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Identification of Neuroregenerative Protein Networks

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HIGHLIGHTS

- Some genes encode proteins with ambiguous or less known functions in nervous system regenerative processes, like *IL6*, *IL1B*, and *CXCL12*.
- PPI networks were effectively used to analyze nervous system regeneration, providing insights into centrality and protein connections.
- Cytoscape is a platform for working with PPI networks.

Abstract: One of the main limitations of Regenerative Medicine was growth factor selection for stem cell culture to obtain optimal differentiation, resulting in functional tissue. For neural regeneration tissue engineering techniques, this choice became even harder due to tissue complexity. This work aimed to use a systems biology approach allied to bioinformatics to identify growth factors for nervous cell regeneration in protein-protein interaction (PPI) networks, potentially resulting in more effective therapy design. Articles were curated using the PubMed database, and protein coding genes related to neuroregeneration and nervous tissue engineering were extracted for PPI network construction, in which enrichment analysis of Gene Ontology, clustering, and protein centrality were applied. The work generated a main network with 64 nodes, 440 edges, and 3 clusters: one with 21 nodes and 187 edges, another with 6 nodes and 12 edges, and a third one with 3 nodes and 3 edges. Some of the proteins identified, such as *NGF*, *BDNF*, *GDNF* and *NTF*s, had known functions in neuroregeneration, validating our method efficiency. Protein-coding genes with ambiguous functions or less described in the literature were also found, such as *IL6*, *IL1B*, and *CXCL12*, suggesting more studies to clarify their roles in neuroregeneration.

Keywords: systems biology; neuroregeneration; proteomics; bioinformatics; regenerative medicine.

INTRODUCTION

Regenerative Medicine's goal is to restore lost cellular functions of damaged organs via cellular regeneration or transplantation using a healthy equivalent [1]. Regenerative Medicine has predominantly used graft implants or tissue restoration, but there is a race to develop therapeutic processes that reduce or eliminate the long wait for an organ donor and decrease the chances of organ or tissue rejection [2].

In vitro tissue engineering needs Pluripotent Stem Cells (iPSC), and a scaffold made of biopolymers that can be integrated or degraded by the organism in the presence of biomolecular inducers of tissue growth and differentiation [3, 4]. Despite the promising results of Regenerative Medicine in epithelial and connective tissues [5, 6], there is still a lot to advance regarding cost reduction and its use for complex tissue regeneration, such as muscular and nervous tissue.

Nervous tissue regeneration is very limited, especially the central nervous system [7, 8], which results in many people having irreversible sequels resulting from traumas, accidents, and degenerative diseases. One of the main hurdles for Regenerative Medicine is to control stem cell fate and phenotype [9, 10], which will be greatly improved upon a better understanding of signaling pathways involved in the desired differentiation [11, 12].Given the complexity of protein-protein interaction networks, its analysis requires robust bioinformatics tools, such as Cytoscape [13, 14].

Nerve tissue regeneration needs better scaffold matrixes and better selection of stem cell lineages [15–19]. To our knowledge, there is no *in silico* study of nervous system growth factor interaction applied to tissue engineering. Here we seek to find relevant proteins involved in nervous tissue regeneration that could be better evaluated *in vitro* and *in vivo*, for their potential therapeutic application.

MATERIAL AND METHODS

Manual Curation

The assembly of protein-protein interaction networks, to carry out *in silico* analyses of growth factors involved in nervous system regeneration, started by using a PubMed search tool to find articles about proteins with neural regeneration potential. This step was divided into two secondary procedures: a search for articles and data analysis, directly applying the methodology for building protein-protein interaction networks developed by our research group [20, 21].

Keywords used for the search were: "((regeneration AND nervous system) OR neuro regeneration) AND tissue engineering AND growth factors"; filters applied in the search were: Species "Human" | Text Availability: "Free full text". We aimed to obtain articles about nervous tissue regeneration and human tissue engineering, resulting in growth factors and proteins that could be applied to *in vitro* studies.

The construction of protein-protein interaction networks utilized the "STRING App," an extension of the "Cytoscape 3.9.1" software. Proteins were added with the data source adjusted as "protein query" and the species configuration set as "*Homo sapiens*" to ensure compatibility. Considering the large number of proteins, the interaction reliability cutoff value was raised to 50% to increase accuracy. Maximum number of additional interactors was set to 0.

To identify the most relevant proteins within this cluster, two measures of centrality were used: the betweenness centrality (BC), which calculates how central a protein is in the network based on the number

of shortest paths through any given vertices that pass through a node; and Closeness Centrality (CC), which takes into account the sum of the shortest paths between a node and all other network nodes.

Subsequently, the "MCODE App" was used to calculate the weight of certain nodes and the clustering coefficient of correlated molecules within the network [22]. To increase specificity and associate PPI network regions with biological processes and molecular functions, pathway enrichment analysis was conducted. The "STRING enrichment" tool was utilized to display different divisions within GO databases, KEGG Pathways, and WikiPathways metabolic pathways databases, providing insights into biological processes, molecular functions, metabolic pathways, tissue specificity, and cellular localization of protein activity.

Furthermore, the significance of the correlation between interacting proteins and categories was assessed using False Discovery Rate (FDR) value calculated by the STRING App. Lower FDR values indicated lower chances of false positives, enabling result ranking based on significance, with the most important associations listed first.

RESULTS AND DISCUSSION

We used 77 articles to extract 64 genes encoding proteins associated with neuroregenerative processes. The list of protein-coding genes is shown in the table below.

Table 1. Proteins obtained after curation of PubMed articles. The table lists a series of proteins associated with neural regeneration and tissue repair. For each protein, three types of information are provided: the protein name, the associated code in the UNIPROT database, and the corresponding gene name. The listed proteins include growth factors, neurotrophins, interleukins, fibroblast growth factors, vascular endothelial growth factors, as well as other proteins and factors associated with neural regeneration and cell growth.

PROTEIN	CODE IN UNIPROT	GENE
Beta-nerve growth factor	P01138	*NGF*
Brain-derived neurotrophic factor	P23560	*BDNF*
Neurotrophin-3	P20783	*NTF3*
NT-4/5	P34130	*NTF4*
Glial cell line-derived neurotrophic factor	P39905	*GDNF*
PSP	O60542	*PSPN*
NTN	O95631	*NTN1*
ART	Q5T4W7	*ARTN*
Interleukin-6	P05231	*IL6*
Ciliary neurotrophic factor	P26441	*CNTF*
TGF-β3	P10600	*TGFB3*
Fibroblast growth factor 1	P05230	*FGF1*
Pro-neuregulin-1	Q02297	*NRG1*
Fibroblast growth factor 2/Basic fibroblast growth factor	P09038	*FGF2/bFGF*
Hepatocyte growth factor	P14210	*HGF*
Leukemia inhibitory factor	P15018	*LIF*
Osteopontin	P10451	*SPP1*
SPARC	P09486	*SPARC*
Clusterin	P10909	*CLU*
Chondroitin sulfate proteoglycan 4	Q6UVK1	*CSPG4*
Chondroitin sulfate proteoglycan 5	O95196	*CSPG5*
Vascular endothelial growth factor A	P15692	*VEGFA*
Vascular endothelial growth factor B	P49765	*VEGFB*
Nestin	P48681	*NES*
Pro-epidermal growth factor	P01133	*EGF*
Neurogenin-1	Q92886	*NEUROG1*

Cont. Table 1

Neurogenic differentiation factor 1	**Q13562**	***NEUROD1***
Neuromodulin	P17677	*GAP43*
Tyrosine 3-monooxygenase	P07101	*TH*
Choline O-acetyltransferase	P28329	*CHAT*
Fibroblast growth factor 4	P08620	*FGF4*
Interleukin-7	P13232	*IL7*
Macrophage migration inhibitory factor	P14174	*MIF*
Platelet-derived growth factor subunit B	P01127	*PDGFB*
Ciliary neurotrophic factor	P26441	*CNTF*
Thrombomodulin	P07204	*THBD*
Insulin-like growth factor I	P05019	*IGF1*
Protransforming growth factor alpha	P01135	*TGFA*
Interleukin-3	P08700	*IL3*
Kit ligand	P21583	*KITLG*
Thyroid peroxidase	P07202	*TPO*
Bone morphogenetic protein 4	P12644	*BMP4*
Interleukin-8	P10145	*IL-8*
Fibroblast growth factor receptor 3	P22607	*FGFR3*
Vinculin	P18206	*VCL*
Profilin-1	P07737	*PFN1*
Focal adhesion kinase 1	Q05397	*PTK2*
Mitogen-activated protein kinase 1	P28482	*MAPK1*
CDC42 small effector protein 1	Q9NRR8	*CDC42SE1*
Interleukin-2	P60568	*IL2*
Tumor necrosis factor	P01375	*TNF*
Transforming growth factor beta-1 proprotein	P01137	*TGFB1*
Interleukin-1 beta	P01584	*IL1B*
Sonic hedgehog protein	Q15465	*SHH*
Interleukin-10	P22301	*IL10*
Neurotensin/neuromedin N	P30990	*NTS*
E3 SUMO-protein ligase EGR2	P11161	*EGR2*
Stromal cell-derived factor 1	P48061	*CXCL12*
C-C motif chemokine 2	P13500	*CCL2*
Interleukin-13	P35225	*IL13*
Growth/differentiation factor 15	Q99988	*GDF15*
Insulin-like growth factor-binding protein 5	P24593	*IGFBP5*
Transforming growth factor beta-2 proprotein	P61812	*TGFB2*
Pleiotrophin	P21246	*PTN*

The result was a network with 64 nodes and 440 edges as shown in Figure 1.

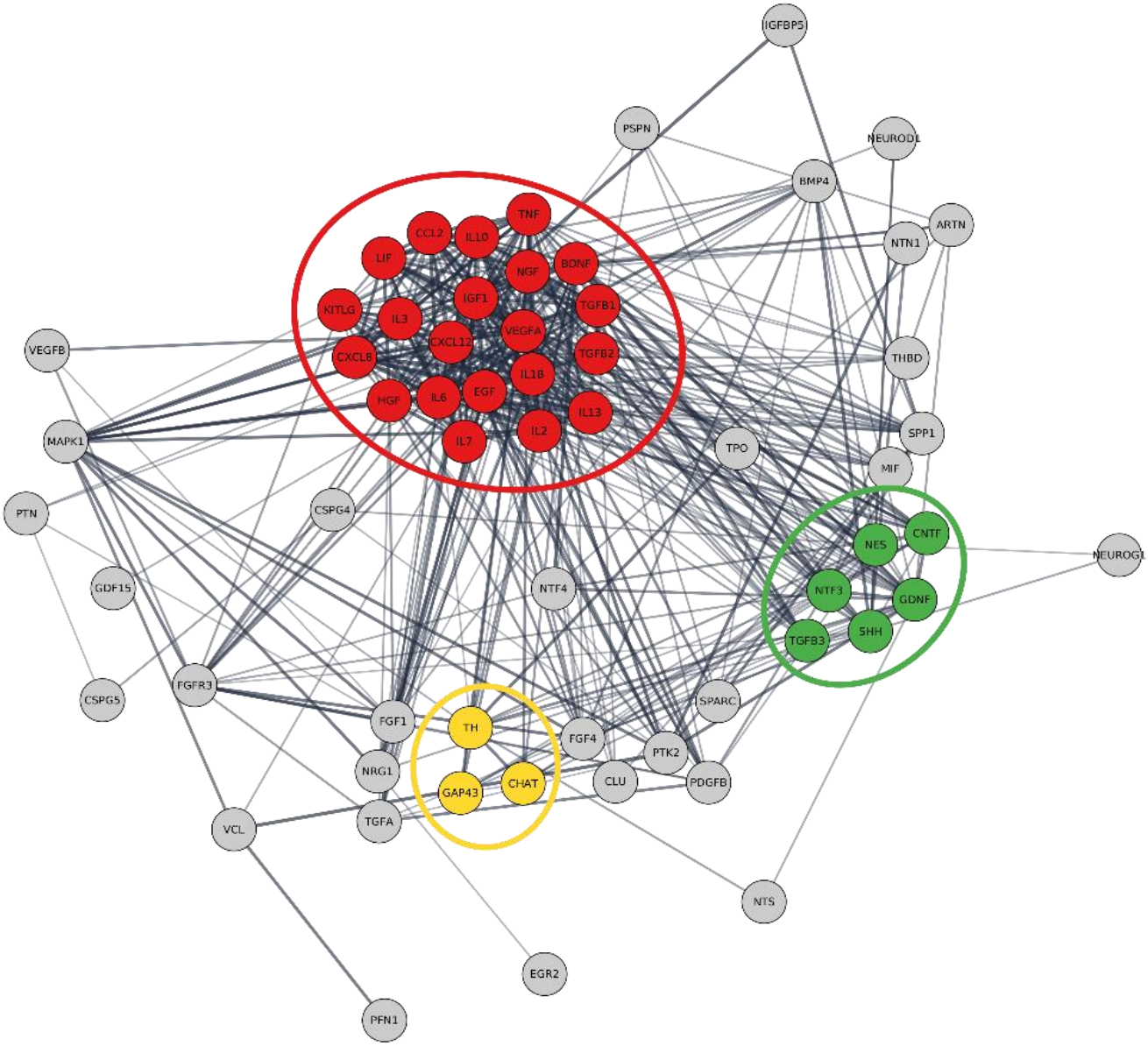

Figure 1. Cytoscape PPI network resulting from curated proteins. In Red: Cluster 1; In Green: Cluster 2 and in Yellow: Cluster 3.

After assembling this network, the "MCODE App" was used to divide the network into three distinct groups, from which new networks were formed. The three networks were based on MCODE algorithms and analyzed by “STRING enrichment,” generating a Gene Ontology database that defines molecular function, called GO Molecular Function; a Gene Ontology database that defines biological processes, called GO Biological Process; and a WikiPathways and KEGG Pathways metabolic pathway database. The three clusters formed, their main proteins, their molecular functions, and the biological processes to which they are linked are described below.

Cluster 1:

The largest cluster had 21 proteins (Table 2), forming a network of 21 nodes and 187 edges (Figure 2). Molecular function most correlated was that of receptor-ligand activity (GO: 0048018), with an FDR of 1,2E-30.

Binding to cytokine receptors (GO:0005126) presented a relevant FDR of 4.24E-28, added to the fact that 18 of 21 molecules are related to such activity and that these molecules are involved in cytokine metabolic pathways and inflammatory processes present on WikiPathways (WP530 FDR of 1.19E-17), suggests that such proteins may be involved in signaling pathways important to nervous system regeneration. [23–25]

Table 2. Proteins present in Cluster 1 after MCODE App analysis.

PROTEIN	CODE IN UNIPROT	GENE
Vascular endothelial growth factor A	P15692	*VEGFA*
Tumor necrosis factor	P01375	*TNF*
Transforming growth factor beta-2 proprotein	P61812	*TGFB2*
Transforming growth factor beta-1 proprotein	P01137	*TGFB1*
Beta-nerve growth factor	P01138	*NGF*
Leukemia inhibitory factor	P15018	*LIF*
Kit ligand	P21583	*KITLG*
Interleukin-7	P13232	*IL7*
Interleukin-6	P05231	*IL6*
Interleukin-3	P08700	*IL3*
Interleukin-2	P60568	*IL2*
Interleukin-1 beta	P01584	*IL1B*
Interleukin-13	P35225	*IL13*
Interleukin-10	P22301	*IL10*
Interleukin-8	P10145	*IL-8*
Insulin-like growth factor I	P05019	*IGF1*
Hepatocyte growth factor	P14210	*HGF*
Pro-epidermal growth factor	P01133	*EGF*
Stromal cell-derived factor 1	P48061	*CXCL12*
C-C motif chemokine 2	P13500	*CCL2*
Brain-derived neurotrophic factor	P23560	*BDNF*

Another important molecular function shared by 16 of the 21 proteins found is their activity as growth factors (GO:0008083), with an FDR of 1.25E-26. Understanding the differences between cytokines and growth factors and their mechanisms of action is relevant for the development of more effective therapies [26]. Cytokines can regulate inflammatory processes, which can lead to neurotoxic processes or even neural cell apoptosis [27]. They can also mobilize glial cell activity as a first response to damage and trauma [27, 28].

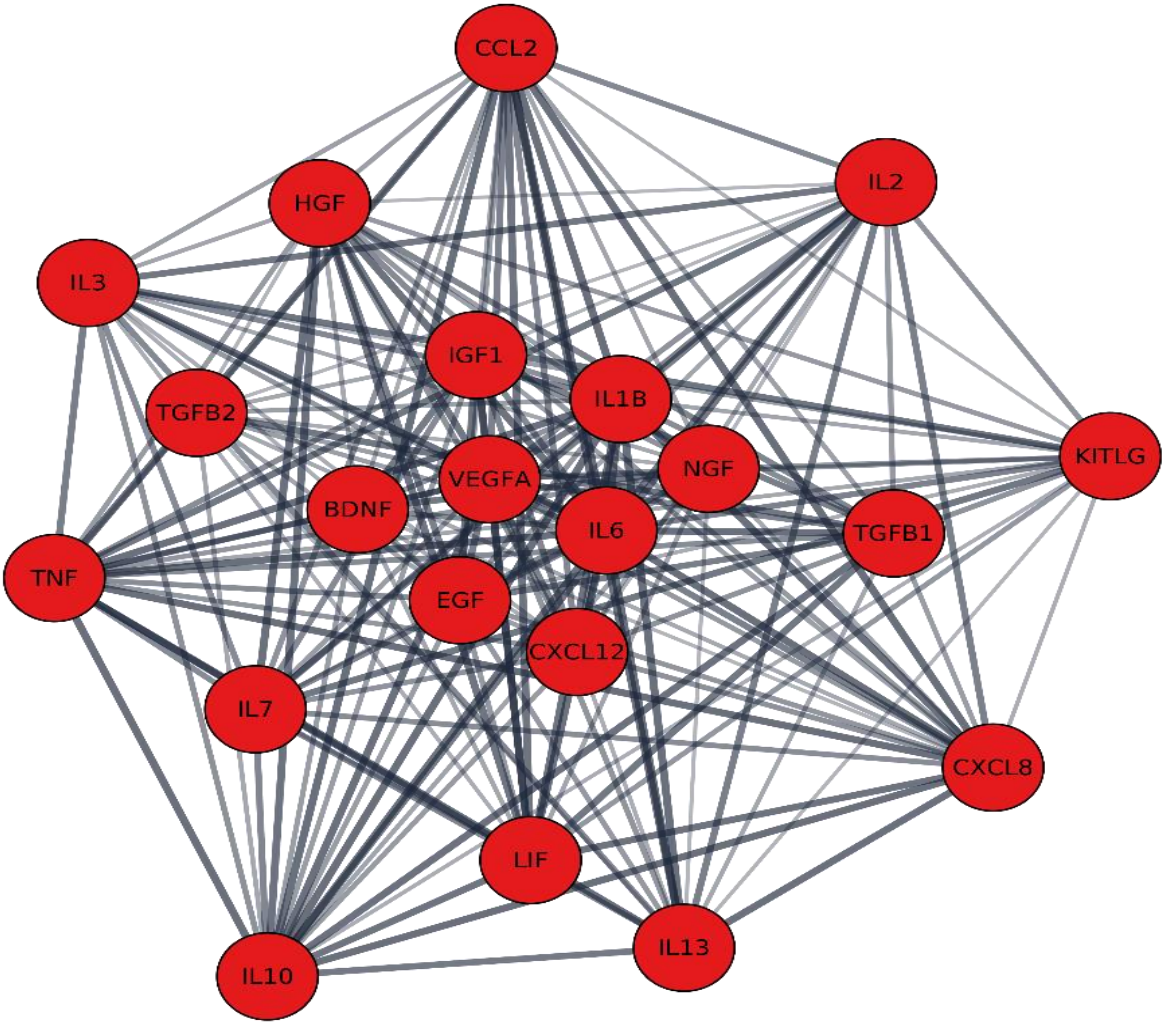

Figure 2. PPI network with proteins that compose the Cluster 1 after MCODE App analysis.

In betweenness centrality (BC) and closeness centrality (CC) analyses, the proteins considered most important within the cluster are those whose genes are listed in Table 3. This suggests that these proteins play crucial roles in the protein-protein interaction network , significantly influencing communication and interaction between cluster components [29]:

Table 3. Coding genes for most central proteins in Cluster 1 and their respective centrality scores.

GENE	CLOSENESS CENTRALITY	BETWEENNESS CENTRALITY
VEGFA	0.7228915662650602	0.1173273572494937
EGF	0.7142857142857143	0.08923373414176952
NGF	0.6818181818181819	0.07543670397092214
IGF1	0.6818181818181819	0.05550419313681339
IL6	0.6666666666666666	0.07207207394254099
BDNF	0.6451612903225806	0.06335408969891736
IL1B	0.625	0.024037823977144834
CXCL12	0.6185567010309279	0.03092741152578663

Vascular Endothelial Growth Factor A (*VEGFA*) is a proangiogenic molecule that induces vascularization, thus allowing the arrival of nutrients, oxygen and other molecules necessary for regeneration [30, 31]. Molecules from the *VEGF* family provide conditions for cell survival, explaining their high degree of centrality.

Epidermal Growth Factor (*EGF*) induces cell proliferation and differentiation with a well-known role in nervous system regeneration, being used in neural regeneration clinical trials [32–34].

Neural Growth Factor (*NGF*) is a member of the neurotrophin family, with more specific actions on nervous tissue cell proliferation, differentiation, and survival [35]. *NGF* increases the number of myelinated axons, matured nerves, myelin sheath thickness, fiber diameter, and population density. In the absence of *NGF*, apoptosis is likely to occur, supporting its role as an inhibitor of programmed cell death [36].

Insulin-Type I Growth Factor (*IGF1*) is a molecule produced in the liver and stimulated by growth hormones acting on cellular growth and development processes, as well as promoting neurogenesis [37].

Interleukin-6 (*IL6*) is an inflammatory cytokine that shows ambiguous effects on the nervous system. Despite appearing as a harmful cytokine in nervous development [38], there are studies that suggest a role in differentiation of oligodendrocytes and astrocytes, with potentially neurotrophic action [37].

Brain-Derived Neurotrophic Factor (*BDNF*), another growth factor of the neurotrophic family, has *NGF* like properties, sharing with it a genetic homology of 54% [39]. *BNDF* can help axon functional recovery, promoting survival and differentiation of human neural precursor cells (*HNPC*) [40].

Interleukin 1-beta (*IL1B*) is a pro-inflammatory cytokine that has neurodegenerative effects [41], however, in cases of trauma or neurological damage, *IL1B* can induce reactive astrocytes. Astrocyte reactivity, like the formation of oligodendrocytes and astrocytes induced by *IL6*, has ambiguous effects, and may promote neuroprotection, synaptic loss, or neurotoxicity [38, 42–44].

Stromal Cell-Derived Factor 1 (*SDF-1*) encoded by the *CXCL12* gene is a small chemokine that acts on leukocyte migration (44), also showing nervous system angiogenic, neurogenic and anti-apoptotic properties. [45].

Interestingly, genes encoding proteins with ambiguous functions in the nervous system were centrally interacting cluster proteins, such as *IL6*, *IL1* and *CXCL12*.

Cluster 2:

The second most relevant cluster is composed of 6 proteins, which form a network of 6 nodes and 12 edges, containing the proteins listed in Table 4. Based on STRING Enrichment data, biological processes that presented the lowest FDR value showed a negative regulation of neuronal apoptosis (GO:0043524) with an FDR of 2.09E-6 and a negative regulation of apoptotic processes (GO:0043066) with an FDR of 2.38E-5, compatible with pluripotent stem cell differentiation pathway in the WikiPathways database (WP2848) with an FDR of 2.2E-4.

Table 4. Proteins present in Cluster 2 after MCODE App analysis.

PROTEIN	UNIPROT CODE	GENE
Neurotrophin-3	P20783	*NTF3*
Glial cell line-derived neurotrophic factor	P39905	*GDNF*
Ciliary neurotrophic factor	P26441	*CNTF*
TGF-β3	P10600	*TGFB3*
Nestin	P48681	*NES*
Sonic hedgehog protein	Q15465	*SHH*

Negative regulation of apoptosis can lead to neurodegenerative processes, which have a negative impact in the nervous system [46]. The protein with the highest degree of network centrality shown in Figure 3 was *Glial Cell Lineage-Derived Neurotrophic Factor* (*GDNF*), with a score of BC = 0.0707 and CC = 0.625. *GDNF* acts as a nerve growth factor, promoting axonal growth and neuron myelination [47, 48]. GDNF can induce neuroprotective effects by preventing astrogliosis harmful effects [49].

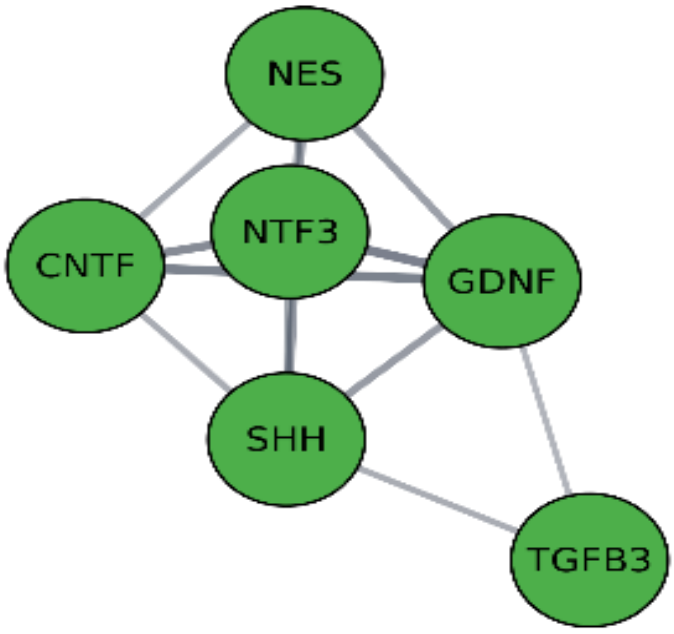

Figure 3. PPI network with proteins that compose Cluster 2 after MCODE App analysis.

Nestin (NES) (BC = 0.0611 and CC = 0.588) can promote neurogenesis, survival, and renewal of neural progenitor cells [50].

The third protein with the highest degree of centrality was *Sonic Hedgehog* (*SHH*) with a score of BC = 0.0361 and CC = 0.576. *SHH* acts as a growth factor in spinal cord development, promoting neurites differentiation and inhibiting astrocyte lineages, preventing pathogenic inflammatory processes [51].

Neurotrophin-3 (*NT-3*), encoded by the *NTF3* gene, belongs to the neurotrophin family (BC = 0.0121 and CC = 0.555). NT-3 has axonal regeneration and neuron myelination action, possibly promoting neuron protection [52, 53]. *NT-3* has chemotaxis properties, guiding axon growth to form nerves [54]. *Ciliary Neurotrophic Factor* (*CTNF*, BC = 0.0080 and CC = 0.550) increases survival of ganglion neurons, preventing neuron degeneration after axotomy [55]. Furthermore, *CTNF* can promote axon regeneration by inducing the proliferation of Schwann cells [56]. The protein with the lowest centrality in the cluster was *Transforming Growth Factor beta-3* (*TGFB3*, BC = 0.0016 and CC = 0.530), which has an effect on differentiation and proliferation of the nervous system [57].

Cluster 3:

The third and smallest cluster resulted in a network of three nodes and three edges, with the proteins listed in Table 5. STRING Enrichment results indicated the category with the lowest FDR of WikiPathways metabolic pathway, biogenic amine synthesis (WP550), with an FDR of 0.0014 and biological processes from the GO database as being related to neurotransmitter biosynthetic processes (GO:0042136), with an FDR of 0.0308.

Table 5. Proteins present in Cluster 3 after MCODE App analysis.

PROTEIN	UNIPROT CODE	GENE
Neuromodulin	P17677	*GAP43*
Tyrosine 3-monooxygenase	P07101	*TH*
Choline O-acetyltransferase	P28329	*CHAT*

For this small network (Figure 4), with only three nodes, the centrality value of each one of them is of little relevance due to high error rates.

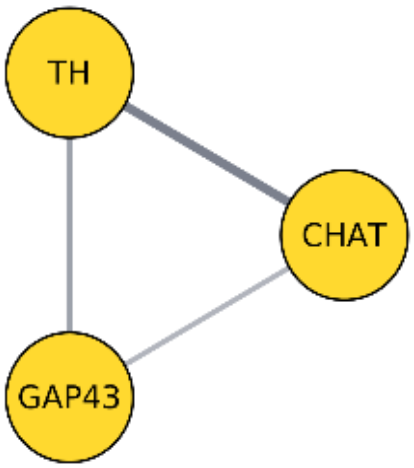

Figure 4. PPI network with the proteins composing the Cluster 3 after MCODE App analysis.

Although these are genes involved in neurotransmitter biosynthesis, their direct effect on nervous tissue regeneration is not clearly known. Some studies indicate that neurotransmitters can contribute to developmental processes as signals and regulators of nerve cell growth and differentiation [58–60].

In this work, the construction of protein-protein interaction networks (PPI) proved to be a valuable tool in the analysis nervous system regeneration data, providing centrality data and degree of connection within a complex system. In addition, enrichment tools added a layer of network functionality. Cytoscape demonstrated its effectiveness as a satisfactory platform, and with the availability of applications (Apps), created by other study groups, it may have unforeseen future use. Open Source tools in science is increasingly present and moves a considerable portion of scientific production.

Several network proteins, such as *NGF*, *BDNF*, *GDNF* and *NTF*s, are already known for their functions in neural regeneration, validating our results. In addition, we also found genes encoding proteins with ambiguous or less known functions in nervous system regenerative processes, such as *IL6*, *IL1B* and *CXCL12*, with a further need to better evaluate their importance in neuroregeneration.

This study proved possible to obtain useful information from noisy data and to interpret it for the benefit of medical treatments.

CONCLUSION

Protein-protein interaction networks (PPI) were used for the analysis of nervous system regeneration, providing information about centrality and protein connections. Cytoscape software and applications confirmed the importance of *NGF*, *BDNF* and *GDNF* in neural regeneration. Ambiguous proteins like *IL6*, *IL1B* and *CXCL12* need further investigation.

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