Developing an Astrocyte Signaling Model to Inform and Improve Stroke Treatment

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Developing an Astrocyte Signaling Model to Inform and Improve Stroke Treatment

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Abstract

Due to the complex nature of ischemic stroke, it is challenging to identify and create targeted therapies following the removal of the obstruction. There is a lack of comprehensive signaling networks that could assist researchers in discerning targets specifically in astrocyte signaling following stroke. The signaling network model was designed to analyze inter- and intracellular communications in stroke and normal physiological conditions and in the presence of mitochondrial delivery. The model highlights outputs of interest including proliferation, different morphologies, and exosomes to elucidate a clearer understanding of cell-cell communications. The model accurately predicts a decrease in resting morphology, and an increase in both reactive morphology and glutamate release when compared to existing literature. The model also predicted an increase in exosomes following stroke and mitochondrial delivery when compared to a normal resting condition. *In vitro* experiments found that the stroke with mitochondria group resulted in a significant increase in exosomes compared to the stroke ($p<0.05$) and control ($p<0.01$) groups, validating the findings of the model.

Keywords: stroke, astrocytes, exosomes, signaling model

Introduction

In 2017, stroke was the fifth leading cause of death in the United States[1]. There are three main types of strokes: an ischemic stroke, a hemorrhagic stroke, and a transient ischemic attack[2]. Ischemic stroke, which comprises 87 percent of stroke incidences, occurs when there is a blockage in a blood vessel that is supplying the brain, resulting in cell death as oxygen and nutrients cannot reach brain cells[2]. In ischemic stroke, the brain's normal metabolic processes and energy levels are severely disrupted. Without available energy, the cells are unable to maintain ion gradients, causing depolarization and the release of amino acids at the synapses. The lack of energy also interferes with the reuptake processes at the synapses in the brain, leading to accumulation of glutamate. As a result, ions flow into the neurons, affecting the osmotic pressures and causing the cells to swell. Ultimately, these events give rise to excitotoxicity and increased intracranial pressure and, therefore, cell damage, necrosis and potential apoptotic processes[3–5]. The effects of the obstructed blood flow during stroke are vast and severe. Studies have found that over 50 percent of patients who survive beyond two days following their first stroke die within five years due to complications from the stroke, including a subsequent stroke and heart disease[6,7]. Treatment of stroke within four and a half hours of its onset is necessary to significantly reduce the risk of long-term disability and mortality; beyond that time, treatment efficacy decreases significantly[8]. Thus, given the high rates of stroke occurrence and the severity of outcomes, effective treatments are critical.

Due to the time-sensitive nature of ischemic stroke, current treatments are limited, and treatments focus predominantly on removing the obstruction. Therapies such as delivery of tissue plasminogen (tPA) activator are used to dissolve clots in order to improve blood flow and prevent ischemic damage[9]. These treatments appear promising; however, many patient populations are not eligible due to restrictions[10]. Additionally, tPA has only been found to prevent disability in six out of every 1000 strokes and also increases the risk of bleeding in the brain[11]. Mechanical devices are designed to retrieve and remove the occlusion. These tools have proven

effective, but they are only used in approximately three percent of hospitals[12]. These tools are also only effective on the first pass in approximately one quarter of patients, despite first pass success being necessary for the best outcomes[13].

There are several existing computational models that relate to stroke, but they typically are not robust enough to be used to identify new therapies. Models have been developed to simulate recovery of motor function following stroke, but these models cannot be used to identify specific treatment targets in the brain[14,15]. Other models have been created to detail intracellular communication following stroke. However, these models depict a limited number of communication pathways, which limits the extent to which the model can be used to understand interactions within cells[16,17].

This lack of an effective treatment and the knowledge gap that limits the development of new treatments have governed our modeling and hypothesis. We hypothesize that delivering mitochondria to astrocytes following stroke will increase the number of factors that promote recovery in the released exosomes. A schematic is given in Figure 1 describing our hypothesis, showing an increase in released exosomes following mitochondrial delivery following ischemic stroke.

Astrocytes are specialized glia cells and are the most abundant cell type in the central nervous system[18]. They play an essential role in maintaining normal brain function. Following an ischemic stroke, astrocytes carry out multiple functions that both benefit and damage neurons, making them an excellent therapeutic target to improve functions in the central nervous system[19].

Exosomes are extracellular vesicles that transport proteins, nucleic acids, and lipids between cells over long or short expanses and are proficient in manipulating target cells[20]. Exosomes released by neural cells play an important role in communication between these cells and the periphery in both normal and disease conditions[20]. Being able to manipulate the phenotype of these exosomes is important for preventing further brain degradation post stroke and developing new treatments. Recent studies in ischemic heart disease have successfully shown that delivering mitochondria to the affected area promotes recovery[21]. Ischemic stroke follows a similar mechanism to ischemic injury in the heart, and thus the impact of mitochondria delivery is

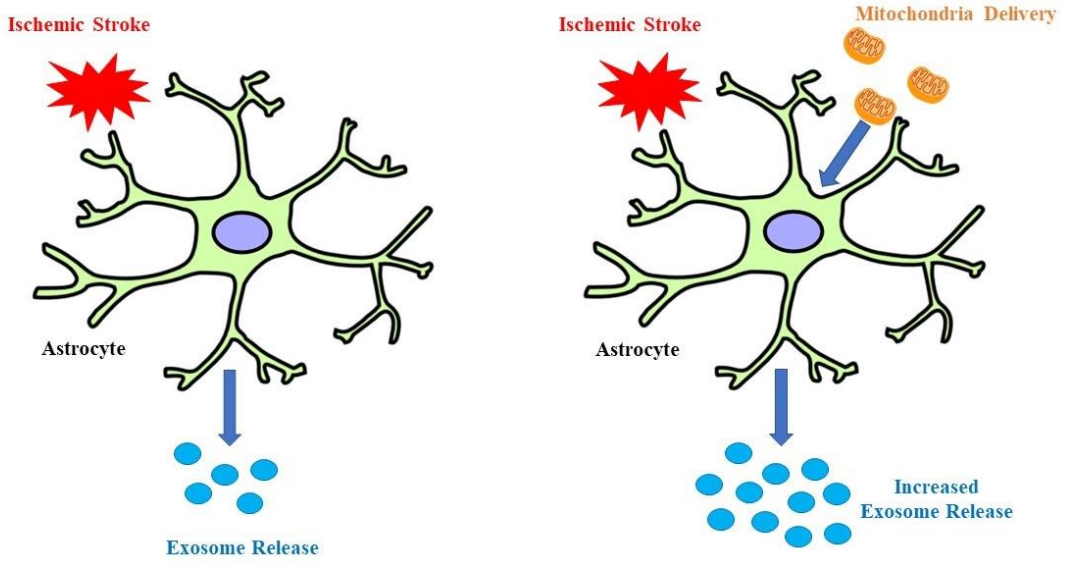

Figure 1. Increased Exosome Release Following Mitochondria Delivery in Astrocytes. The figure depicts an astrocyte in ischemic conditions releasing exosomes on the left. The astrocyte on the right experiences increased exosome release with the delivery of mitochondria during ischemic stroke, which represents a part of the hypothesis.

Input Species	Condition in Stroke	Citation
Extracellular ATP	Increase	Gülke, E., et al. (2018)[25]
GABA	Increase	Głodzik-Sobańska, L., et al. (2004)[26]
IGF1	Decrease	Tang, J., et al. (2014)[27]
JAG1	Increase	Liu, X.S., et al. (2011)[28]
PACAP	Decrease	Fang, Y., et al. (2020)[29]
Acetylcholine	Decrease	Beley, A., et al. (1991)[30]
Extracellular Ca++	Decrease	Kristián, T. & Siesjö, B.K. (1998)[31]
TGFβ	Increase	Krupinski, J., et al. (1996)[32]
Thrombin	Increase	Stein, E.S., et al. (2015)[33]; Carcaillon, L., et al. (2011)[34]
Glt Reuptake	Decrease	Dirnagl, U., et al. (1999)[3]
ALG-2	Increase	Li, W., et al. (2000)[35]
GTP	Decrease	Becerra-Calizto, A. & Cardona-Gómez, G.P. (2017)[36]
Norepinephrine	Increase	Leonardis, L., et al. (1994)[37]

Table 1: Input species and their response following stroke. These changes collectively represent the stroke input condition.

also an area of interest in ischemic stroke research. Mitochondria could aid in recovery from ischemic stroke, potentially by altering the biogenesis of exosomes in astrocytes such that they positively impact the surrounding cells in the brain.

The overarching goal of this project is to understand the signaling involved in astrocytes during stroke and to test the hypothesis that delivering mitochondria to astrocytes following stroke will increase exosome release and increase the number of beneficial neurotrophic factors in the exosomes. To do this, we developed a signal network model of astrocytes in normal and stroke conditions (Aim 1). We then used the model to test the effects of stroke and mitochondria delivery on astrocytes' exosome release (Aim 2). Lastly, we validated our model with literature and *in vitro* experiments (Aim 3).

Results

To achieve the first aim, a computational model was developed following an extensive literature of known signaling pathways within astrocytes. The pathways were combined into one signaling network using Matlab's Netflux software. The Netflux software was developed by University of Virginia's Saucerman Lab and made available to any researchers. Cytoscape was then used to create an image of the complete pathway, seen in Figure S1. The signal networking model contains pathways that control key aspects of astrocytes' response to stroke. Specifically, the outputs of the model are the morphology of astrocytes, proliferation, apoptosis, glutamate release, and exosome release. The model includes 14 input species, five output species, and 81 reactions. There are 68 total species in the model. The computational model was then applied, using both normal and stroke conditions in order to investigate the hypothesis.

A literature review was conducted to determine how inputs varied during an ischemic stroke. The results of the literature review are seen in Table 1, with a number of input conditions altered to represent a stroke condition rather than a single input species of stroke. The diseased state was represented in this way as stroke is highly complex in astrocytes, and this complexity is better highlighted through the change of multiple input species. The model was then used to

Output	Change in Stroke Conditions	Agreement with Literature	Citations
Proliferation	Increase	Inconclusive	Kudabayeva, M., et al (2017)[23]; Ding, S. (2014)[38]
Apoptosis	No Change	Disagrees	Xu, S., et al. (2020)[39]
Resting Morphology	Decrease	Agrees	Acaz-Fonseca, E., et al. (2019)[22]
Reactive Morphology	Increase	Agrees	Acaz-Fonseca E., et al. (2019)[22]; Kudabayeva, M. et al (2017)[23]
Glutamate	Increase	Agrees	Fogal, B., et al. (2007)[24]

Table 2: Outputs of interest and their changes from the stroke condition. The model accurately predicted changes in morphology and glutamate when validated with published results. The model was inconsistent with changes in apoptosis and proliferation.

measure how varying these inputs to simulate a stroke would affect the outputs.

The outputs of interest from the model are proliferation, apoptosis, resting and reactive morphologies, glutamate, and exosome release. While the change in exosomes with stroke and mitochondrial conditions was evaluated via our own *in vitro* experiments, the first four outputs were validated against the literature as seen in Table 2. Our model found a decrease in resting morphology of astrocytes and an increase in both reactive morphology and glutamate release. This is consistent with published data in the literature[22–24]. However, there was inconsistency with published data for proliferation and apoptosis. The model predicted an increase in proliferation following stroke. Published data suggests that proliferation varies spatially from the infarction or obstruction in the brain tissue[23,38]. Proliferation also depends on the morphology of the astrocytes, as astrocyte reactivity and glia scarring affect proliferation[38]. The model itself is not spatially dependent and does not account for the potential of scarring in the tissue. Thus, this validation is inconclusive. Our model predicted no change in apoptosis, straying away from published data[39]. Although this result was surprising, it has been recently documented that a majority of astrocytes can survive ischemia, but just function less efficiently. Therefore, the documented change is fairly small.

The model was further investigated via sensitivity analysis, in which each node was knocked down and the changes in activity of the other species were measured. The sensitivity analysis identified several influential nodes in the model (Fig 2). Specifically, the knockdown of intracellular calcium, P2Y1, glutamate and its receptor, the alpha subunit of the G-protein coupled receptor, and the nodes in the endosomal sorting complex. Given that calcium and glutamate are key signaling molecules in the brain, it was expected that they would be influential nodes. Further, the endosomal sorting complex is a linear set of reactions, and as such,

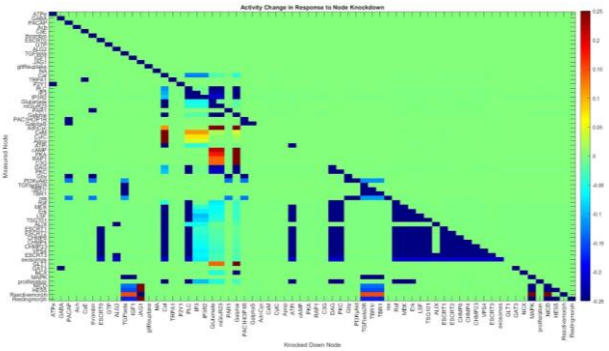

Figure 2. Activity change in response to node knockdown. Each node in the model was knocked down and the resulting change in each node's activity was measured.

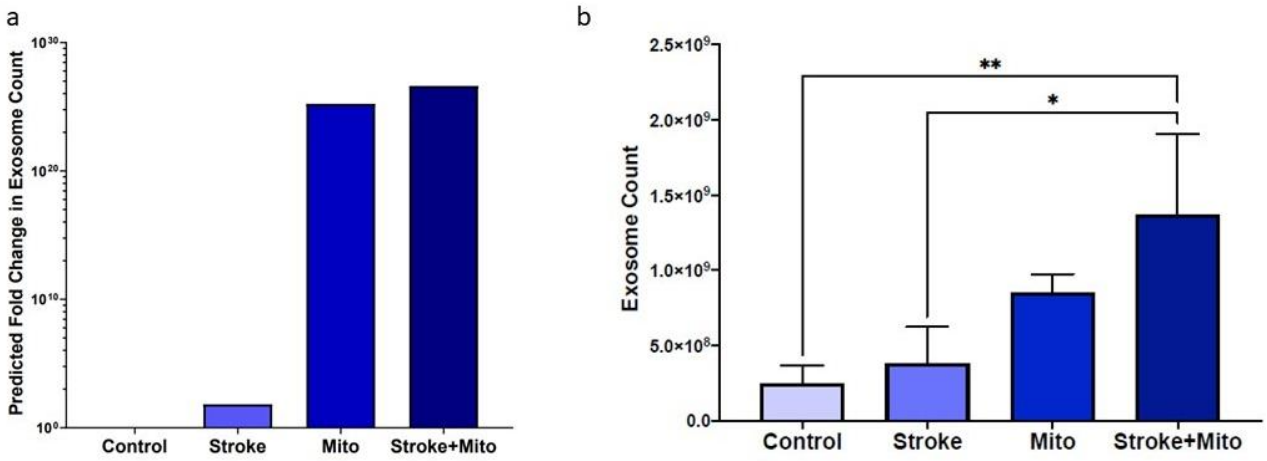

Figure 3. The model predictions of change in exosome concentration validated by *in vitro* experiments. The model predicted that exosome concentration would increase in stroke, mitochondria and stroke and mitochondria conditions in that order. The graph shows the fold change in the activity of the exosome production pathway; the activity in all conditions was normalized to the control, making the value for the control condition 1 (a). *In vitro* experiments found the same order of increase in the number of exosomes released by astrocytes (n=3 per group). Two-way ANOVA and Tukey's multiple comparisons test were used to perform the statistical analysis (b). **p<0.01 *p<0.05

the knockdown of an upstream node directly affects each of the downstream species.

When stroke conditions were applied to the model, it predicted that the exosome release from astrocytes would increase compared to normal conditions. Modeling mitochondrial delivery both with and without stroke predicted that exosome concentration would increase from normal levels more than what was predicted after stroke. The stroke with mitochondria condition was predicted to have the greatest increase in exosome concentration (Fig. 3a). *In vitro* experiments found similar results (Fig 3b). The stroke with mitochondria group resulted in a significant increase in exosomes compared to the stroke ($p<0.05$) and control ($p<0.01$) groups. While not significant, there was an observed increase in exosome release from the mitochondria-only to the stroke with mitochondria group.

Discussion

Analysis of the results show that the astrocyte computational model predominantly aligns with published data. Design constraints and assumptions made when making the model, referenced in the materials and methods, reduced the accuracy of the model; however, the model was able to effectively predict several outcomes that were observed in published data. Additionally, the model was able to effectively predict a change in released exosome concentrations. The use of our model supports our hypothesis that the delivery of mitochondria during an ischemic stroke will significantly increase the number of exosomes released from the astrocyte cells. The increase in released exosomes may offer a possible route for more signaling to surrounding neurons during ischemia, which could prove to be a potential stroke therapy target. The released exosomes can be either beneficial or harmful for cellular recovery depending on the phenotype.

In order to investigate the characteristics of these released exosomes, mass spectrometry is being conducted on samples. Exosomes isolated following *in vitro* experiments will be analyzed in order to study the factors present and determine if the exosomes are beneficial or detrimental. This and future experiments will reveal how the mitochondria affects released exosomes from astrocytes during an ischemic stroke. This could indicate if mitochondria can be used as a potential therapy for strokes. Additional experiments should also be carried out using mitochondria from mouse adipose tissue, instead of mitochondria harvested from muscle. The use of

mitochondria from adipose tissue would allow this treatment to be more readily translated into clinical use, as adipose tissue samples from patients are more easily acquired than muscle tissue samples.

Finally, the ultimate goal of the project is to access the mitochondrial treatment during an ischemic stroke *in vivo.* Preliminary experiments will continue to be conducted *in vitro*, but the motivation is to translate this mitochondrial delivery in mouse subjects, in order to determine the effects of mitochondrial treatment on astrocytes and other neural cells in their native environment. Additional constraints will be considered, such as the delivery method with focused ultrasound, as well as the method for harvesting the species mitochondria from tissue.

Ultimately, the model developed during this project has added to the understanding of signaling in astrocytes following stroke and the ways in which these signaling pathways impact astrocyte morphology and exosome release. Thus, this model can continue to be used to evaluate potential treatments for stoke. This project has also made strides in identifying mitochondrial delivery as a potential treatment for stroke, which could help to reduce its catastrophic impacts of stroke on patients worldwide.

Materials and Methods

Computational Model: Matlab's Netflux software, created and open sourced by the Saucerman Lab, was used to build the model. The details of this software are described in Kraeutler, et. al.'s 2010 paper[40]. The literature survey yielded a total of 51 papers detailing astrocyte signaling pathways in normal and stroke conditions. The following terms were used to gather our sources: 1) "astrocyte signaling pathways," 2) "astrocyte energetic signaling," 3) "astrocyte extracellular vesicle pathways," 4) "astrocyte inflammatory pathways," 5) "astrocyte signaling ischemic stroke," 6) "astrocyte signaling disruption stroke," and 7) "astrocyte signaling stroke." These papers provided the bulk of our base model, specifically focusing on cell energetics and exosome biogenesis to address our hypothesis. We also focused on stroke markers such as glutamate, ATP, and thrombin to elucidate the role of mitochondria, and exosomes. The model was then simulated to model stroke conditions by changing a multitude of inputs based on the literature. Mitochondrial delivery was also simulated by increasing the weight of the ATP pathway in the model.

Model Simulations: To simulate normal physiological conditions, all inputs and the ATP production pathway were given weights of 0.5. To simulate stroke, inputs that were found to decrease were weight to 0.25, inputs that were found to increase were weighted to 0.75. To simulate the delivery of mitochondria, the ATP production pathway's weight was increased to 0.75.

Sensitivity Analysis: To analyze the sensitivity of the model, MATLAB was used to evaluate the change in activity in the model when each individual node was knocked down. The activity of all of the species was set at 0.5. Then, each individual species' activity was reduced to zero and the change in activity of the downstream species were measured.

Constraints and Assumptions of the model: The literature review performed to create the astrocyte signaling model was carried out for a wide variety of pathways. To minimize interference and hours of research, we selected pathways that related to our outputs of interest for the final model. For example, a distinct pathway was included to show the biogenesis of exosomes. Additionally, it was assumed that specific pathways were conserved across all cell types, and therefore could be included in the astrocyte model. An additional constraint of the model is the assumption that the effects of many reactions are equally weighted. The model does not differentiate between the weights of different reactions, and therefore is not completely representative of certain reactions in astrocytes. Lastly, the results of the Netflux model represent the model activity in arbitrary units.

Cell Culture: Human astrocytes were cultured in collagen-coated tissue culture flasks in Astrocyte Medium (ScienCell Research Laboratories) supplemented with 2% FBS and 1% astrocyte growth serum. The media was changed approximately every three days and the cells were passaged approximately every seven days. The cells were seeded at densities between 4,000 cells/cm^2 and 8,000 cells/cm^2. The astrocytes were maintained through passage seven.

Mitochondrial Isolation: Skeletal muscle was harvested from the hindlimbs of one mouse per flask of cells being treated. The muscle was harvested while the mouse was anesthetized and prior to euthanizing to ensure minimal tissue death and damage to the sample. The tissue was homogenized using the gentleMACS Dissociator (Miltenyi Biotec). The Mitochondria Isolation Kit for Tissue (Thermo Scientific) was used to extract mitochondria, and the associated protocol was followed. The reagents in the kit were supplemented with 1% EDTA-free HaltTM Protease Inhibitor Cocktail (Thermo Scientific). Mitochondrial pellets were resuspended in sterile DPBS.

In vitro hypoxic chamber to simulate hypoxia: Twelve flasks of human astrocytes were plated at 7,000 cells/cm^2 approximately twelve hours prior to the hypoxic conditions. To simulate the hypoxia associated with ischemic stroke, a hypoxic setup was adapted from the protocol developed by Wang, *et al* [12]. Six of the flasks were placed in individual airtight plastic bags that were sterilized with ethanol. Each bag was heat sealed, and a corner was cut to fill the bag with nitrogen equilibrated gas of 5% carbon dioxide and 1% oxygen (Cal Gas Direct). Once the bag had inflated, it was sealed and checked for leaks. The bags were placed in the cell culture incubator for three hours. The remaining six flasks remained in normoxic conditions during this time.

Mitochondria treatment: After being removed from the hypoxic chamber, 800 uL of the mitochondria suspension was added to three hypoxic and three normoxic flasks. After a two hour incubation, the media was replaced in all twelve flasks.

Exosome Isolation: Supernatant from the human astrocyte cultures were taken 18 hours after stroke and clarified at 300 x g for 10 minutes at 4 degrees Celsius to remove any cells / cellular debris. Clarified supernatant was then treated with the ExoQuick-TC density gel (System Biosciences, Palo Alto, CA) at a 1:5 ExoQuick-TC to supernatant ratio, and refrigerated overnight at 4 degrees Celsius. After an overnight incubation, the ExoQuick-TC / biofluid mixture was centrifuged at 1500 x g for 30 minutes at 4 degrees Celsius. The supernatant was aspirated, and the remaining mixture was centrifuged at 1500 x g for an additional 5 minutes. The exosome pellet was then resuspended in 500 µL in non-sterile PBS and refrigerated at 4 degrees Celsius until future use.

NanoSight: After isolation, the exosome samples were resuspended to a total of 1mL, and used for Nanoparticle Tracking Analysis (NTA), using the NanoSight NS300 module. Exosome concentrations, mean and mode size, and standard deviation were taken over 5 runs and averaged together for each sample.

End Matter

Acknowledgments

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Supplementary Figures

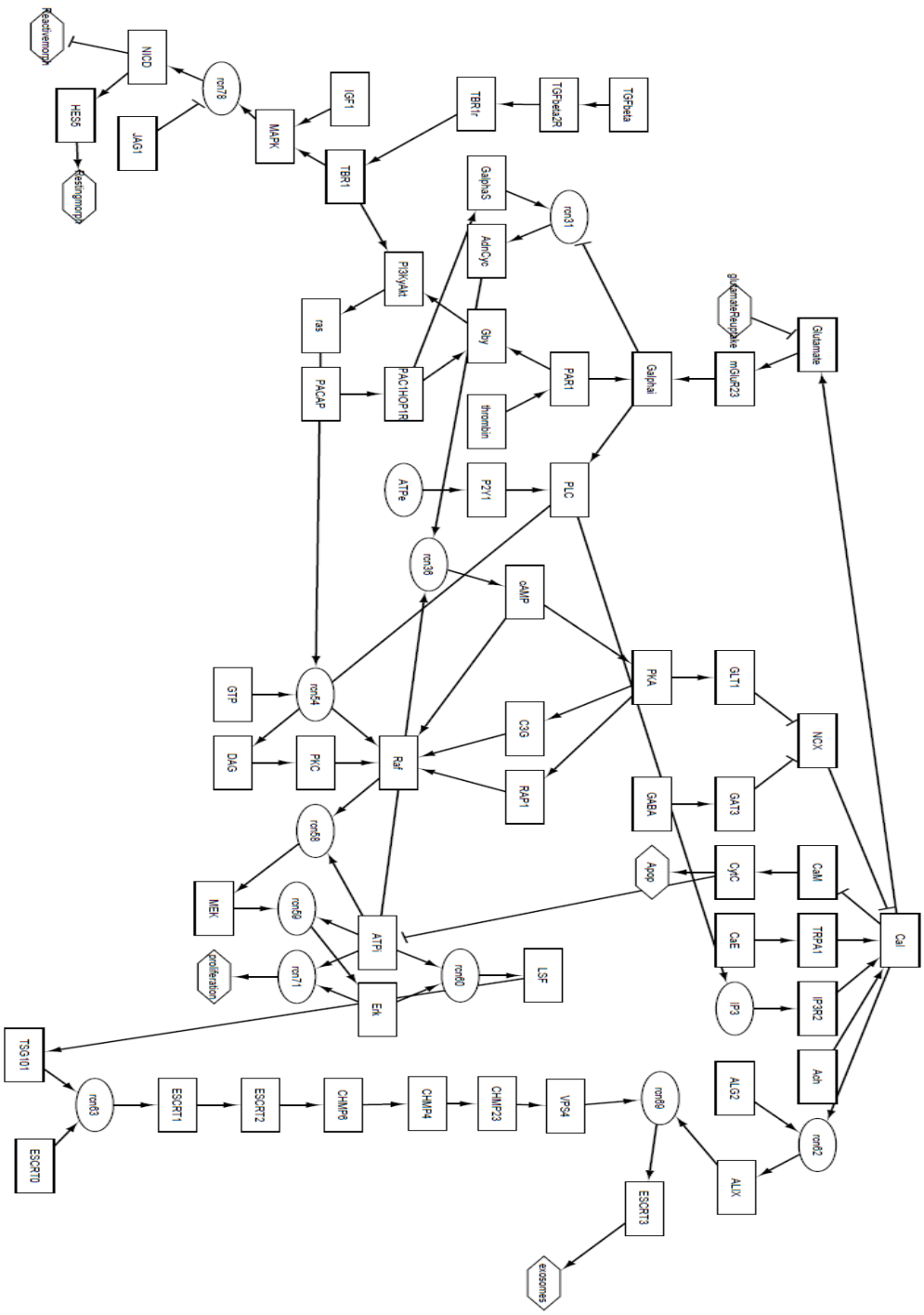

Figure S1. Cytoscape Model of Signaling Pathways in an Astrocyte Cell. The model was constructed following an extensive literature review of known signaling pathways. Matlab's Netflux software was used to combine the pathways into one complete, robust model that informs researchers what happens when certain inputs are introduced to astrocytes. The model was then exported to cytoscape in order to create a visualization.