumulations of neurofibrillary tangles of the hyperphosphorylated Tau protein. These AB peptide deposits and neurofibrillary tangles are the histopathological characteristics of the disease.

Microglia

Microglia are immune cells of the central nervous system (CNS), which guarantee tissue homeostasis, mediate the defense against pathogens and constitute the sentinel immune network[5,6,7]. Unlike other tissue macrophages, the microglia do not originate from a monocyte progenitor, but derive from a macrophage lineage of the sac primitive yolk[8]. In addition, the microglia are distinct from the myeloid cells which may infiltrate the nervous system under inflammatory conditions[9]. As specialized macrophages, microglia actively support and protect the neuronal environment.

Microglia perform various functions in the CNS of the healthy adult: patrol the brain to detect pathogens, contribute to neuronal health at synapses, modulate synaptic transmission, and promote neurogenesis. Under normal conditions, microglia actively monitor their environment with projections[10], with the ability to react immediately to homeostasis imbalances.

It has been suggested that, under small disturbances, the microglia provide trophic support and protection[11]. Infection, neurodegeneration, and other conditions associated with loss of homeostasis, induce changes in microglial morphology, gene expression and function - referred generically as "activation"[5,6,7,12]. Activated microglia stimulate phagocytic activity, release chemokines and neurotrophic factors and present T-cell antigens[6].

Microglia and Alzheimer's disease

There is no consensus on the contribution of the systemic immune response and the recruitment of monocyte and tissue-resident microglia for the onset and progression of AD[13-18]. Some studies have shown that, under certain conditions, microglia acquire proinflammatory activity, which was associated with the evolution of the disease[19,20].

The debate regarding the involvement of microglia as a response to brain pathologies exists because of technical limitations in the analysis of heterogeneous cellular populations, which make it difficult to define the cellular immune types involved in the homeostasis and pathology of the brain. As a result, several innate immune functions were attributed either to microglia or to the monocyte infiltrated into the blood. For example, in AD, as well as in other neurodegenerative diseases, local inflammation associated with cytotoxicity or with the evolution of the disease has often been attributed mainly to microglia[20,21,22]. In contrast, several studies have attributed an important role to monocytes in the clearance of brain toxins[17,23,24].

New studies involving sequencing single cell RNA in isolated brain immune cell samples identified a protective subtype of microglia, microglia associated with disease (MAD) and their dynamics during the progression of AD. Through immunohistochemistry, this subtype of microglia was associated to brain areas affected by the disease. More detailed analysis of genes expressed by MAD revealed an increase in lipid metabolism pathways and in the expression of genes related to phagocytosis, corresponding, though, to the need for deboning the entanglements in AD.

Immunomodulation between MAD-specific genes and AB peptide identified an improvement in phagocytic activity of MAD cells and a maintenance of their function in the brains of mice and humans. Genomic analysis of post-mortem brains of patients with AD and corresponding non-DA controls will allow the identification of the role of MAD in AD and reveal greater immune heterogeneity.

It is possible, therefore, that there is a compensation in the cerebral homeostasis between the numbers of MAD cells with phagocytic activity and the mechanisms of control with inhibitory signaling on this cellular subtype. Such control mechanisms, while essential for microglial function to ensure their immune activation can become a negative factor when strong phagocytic activity is needed - as in aging or under neurodegenerative diseases.

Microglia and lithium

As suggested, the sustained reactivity of microglia is implicated in the pathology of many neurodegenerative diseases and an increase in its reactivity may lead to apoptosis[25-28]. Apoptotic microglia impairs the CNS protection and its repair function, and has been found in several neurological diseases, such as AD[29,30]. P53 is a transcription factor whose alteration promotes apoptosis through the normal cell cycle, particularly during the enhancement of neural networks in the development. P53 also activates the expression of genes involved in stress-induced apoptosis and the apoptotic pathways involved in neurodegenerative diseases[31-33] that may arise from inappropriate activation of p53[34,35]. Changes in p53 expression occur in the glial cells in neurological conditions: p53 expression increases predominantly in glial cells in AD[36]. Also, positive regulation of p53 in AD can induce Tau phosphorylation[28], which allows to establish a relationship between p53, microglial activation, apoptosis and neurodegenerative disease.

In this context, treatment with lithium chloride may decrease both levels of messenger RNA (mRNA) and p53 transcripts in cultured neurons. Exposure of the microglial (5 mM) for 24 hours before the microglial activation significantly reduced the expression induced by activation of p53 in the primary microglia. In addition, the lithium chloride directly inhibits the serine / threonine kinase and the glycogen synthase kinase-3[37], whose activation has pleiotropic effects, including those in p53 and apoptosis[38]. At concentrations of 0.1-10 mM lithium chloride was observed an increase in the number of microglial cells in mixed neuronal-glial cultures. Treatment with lithium can reduce microglial activation[39] and promote a neuroprotective microglial phenotype[40]. In summary, these findings suggest that the prevention of microglial and posterior stress apoptosis and the maintenance of its proliferation capacity is therefore neuroprotective. Inhibition of p53 prevented the expression of apoptotic markers induced by peptide AB.

In addition to its effect on p53 expression, further research has demonstrated the effects of treatment with lithium on the production of cytokines in differentiated monocytes, such as microglia. The microglia produces the third component of the complement system (C3) and expresses the C3 receptors[41] and is involved in synaptic elimination because of the expression of these proteins[42]. It has been suggested that the therapeutic concentration of lithium modulates neuronal connectivity and neuroprotective effects on the human brain[43], inducing the production of C3 and pointing to C3 as a biomarker of peripheral dendritic cells and microglia in the CNS.

Maturation of neuron / glia

Microglia is known, mainly, for its role in neuropathology. However, such immune cells interact actively with neurons under physiological conditions, being able to modulate Synaptic functions. Originated from myeloid precursors of the yolk sac, microglial cells invade the CNS during early embryonic development, and may influence the proliferation, migration and neuronal differentiation, as well as the formation of mature neural networks thus contributing to the development of the CNS. These microglial cells are involved in crucial stages of neuronal survival and apoptosis, axonal growth, neuron migration, supernumerary synapses and functional maturation of developing synapses.

During their monitoring of the cerebral parenchyma, the microglial cells process the contact of the synapses. The occurrence and duration of these contacts are regulated by the activity of the neural network and can influence the fate and activity of the synapses[44,45,46]. These observations suggest that changes in the microglial phenotype in pathological conditions will have the following consequences: the loss of the constitutive influence of topographical microglia and the emergence of new interactions with the immune nature of these cells. It becomes relevant, therefore, to understand the interaction between microglia and other developing brain cells and their influence on formation of neural and synaptic networks.

In primary cultures of mesencephalic and cortical embryonic neurons, the microglial medium increases the absorption of neurotransmitters and the number of mature neurons. This suggests that the microglial cells have a neurotrophic effect through the release of factors, probably derived from plasminogen[47]. Microglia purified from brain of embryo rat promotes the proliferation of cultured neuronal cells by releasing the extracellular matrix - thrombospondin[48]. Thrombospondin is also highly expressed by astrocytes and promotes synaptogenesis.

The discovery of microglial cells that regulate synapses under physiological conditions has changed the importance of these cells in CNS physiology and motivates studies. The ability of these immune cells of remodeling its phenotype according to environmental changes makes them the most plastic cells of the CNS.

ACADEMIC CONTRIBUTION

In summary, AD is an age-related neurodegenerative disease characterized by progression of memory and cognitive dysfunction, manifested histologically with parenchymal deposition of AB peptide, neurofibrillary tangles formation and neuroinflammation. It has been highlighted the role of some cells of the brain defense system, such as microglia, in the pathophysiology of AD and in the influence on the progression of the disease. However, there is no consensus among the specific contribution of microglia in these processes. Select genes related to neuron-glia interaction and verify the operation of these selected genes in culture of lithium-treated

hippocampal neurons could reveal signaling pathways and provide a better understanding of the neuroprotection pathway in the neuroinflammation of AD.

OBJECTIVES

The objective of this research is to determine in the literature genes related to the neuroprotection of the neuronal-glia interaction. After identifying the genes, to analyze the influences of different doses of lithium on cellular processes pathways.

Specific objectives

1. To identify, according to data in the literature, genes of neuron-glia interaction present in AD;
2. Analyze the genes in the microarray data and compare lithium treatment (0.02mM; 0.2mM and 2mM) with the control condition (no treatment with lithium).
3. Identify the signaling pathways present in the different treatment doses of the cell cultures with lithium.

MATERIAL AND METHODS

Primary cultures of neurons

Hippocampal neurons cell culture

Cultures of post mitotic neurons from rat embryos were obtained according to the following protocol. Wistar rats provided by the Center of Faculty of Medicine of the University of São Paulo (FMUSP) were sacrificed on the 18th day of gestation, thus obtaining embryos of the desired age (E18).

The method used for induction of death was guillotine. After abdominal alcohol asepsis diluted in water (70% v/v), using sterile surgical instruments and gloves, the uterine sac containing their embryos (usually 10 to 16) was extracted by laparotomy. Then it was conditioned in a sterile Petri dish and taken to a laminar flow chamber, where tissue dissection followed, in an aseptic environment. After removal of the gestational membranes, the fetuses obtained were submitted to occipitofrontal craniotomy - the respective brains were removed and packed in sterile petri dish. The brains were immersed in Neurobasal medium solution (Gibco) containing penicillin and streptomycin, 20mM glutamine and supplement B-27 (Gibco) heated to 37°C. Because of the large number of brains to be dissected, and the time required for this procedure, half of the brains were kept in a 5% CO_2 perfused oven at 37ºC, while hippocampal of the other half was dissected, in order to preserve the viability of the dissected tissues after time of procedure.

The dissection steps were done under a medium magnifying glass (40_{ϵ}), using microsurgical instruments. The cerebral hemispheres were separated, the membranes removed and then the dissection of hippocampal formations. The tissues obtained were reduced to smaller fragments (approximately $1mm^3$), transferred to the test tube, the HBSS solution was removed, and trypsin solution (0.5 mg/mL in HBSS) was added for 20 minutes in a water bath at 37°C.

After incubation, the fragments of nerve tissue were washed again three times in 5mL of Hanks solution containing additives (HBSS +), namely: magnesium chloride 8mM (MgCl2 .6H2O) (Sigma), serum 5% (v/v) inactivated equine (Sigma), 10mM Hepes buffer (Gibco) and 1μg/ml deoxyribonuclease (DNase I).

The tissue fragments were dissociated by hydro mechanical action in 2-4 mL of HBSS +. To do this, two Pasteur glass type pipettes were used successively, with their respective passage reduced by fire to approximately 1.0 and 0.5mm in diameter. Suspension of isolated cells was then obtained by dissociating the tissue fragments, aspirating it with the aid of a bulb of the opposite end of the pipette, and exhausting its contents repeatedly (20 to 30 times with each pipette) until a cloudy and homogeneous solution is obtained with the naked eye.

For counting and evaluating cell viability, a 10uL aliquot of this suspension was withdrawn and diluted 20-fold in solution containing 90μL of HBSS and 100μL of Trypan blue dye. A 10μL aliquot of this new solution was transferred to a hemocytometer (Neubauer chamber), under a glass cover, and observed in a magnifying microscope 100 (Eclipse E200®; Nikon), with an objective lens of 10 magnification. It was made an average estimate of the number of uncolored cells in the four fields of the counting chamber. Each field has an area of $1mm^2$ (corresponding to the set of 16 squares whose sides measure 0.25 mm) and gives a number of cells in 10^{-4} mL of suspension. Multiply the number obtained in the count by the respective dilution factor (number x 20 x 10^{-4}), thus inferring the approximate number of viable cells per milliliter of the cell suspension.

For the preparation of the culture vessels, Petri dishes (Sarstedt Cell +) made in treated sterilized polystyrene ready to use; and for the sensitization of the culture wells, polyd-lysine (PdL). Two days prior to dissection, 5μg/mL PdL solution was applied in sterile deionized water, in sufficient volume to cover the culture surface. The PdL solution was maintained for two days and removed just prior to seeding the cell suspension.

The cell suspension was diluted in growth medium at the ideal density of 1.0 or 1.5x105 cells/mL, for immediate seeding in culture vessels. Using Petri dishes, 5mL of the cell suspension was discharged into each well, thus reaching a density of approximately 1.5x105 cells per cm^2 of culture surface. The usual yield of the procedure is approximately 500,000 cells per hippocampus - from 1.0 to 1.5x105 cells per animal. Despite the low need of neurons

for oxygen, the survival of the cells is a function of the total time of the procedure, which should not exceed two hours, from the sacrifice of the animal to the sowing and conditioning of the cultures in the incubator.

Cultures were maintained at 37°C in a humidified air-CO_2 incubator (5% v/v). As a growth medium, *Neurobasal* medium plus 0.1mM l-glutamine (Gibco 25030-024), antibiotics (50 IU/ml penicillin solution and 50 µg/ml streptomycin), and B-27 supplement (Gibco 17504-036) (1:50 v/v).

In the first 24 hours in culture only, the growth medium was enriched with fetal bovine serum (FBS). On the second day of incubation, the growth medium was replaced in its entirety by means of fresh culture without FBS. Thereafter half the volume of supernatant from each culture well was changed every 48-72 hours to fresh means; always without FBS. Low density hippocampal neurons tend to degenerate rapidly if the growth medium is completely changed, since the secreted factors by glial cells present in culture are required for neuronal development and survival.

Treatments

Cells were treated with ClLi (Lithium Chloride) at concentrations of: 0.02 mM, 0.2 mM and 2 mM for 7 days. All samples were compared with the control condition, that is, condition without administration of the drug. The treatment started on the fourth day in culture until the tenth day.

Microarray Analysis

Cultures were carried out in a 6-well petri dish, with 20 plates for each treatment (control, 0.02mM, 0.2mM and 2mM). At the end of the culture the RNA was extracted by RNeasy Mini Kit (Quiagen).

This kit allows to extract RNA from a small number of cells (maximum of 5x105). Briefly, the cells are lysed and the RNA is bound to the silica membrane of the column. Following is a treatment with DNase I to remove traces of genomic DNA. RNA was eluted with RNase-free water. The RNA concentration was determined spectrophotometrically using Nanodrop.

The quality of the samples was determined by the Agilent 2100 Bioanalyser (Agilent Technologies, U.S.A.) through the RNA Integrity Number (RIN) obtained by the Agilent RNA 6000 Pico and Nano Kit (Agilent Technologies, U.S.A.) according to the amount of sample. This platform allows the analysis of samples of RNA on a chip through capillary electrophoresis. Samples are considered good when RINs are with values equal to or greater than 7. After determining their concentration and quality, the RNA was stored at -80°C.

Bioinformatics Analysis

For the formation of PPI (Protein protein interaction) networks we will consult three databases of human interatomic: HPRD[49], MINT[50] and Intact[51]. We will do a cut-out of this network comprising up to the second neighbors from the genes selected from the literature. We will look for in the literature public data of microarray comparing cases and controls in the hippocampus. To assess the difference in co-expression of the differentially expressed in the microarray, we will perform an analysis of gene co-expression networks. The correlation of each gene and its partners will be calculated by Pearson's Coefficient of Correlation (PCC). Differences in PCC between groups will be compared to 1000 lists of coexpression differences mixing cases and controls, that is, 1000 random sets (p-value ≤ 0.05). We will also use the WGCNA tool[52] to search for co-expression module.

The network will be viewed using Cytoscape[53]. We will analyze the differences topological properties of gene networks seeking to prioritize AD-related partners and to compare findings in the hippocampus. For identification of broker and bridge genes, we will use the Interatomic Graph website (http://bioinfo.lbhc.hcancer.org.br/interactomegraph).

Methods of analysis

The data will be analyzed in software R or SPSS 18.

Publication Search

A systematic literature search was conducted in PubMed databases up to April 11, 2018. The combination of terms "neuronal", "glia" and "interactions", "cell culture", "gene interactions", "lithium influence" was used with language restriction - English - and publication date (from 2013 only). Relevant studies were retrieved, and their references were checked to find other relevant publications.

The inclusion criteria were: (1) evaluation of the association between neuronal glia and genes interactions, lithium influence and interactions cell cultures; (2) studies with animals; (3) RNA/DNA analysis. The exclusion criteria were: (1) protein analysis only; (2) studies with humans.

ETHICAL ASPECTS

The approval by the Ethics Committee of the Hospital was obtained before beginning of the studies described in this plan, which are part of the Regular Project CNPQ 2009 / 52825-8, entitled "Expression and activity of APP-secretases and GSK3B in human platelets and cultures effects of treatment with donepezil and lithium ".

Handling and slaughter of animals shall be conducted in accordance with the principles set in the "Guide for the care and use of laboratory animals"(ISBN 0-309-05377-3), National Institute Health (NIH publication No. 86-23, revised 1985).

RESULTS

Microarray Analysis

It was obtained from microarray analysis: 44 genes at 0.02mM; 1256 genes at 0.2mM and 2396 genes at 2 mM of Lithium treatment. To identify the genes differentially expressed in relation to the control condition, the t test and corrected by Bonferroni.

Literature Search and Database Analysis

Following the criteria, were found:

- "Neuronal glia interactions cell culture": 50 articles in the last 5 years;
- "Neuronal glia genes interactions": 25 articles in the last 5 years;
- "Lithium neuronal glia interactions": 4 articles in the last 5 years;
- "Lithium influence in Neuronal glia": 1 article in the last 5 years;
- "Lithium influence in Neuronal glia interactions": 0 article in the last 5 years.

From the remaining articles, the abstracts were read and, again, more articles were filtered. To compose the references to the work, the last filter was the reading of the whole material and methods. In short: search for the descriptors → reading the titles → reading the abstracts → reading the material and methods → obtaining the articles to be used in the review.

The search of databases retrieved many articles giving a total of 80 relevant titles and abstracts for review. A further unrelated 52 articles were excluded based on abstracts. After a material and methods review, 4 studies remained[54-57]. The main characteristics of these eligible studies and the selected genes are listed in Tables 1 and 2.

Table 1. Characteristics of the eligible studies

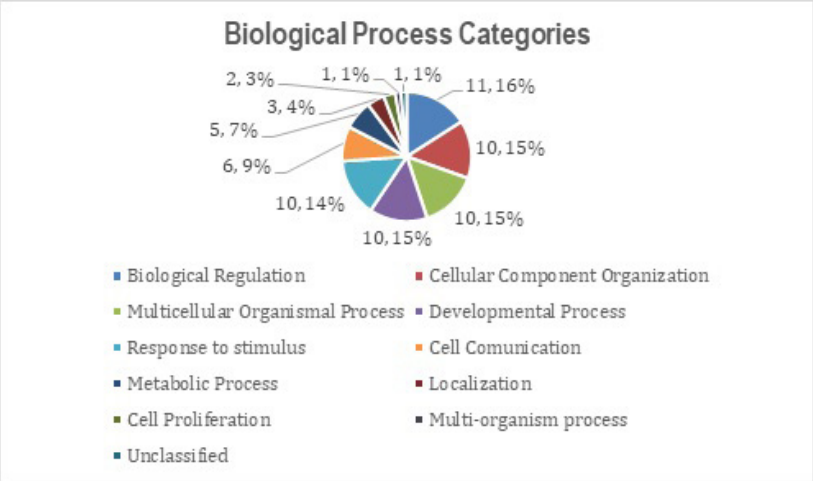
Article	Specie/Model	Biological matrix	Drug treatment	Experiment	Selected genes
Kangas SM, et al., 2016	13.5-day-old mouse embryos Mice (C57BL/6JOlaHsd, Harlan Holland)	Dorsal root ganglions	—	Hybridization Kit (Agilent) into SurePrint G3 (Mouse GE 8 x 60K arrays (Agilent, Design ID 028005).	**6 genes***: Ngfr,* P*ou3f1 Nefm Mag, Mpz , Mbp*
Emamghoreishi M, et al., 2015	18-day embryos of Sprague-Dawley rats	Embryonic cortices	1mM Lithium	PCR	**2 genes**: *S100ß* and *GAPDH*
Chen SH, et al., 2015	Timed-pregnant Fisher 344 rats at day 14 of gestation	Cortices and midbrain	—	PCR	**7 genes:** *GDNF F2, BDNF F1, GAPDH F2, TNFalfa F4, Inos F1, INOS R1, GDNF R2*
Yang J, et al., 2014	P0 or P1 Adult male Sprague–Dawley rats	Hippocampi	—	PCR	**6 genes:** *GAPDH, ephrin A1,ephrin A2,ephrin A3, ephrin A4, ephrin A5*

Table 2. Selected genes

Gene Name	Gene ID *Rattus norvegicus*
Ngfr	24596
Pou3f1	192110
Nefm	24588
Mag	29409
Mpz	24564
Mbp	24547
S100ß	25742
GAPDH	24383
Ephrin A1	94268
Ephrin A2	84358
Ephrin A3	170901
Ephrin A4	310643
Ephrin A5	116683

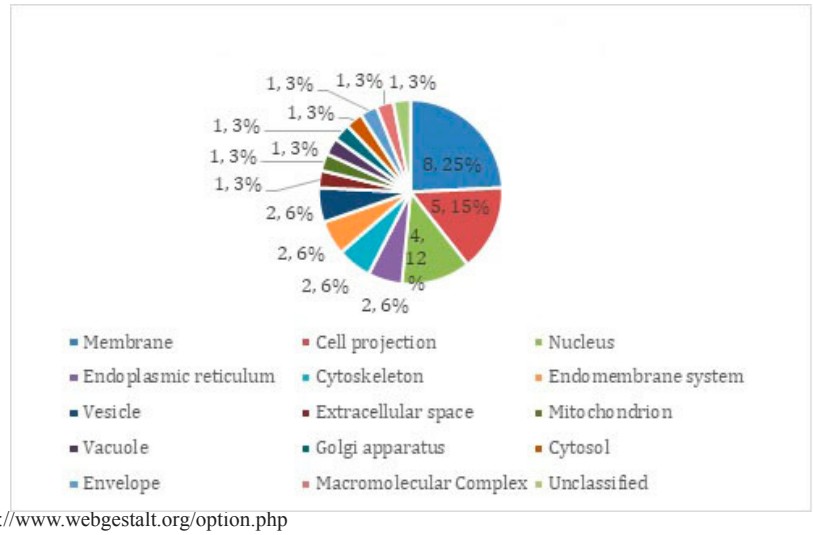
The following genes GDNF F2, BDNF F1, GAPDH F2, TNFalfaF4, INOS F1, INOS R1, and GDNF R2 were excluded because it was not found relation with *Rattus norvegicus* organisms.

The remained selected genes were translated into biological pathways using WebGestalt (WEB-based Gene SeT AnaLysis Toolkit) - a functional enrichment analysis web tool (http://www.webgestalt.org/option.php). For this study, the basic parameters were *Rattus norvegicus* for organisms, Over-Representation Analysis (ORA) as method for enrichment analysis, gene ontology for functional database class and biological process, cellular component and molecular function for functional database categories. For the gene list showed above, their main related biological pathways plotted in charts are presented in Figures 1, 2 and 3.



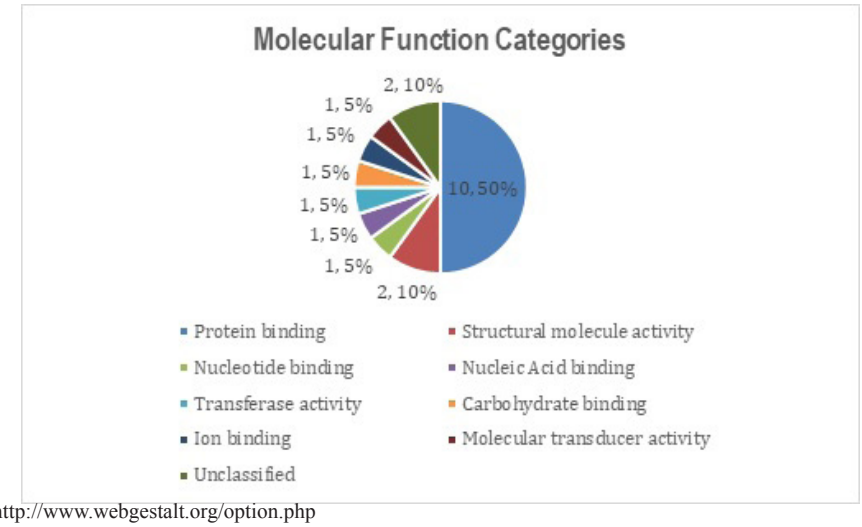
Fonte: http://www.webgestalt.org/option.php

Figure 1. Biological process categories



Fonte: http://www.webgestalt.org/option.php

Figure 2. Cellular components categories

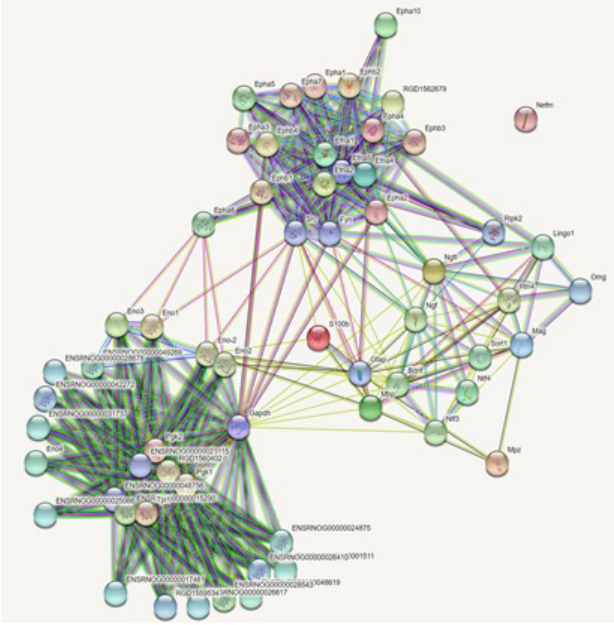


Fonte: http://www.webgestalt.org/option.php

Figure 3. Molecular function categories

In addition, these genes were submitted to STRING, a functional protein association networks analysis web tool (https://string-db.org/). With this database of known and predicted protein-protein interactions, including direct (physical) and indirect (functional) associations from computational prediction, from knowledge transfer between organisms, and from interactions aggregated from other (primary) databases, it was obtained a grown network. Results with no more than 50 interactions between the genes selected from literature and the genes from STRING database are presented in Figure 4.

The remained total genes are listed in Table 3.



Fonte: https://string-db.org/

Figure 4. Grown network with no more than 50 interactions

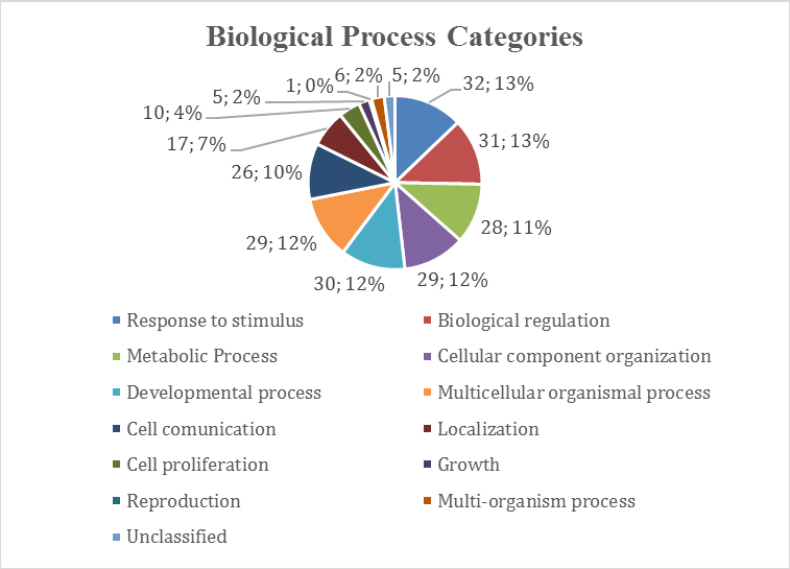
For these second gene list showed above, their main related biological pathways, according to WebGestalt database, plotted in charts are presented in Figures 5, 6 and 7. The basic parameters were *Rattus norvegicus* for organisms, Over-Representation Analysis (ORA) as method for enrichment analysis, gene ontology for functional database class and biological process, cellular component and molecular function for functional database categories.

Comparative Results

The total genes and the genes from microarray analysis for each treatment with Lithium (0.02mM, 0.2mM and 2mM) were compared using a Venn's diagram drawing web tool (http://bioinfogp.cnb.csic.es/tools/venny/). The result for the four lists of elements is presented in Figure 8.

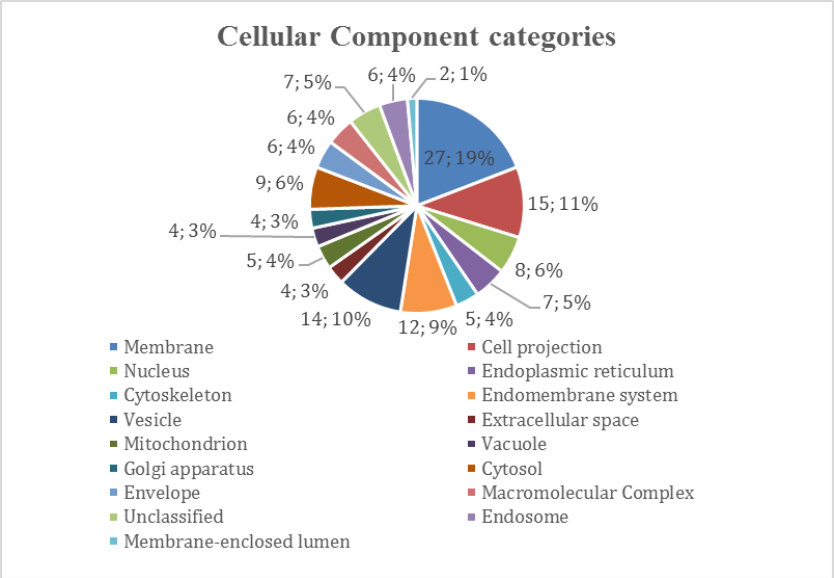
Table 3. Total selected genes

Gene Name	Gene ID *Rattus norvegicus*
Efna1	94268
Tpi1	24849
Eno1	24333
Epha3	29210
Epha4	316539
Pgk2	316265
Eno-2	24334
Eno3	25438
Gapdh	24383
RGD1560402	499525
Ephb3	287989
Efna2	84358
Ephb4	686310
Efna5	116683
RGD1562679	298528
Mag	29409
Epha1	312279
Efna4	310643
Ngf	310738
Epha2	366492
Ephb2	313633
Ephb1	24338
Ngfr	24596
Mbp	24547
Pgk1	24644
Epha7	171287
Rtn4	83765
Ephb3	287989
S100b	25742
Mpz	24564
Bdnf	24225
Eno4	292138
Ntf3	81737
Epha10	298528
Lingo 1	315691
Sort 1	83576
Ntf4	25730
Omg	450224
Ripk2	362491
Gfap	24387
Src	83805
Epha5	79208
Epha8	60589
RGD1559534	287338
Fyn	25150



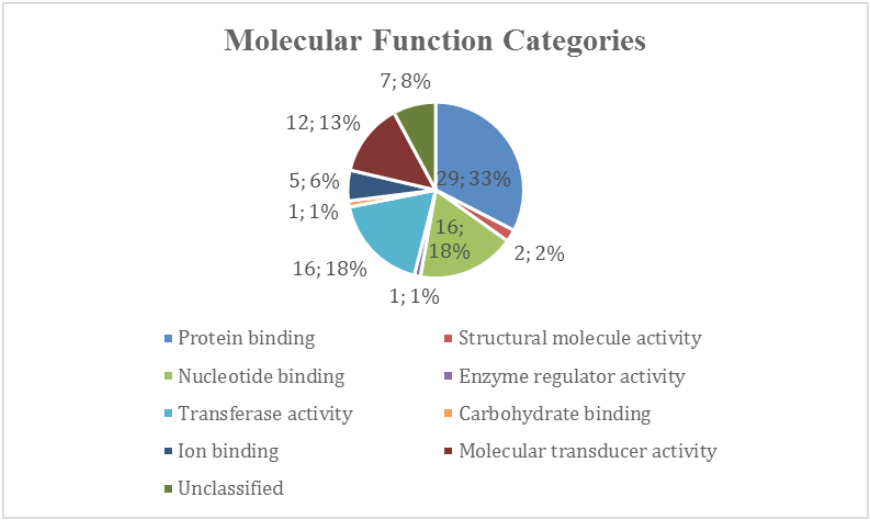
Fonte: http://www.webgestalt.org/option.php

Figure 5. Biological process categories



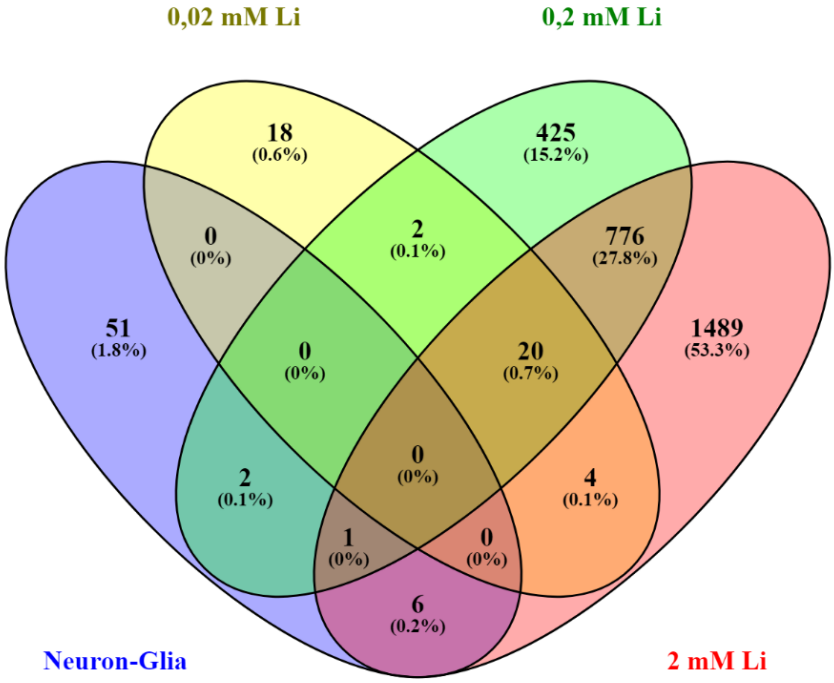
Fonte: http://www.webgestalt.org/option.php

Figure 6. Cellular component categories



Fonte: http://www.webgestalt.org/option.php

Figure 7. Molecular function categories

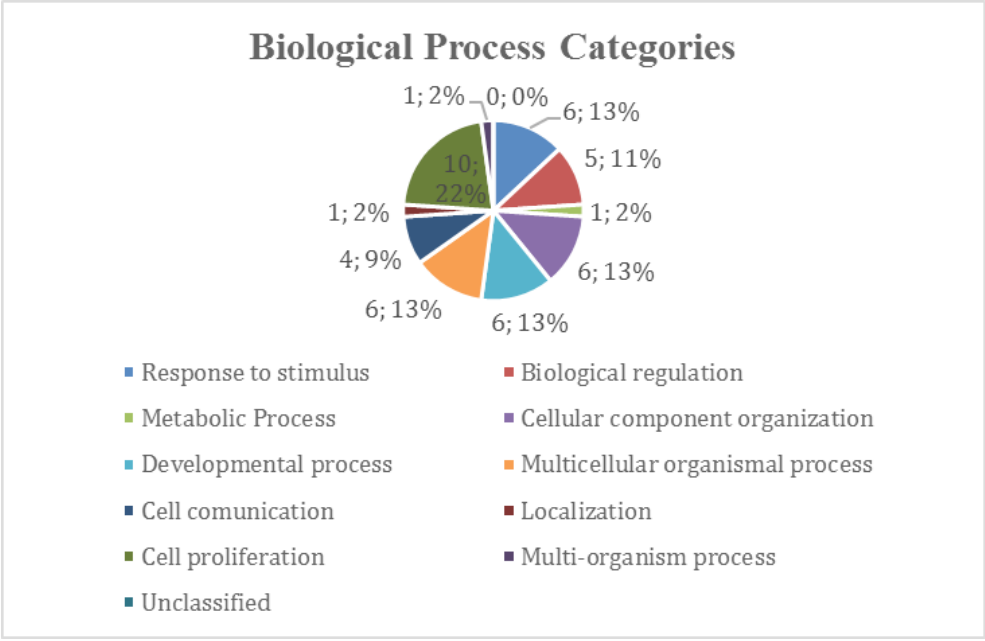


Fonte: http://bioinfogp.cnb.csic.es/tools/venny/

Figure 8. Compared lists of genes with Venn diagram

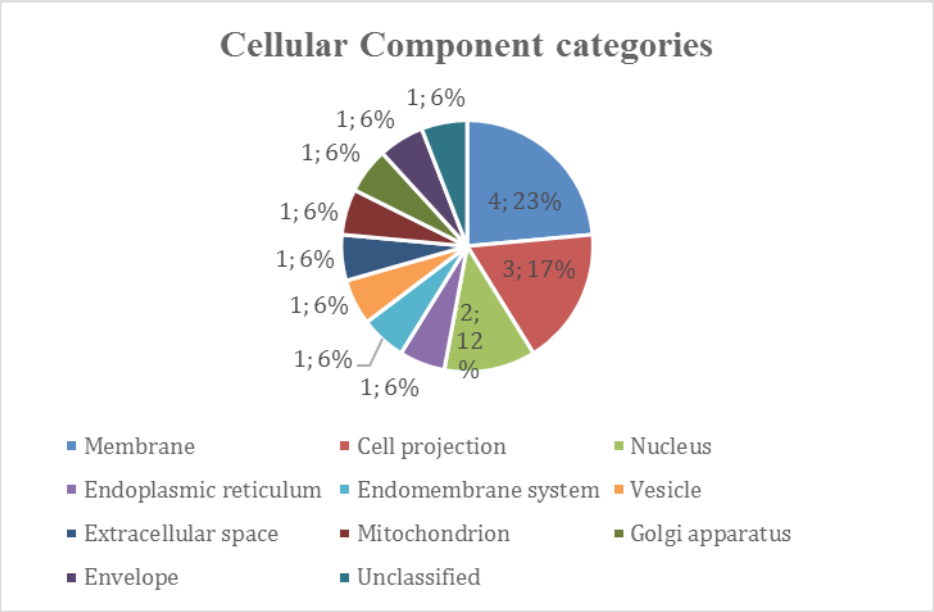
It were found 6 common elements in "Neuron-Glia" and "2 mM Li": Efna2, Efna4, Omg, Ngfr, Mbp, S100b.

The main related biological pathways with the genes above, according to WebGestalt database, plotted in charts are presented in Figures 9, 10 and 11. The basic parameters were *Rattus norvegicus* for organisms, Over-Representation Analysis (ORA) as method for enrichment analysis, gene ontology for functional database class and biological process, cellular component and molecular function for functional database categories.



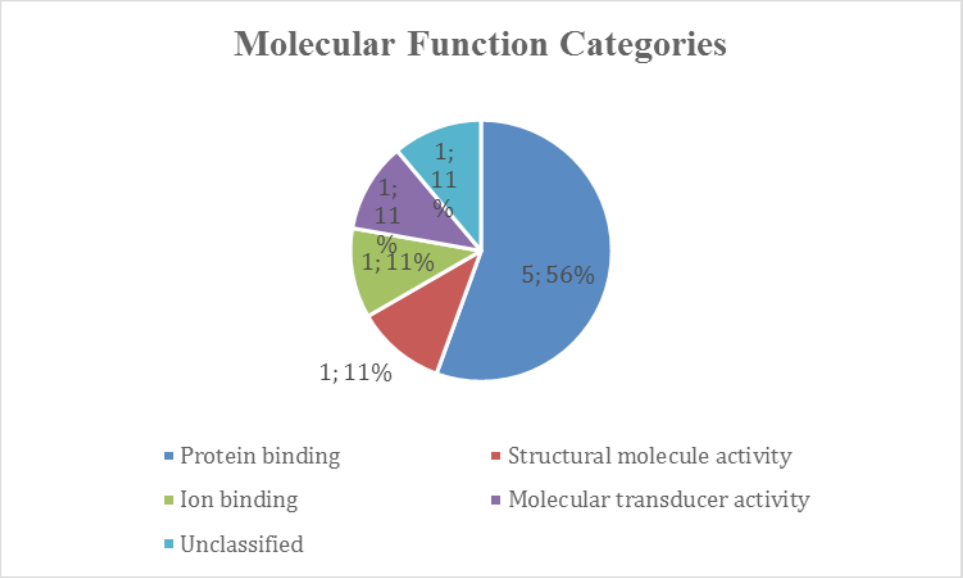
Fonte: http://www.webgestalt.org/option.php

Figure 9. Biological process categories



Fonte: http://www.webgestalt.org/option.php

Figure 10. Cellular component categories



Fonte: http://www.webgestalt.org/option.php

Figure 11 - Molecular function categories

DISCUSSION

We analyzed 4 articles in order to identify genes related to the neuroprotection and the neuronal-glia interaction. These data were used to compare with the ones obtained from the microarray analysis, which includes the genes differentially expressed after a treatment with 0.02, 0.2 and 2mM doses of Lithium, to identify if there is any influence of these doses of treatment into the neuronal-glia interactions pathways.

The main related biological pathways with the genes found in the selected articles were Biological Regulation (11.6%), Membrane (8.25%) and Protein Binding (10.5%) considering, respectively, Biological Process, Cellular Components and Molecular Function categories. For the grown network after STRING database interactions analysis, the results were: Response to Stimulus (32.13%), Membrane (27.19%), Protein Binding (29.33%). At last, for the in common genes between the neuron-glia grown network and genes differentially expressed after a 2mM

Lithium treatment, the main related biological pathways were: Cell Proliferation (10.22%), Membrane (4.23%) and Protein Binding (5.56%). Therefore, biological pathways related to cellular membrane and protein binding function were found as main related with the pertinent genes for neuroprotection and neuronal-glia interaction and with the genes differentially expressed after a high dose of treatment with Lithium.

In addition, there were no overlapping results between the different treatment doses of Lithium and genes linked to neuronal-glia interaction but only between these genes and 2mM of Lithium treatment. High doses, though, can influence the biological pathways related and low doses cannot.

CONCLUSION

Genes with neuron-glia interaction were found in the literature but few of these genes were expressed in cultures treated with Lithium. Lithium presented a dose-dependent effect: sub therapeutic doses showed little or no pathway related to neuroprotection. The project suggests that the therapeutic dose (2mM) is the only one that changes genes related to neuronal-glia interaction.

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