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"Caracterización de la Vía de señalización Wnt en astrocitos y su papel en la neuroprotección"

TESIS

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Resumen

La señalización Wnt se ha estudiado ampliamente en el cerebro adulto, particularmente asociada con procesos de plasticidad sináptica y con el mantenimiento de precursores neuronales. Sin embargo poco se sabe de cómo esta vía se regula en los astrocitos, los cuales participan en una variedad de funciones de mantenimiento, de homeostasis y de plasticidad neuronal, así como en neuroinflamación y en los procesos de muerte. En el presente estudio se analizó el efecto de glutamato externo y de citocina TNF-α sobre la expresión de los agonistas Wnt5a y Wnt7a y del antagonista DKK1 en astrocitos en cultivo.

Los resultados sugieren que a las concentraciones en las que el glutamato activa a receptores ionotrópicos este neurotransmisor produjo un incremento en la expresión de Wnt5a, Wnt7a y DKK1 a tiempos largos (24 h), en tanto que el TNF-α, produjo solamente un incremento en la expresión de Wnt7a a las 24 h y una robusto incremento del antagonista DKK1 desde tiempos cortos (6 h).

En este trabajo se encontró que tanto Wnt7a como DKK1 se encuentran localizados con relativa abundancia en los astrocitos, aunque el contenido de Wnt7a es mayor. En conclusión, en este trabajo mostramos la regulación diferencial de la expresión y del contenido proteico de agonistas y antagonistas de la vía Wnt bajo diferentes estímulos lo que podría tener un papel en eventos de plasticidad neuronal y/o de neurotoxicidad.

Abstract

Wnt pathway in neurons has been studied extensively because of its importance in neurogenesis and neural plasticity. Yet, there is only little evidence about Wnt ligands expression and their action in astrocytes. These are major glial cells known to have many supportive roles in the nervous system under both physiological and pathological conditions. In this study we investigated the effects on Wnt ligands expression and production in astrocytes in response to different stimuli, as well as its cellular location. To accomplish this, astrocyte primary cultures were prepared and treated with either glutamic acid or TNF-α and collected after several time intervals. The RNA expression and protein level of the Wnt agonists, WNT5A, WNT7A and the Wnt antagonist, DKK1 was determined in this time course. Another astrocyte primary culture was prepared for detection of WNT5A,WNT7A/B and DKK1 by immunofluorescence imaging. We found that the mRNA expression of all the Wnt ligands under study changed with the time of exposure to glutamic acid and TNF-α. We also found that WNT7A/B and DKK1 ligands are located at different levels in the cytoplasm of the astrocyte depending on the treatment and the time of exposure. Differential expression of the Wnt pathway in activated astrocytes may contribute to neuronal homeostasis in the central nervous system.

Introduction

Astrocytes or astroglia are the most abundant glial cells in the central nervous system (CNS)[1]. It is almost fivefold more than neuron[2]. There are two main subtypes of astrocytes that are protoplasmic and fibrous[2]. This subdivision was based on the differences in their cellular morphologies and anatomical locations. Protoplasmic astrocytes are located in the gray matter whereas fibrous astrocytes are located in the white matter[2]. While protoplasmic astrocytes exhibit several stem branches that give rise to many finely branching processes, the fibrous astrocytes have long fiber-like processes[2].

These special star shape glial cells play multiple functions in the nervous system under both physiological and pathological conditions. Under physiological condition, astrocytes play roles in organizing, maintaining, supporting and repairing the nervous tissue[3]. They provide trophic and metabolic support to neurons, they participate in detoxification of oxygen species, and they are required to maintain the blood brain barrier (BBB) integrity[3]. In addition, they are responsible for the plasticity and the synaptic transmission in the nervous system by controlling the levels of glutamate, which is the main excitatory neurotransmitter[5].

Astrocytes modulate glutamate levels at the synaptic cleft through six known mechanisms: (i) reversal uptake by glutamate (ii) anion channel opening induced by cell swelling, (iii) Ca^{2+}-dependent exocytosis, (iv) glutamate exchange via the cystine-glutamate antiporter, (v) release through ionotropic purinergic receptors and (vi) functional unpaired connexons, 'hemichannels', on the cell surface[4]. Besides that these pathways have been well defined, it is not clear how often and to what extent astrocytes employ these different mechanisms[4]. It is also unknown whether the mechanism of glutamate release that operates under physiological conditions is also functioning during pathological conditions[4].

Under pathological conditions, astrocytes respond to all forms of CNS (central nervous system) insults through a process referred to as reactive astrogliosis, which has become a pathological hallmark of CNS structural lesions[2]. Astrogliosis is an abnormal increase in the number of astrocytes due to the destruction of nearby neurons induced either by CNS trauma, infection, ischemia, stroke, autoimmune responses, or neurodegenerative diseases[2]. Astrocytes response trigger mechanisms that maintain the cell viability since the activated astrocytes are able to influence the filtration of leukocytes, the activation of innate and adaptive immunity, excitotoxicity, homeostasis, angiogenesis and regeneration of the axons. Astrocytes are also the main cellular component of glial scar, which isolates the healthy nervous tissue from the injured neural parenchyma. Although activated astrocytes may provide neuroprotection via the release of neurotrophic factors, they also participate in inflammatory reactions by expressing pro-inflammatory molecules such as cytokines and chemokines[3] that are involved in some neurodegenerative diseases. It has not been clear under what contexts they may be helpful or harmful and many questions remain about their functions.

Wnt signaling plays a key role in neuroprotection and synaptic plasticity. The impairment of Wnt signaling in astrocytes reduces the production of neurotrophins such as BDNF and NT-3[7]. In contrast to the large amount of evidence that exist on the role of Wnt signaling in neurons[8] there is scarce information about the presence of Wnt-related ligands acting as neuroactive molecules secreted by astrocytes as well as on the stimuli that modify their production and secretion.

Wnt signaling

The Wnt pathway is essential for development, tissue homeostasis and disease. Wnt signaling is a highly conserved pathway present in all metazoans[6] . Both the Wnt ligand and its

corresponding Frizzled receptor genes have under-gone extensive expansion during the metazoan evolution[6]. The Wnt pathway participates in multiple aspects of cellular function during development and in adults. In the brain, it contributes to synapse formation, axonal remodeling, dendrite outgrowth, synaptic activity, neurogenesis, remyelination and behavioral plasticity. There are two major Wnt pathways, the canonical and the non-canonical pathways. In the canonical pathway one of the nineteen Wnt ligands activate one out of ten frizzled (Fz) receptors, except the Frizzle nuclear import (FNI). The ligands bind to the N-terminal extra-cellular cysteine-rich domain of Fz receptor[7]. The Fz receptor then combines with a low-density lipoprotein 5 and 6 co-receptor (LPR5/6)[8]. After the binding, the signal is transduced to the cytoplasmic phosphoprotein Disheveled (Dsh/Dvl)[8]. This will recruit the Axin and the APC, which will disturb the destruction complex of β-catenin[8]. As the destruction complex does not exist anymore, the transcriptional co-activator β -catenin accumulates and enters into the nucleus to activate transcription factors belonging to the T-cell family (TCF) that will regulate target genes[8] (*Figure 1).* TCF factors can act most of the time as transcriptional switches[9]. They repress Wnt targets in the absence of signaling through recruitment of the transducing-like enhancer of split (TLE)/Groucho (Gro) co-repressors. Under Wnt activation, Groucho is displaced by β-catenin, which recruits co-activators such as the histone acetyltransferases CREB-binding protein (CBP) and p300[9] (*Figure 2).*

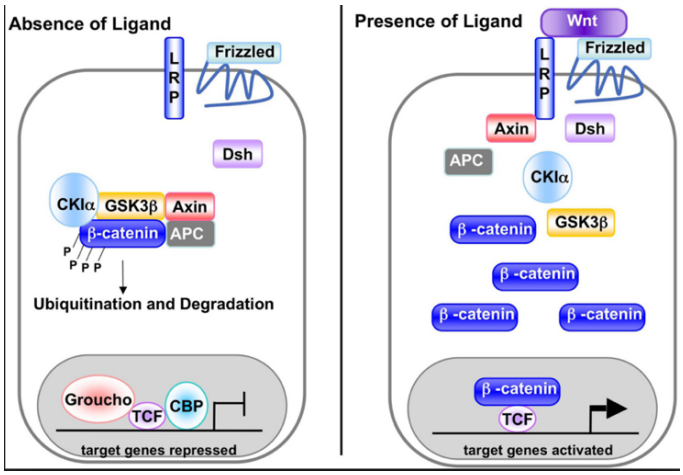

Fig 1. The canonical pathway[10]

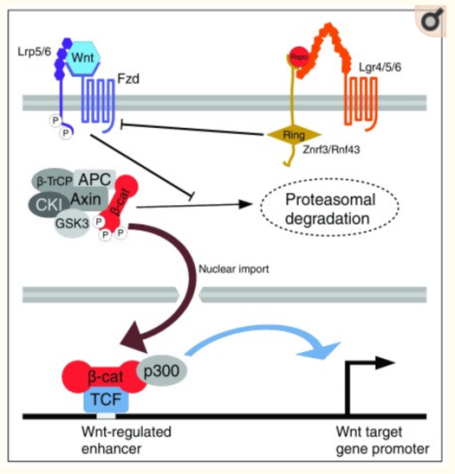

Fig 2. Wnt/β-catenin signaling in vertebrates [9]

TCF/β-cat can bind to enhancers that lie hundreds of kilobases away from the proximal promoters of Wnt targets[9]. The influence of the enhancers on the proximal promoter can be explained by chromatin looping[9]. A signal-dependent recruitment of cohesion subunits (which are strongly associated with chromatin loops), to Wnt-regulated enhancers was shown using chromatin immunoprecipitation sequencing (ChIP-seq)[9]. Activation of the canonical pathway increases the phosphorylation of the C-terminal domain of the RNA polymerase II (Pol II), but does not greatly increase Pol II binding at promoters of Wnt targets[9]. This indicates that Wnt/β-cat signaling stimulates transcriptional elongation[9].

The non-canonical pathway on the other hand, is also divided into different sub-pathways[12] (*Figure 3)*. The most conspicuous are the planar cell polarity (PCP) and the Wnt/Ca^{2+}- pathways[12]. The non-canonical pathways are activated through the binding of a Wnt ligand to other receptors than the Fz receptor such as: the tyrosine receptor kinase–like orphan receptor (ROR) 1 and 2, or the protein tyrosine kinase 7 (PTK7) receptor[12]. In the non-canonical pathway, it appears that the nucleus is not always involved, however, there are changes in the actin skeleton and in the intracellular Ca^{2+} storage[12].

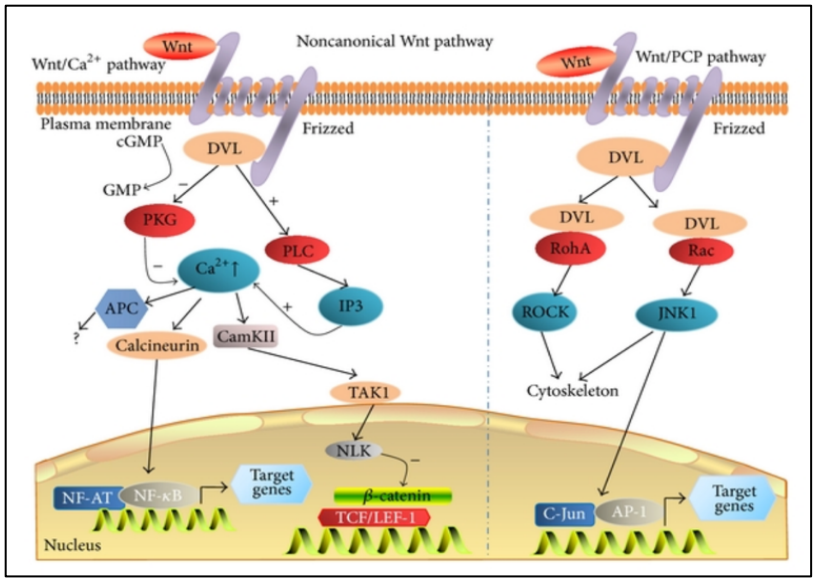

Fig 3. The non-canonical pathway (see text)[13]

The PCP pathway was detected through genetic studies in Drosophila[12]. Mutations that randomize the orientation of epithelial structures such as the cuticle hairs and sensory bristles were found in Wnt signaling components including Frizzled and Dishevelled[12]. In vertebrates, organization and orientation of stereo-cilia in the sensory epithelium of the inner ear, the organization of hair follicles, and the morphology and migratory behavior of dorsal mesodermal cells undergoing gastrulation were found to be regulated by the Wnt pathway[12]. This pathway functions through regulation of actin cytoskeleton to polarize the organization of structures and to direct migration[12]. This pathway seems to function independently of transcription[12].

The Wnt signal in the non-canonical pathway is mediated through Fz receptor independent from the LRP5/6 co-receptor[12]. The Fz co-receptors for the non-canonical pathway are still not well defined. Suggested candidates that can act as co-receptors are NRH1, Ryk, PTK7and ROR2[12]. When the signal is transduced, Dsh is activated[12]. Two domains of Dsh, PDZ and DEP, are utilized to activate two parallel pathways that in turn activate the small GTPases Rho and Rac[12]. When Rho branch is activated, the Dsh-Daam1 complex is induced, this leads to the

activation of Daam1 (Dishevelled associated activator of morphogenesis 1) and consequently to the activation of the Rho GTPase[12]. The Forming-homology protein Daam1 can bind to Dsh and RhoA suggesting the existence of a positive feedback loop for amplification of the signaling pathway[12]. The activation of Rho GTPase will activate Rho-associated kinase (ROCK) and myosin[12]. This will modify the actin cytoskeleton and will lead to cytoskeletal rearrangement[12]. The second branch of the pathway activates the Rac GTPase through the DEP domain of Dsh, and this is independent of Daam1[12]. Activated Rac stimulates JNK activity[12]. JNK downstream factors regulated by this branch are still poorly resolved. In addition, it is not clear yet whether the non-canonical pathway regulates gene transcription through the Rho and Rac proteins, and how do they coordinate cell polarization or direct migration[12].

The Wnt/Ca^{2+} pathway and the Planar Cell Polarity pathway share many components. Yet, they have clear differences that allow them to be considered as two separated branches[12]. The Wnt/Ca^{2+} pathway is dependent on G-proteins activity, which stimulate Ca^{2+} release from the endoplasmic reticulum (ER) as demonstrated in zebrafish and *Xenopus* embryos[12]. In Wnt5a, Wnt11 and rat Fz2 (RFz-2), the intracellular Ca^{2+} can be released without affecting the β-catenin stabilization[12]. Calcium release upon overexpression of Wnt5a or RFz-2 in zebrafish embryos can be inhibited by pertussis toxin and the α-subunit of transducing. This in turns inhibit the G protein signaling[12]. Cytosolic calcium accumulation activates several Ca^{2+} sensitive proteins, such as protein kinase C (PKC) and calcium/calmodulin-dependent kinase II (CamKII)[12]. It has been shown that CamK11 in the *Xenopus* embryo, activate the transcription factor NFAT in order to promote ventral cell fates[12]. CamK11 also activate TGFβ activated kinase (TAK1) and Nemo-like kinase (NLK) [12]. This activation can antagonize the β-catenin/TCF signaling.

Moreover, Ca^{2+} can activate PKC to regulate the tissue separation process during gastrulation, through the small GTPase CDC42 activation[12].

While Planar Cell Polarity and Wnt/Ca^{2+} pathways are the most studied branches of the non-canonical pathway, there are number of other branches that have emerged[12]. These branches seem to have distinct outcomes, although they share many components of the Planar Cell Polarity and Wnt/Ca^{2+} pathways[12]. One example is the pathway that regulates the microtubule cytoskeleton during neuronal migration and synaptogenesis, which depends on the activity of Dsh and GSK [12]. Another example is the pathway that regulates repulsion of neurons during axonal guidance via the Ryk receptor and the proto-oncoprotein Src[12]. Also, the casein kinase inhibits the Rap1-GTPase for cytoskeletal modulation during gastrulation in a different pathway[12].

In vertebrates, Wnt pathway can be activated through one of nineteen ligands[8]. The Wnt ligands are considered soluble, secreted, hydrophobic, heavily modified glycoproteins[8]. These ligands act in short and long range signaling in an autocrine and a paracrine manner. They lie down the Frizzle receptor and activate the scaffolding protein Dishevelled but not the Frizzle nuclear import (FNI)[8]. Looking at these ligands, one can find that some of them can activate both the canonical and non-canonical pathways depending on which tissue or what function they are participating in. Examples of that is the Wnt5a ligand that is known to activate the non-canonical pathway, but has been found to activate the canonical pathway depending on receptor context[10]. An interesting fact is that in some biological processes cells may integrate different Wnt signals and trigger appropriate responses that consider the total net balance. In other biological processes, one Wnt ligand can selectivity activate the pathway[15]. This rise a question of how does the cell selectively activate one pathway rather than another? A recent paper has

explained the ligand-selective signaling of WNTA/B[15]. They found that cerebral endothelial cells, in the neurovascular unit, must express a membrane protein complex[15]. This complex consists of the G protein-coupled receptor 124 and Reck[15]. The expression of this complex form a Frizzled signalosome that leads to activation of the Wnt/β-catenin signaling[15] (*Figure 4).*

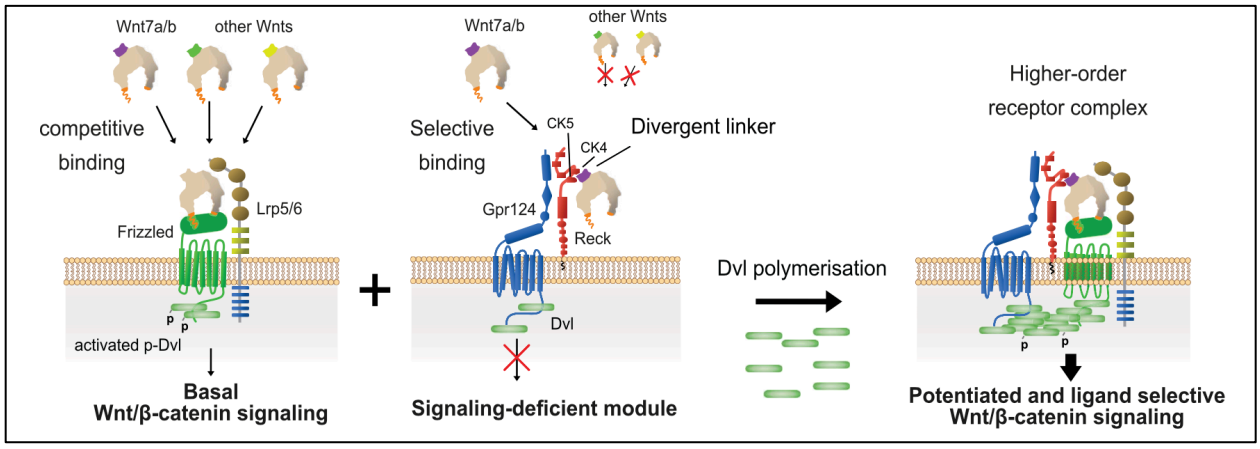

Fig 4. ligand-specific Wnt pathway signaling, linking Fz and Gpr124[15]

The Wnt pathway is inhibited through binding to one of two classes of antagonists[16]. One class blocks the association with the Wnt receptor, like the Wnt Inhibitory Factor-1 (WIF) and secreted Frizzled-Related Proteins (sFRPs), thus inhibiting both the canonical and non-canonical pathways[16]. The other class of antagonists blocks the LRP5/6 co- receptor, like Dickkopf (DKK1) and Wise/ SOST and therefore these class only inhibits the canonical pathway[16]. DKK1 interact with LRP5/6 and the co-receptor Kremen1/2[16]. This triggers LRP5/6 endocytosis, thereby preventing the formation of the LRP5/6-Wnt-Fz complex[16].

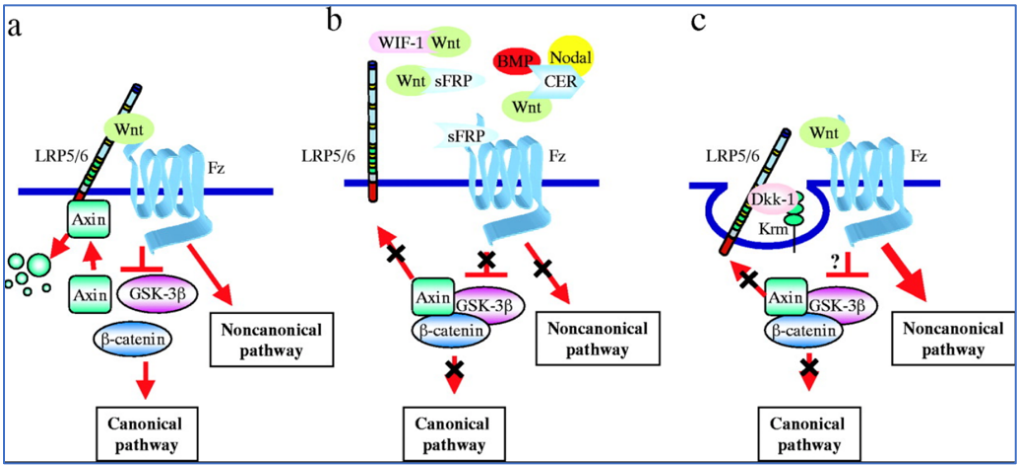

Fig 5. Wnt pathway antagonists[16].

Wnt signaling in brain

Cumulative evidence points the participation of the Wnt signaling in the maintenance of neuronal morphology and synaptic connectivity in the adult brain. Wnt signaling plays critical roles in the CNS through life, participating in the proliferation of neuronal progenitors, in dendrite and axon development and in circuitry establishment. Based on published studies, here are some summarized functions of some Wnt ligands that are responsible for neurogenesis and plasticity. Those studies show that WNT5A loss leads to a decreased number of pre-synaptic boutons[17]. WNT5A also inhibits the cell-cycle activation, which increases the cortical neuron survival in Aβ (amyloid beta) treated cortical neurons[18]. Downregulation of *Wnt5a* can lead to Aβ toxicity in neuronal cultures[18]. Moreover, WNT3 and WNT11 augment the acetylcholine receptor (AChR) clustering in the chick wing and in zebrafish[17]. It is also mentioned that WNT7A induces clustering of the pre-synaptic protein synaptophysin I in the granule cell of dentate gyrus. Additionally, WNT3A increases neurotransmitter release in neurons of the mice hippocampus, WNT5A increases synaptic transmission and signaling, and WNT7A is responsible of the increased number of synapses after exposure to enriched environment[17].

Cultured hippocampal neurons exposed to WNT7A for 1 h showed an increase in the clustering of the presynsaptic synaptophysin without changes in its concentration nor the concentration and organization of the neurotransmitter receptors in the synaptic cleft[19] *(Figure 6*).

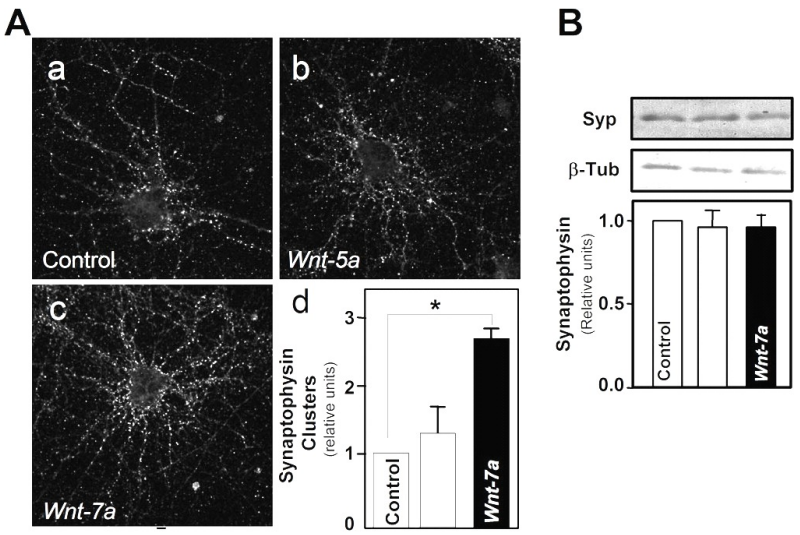

Fig 6. (A) Immunofluorescent images and western blot results of synaptophysin clusters in hippocampal neurons and (B) Western blot of synaptophysin protein levels after WNT5A and WNT7A treatment[19].

On the other hand, the upregulation of the Wnt antagonist DKK1, has been associated to Alzheimer's disease and to brain ischemia[13]. Local infusion of DKK1 in rats caused neuronal cell death and astrocytosis in the hippocampus. It also caused death of the cholinergic neurons of the nucleus basalis[20]. The neuronal death was accompanied with the upregulation of the apoptosis regulatory protein, BAX [20].

Wnt signaling in astrocytes

Even though there are many studies on the participation of Wnt pathway in adult neurons, there are only few done on astrocytes. The information about whether Wnt pathway is expressed in astrocytes is scarce, and there are few evidences that point to the presence of some Wnt ligand expression in these cells and their possible role on neuronal survival or neuronal death. In this

regard, one study proved that the Wnt pathway components are expressed in activated astrocytes [21]. In this study it was shown that the mRNA expression of some Wnt ligands and ten Frizzled receptors was increased with the activation of astrocytes via two different methods[21]. One is by using 100ng/ml of the lipopolysaccharide (LPS) and the other by using 100-200 μg/ml of extracts from lesioned spinal cord (SC100-SC200)[21]. From these studies it was evident that an inflammatory extract from lesioned spinal cord produced an increase of *Wnt5b* and *Wnt7a* mRNA. It has also been found upregulation of the antagonist *Dkk1* in astrocytes surrounding the amyloid plaques from Alzheimer´s disease brain[22].

Astrocyte activation

Activated astrocyte can secrete different molecules in response to different stimuli. For example, it can release glutamic acid and TNF-α under physiological or pathological conditions. Glutamic acid is an excitatory neurotransmitter and a metabolic compound. 70% of the excitatory synapses in the CNS of mammals utilize glutamate as a neurotransmitter[23]. Glutamate is taken up by astrocytes to maintain low concentration at the synaptic cleft avoiding its neurotoxicity[24]. They clear about 90 % of excess glutamate through the excitatory amino acid transporters (EAATs) 1 and 2. Like in neurons, glutamate receptors present on astrocytes include metabotropic and ionotropic receptors[31]. It has been found that glutamate at 100 μM concentration is able to activate the AMPA and KA receptors, which increases the internal Ca^{2+} concentration associated to changes in astrocyte morphology[14].

Astrocytes are highly responsive to pro-inflammatory stimuli. Among different cytokines, TNF-α is a signaling protein involved in many cell activities such as cell proliferation, differentiation, apoptosis, and inflammatory response. In the CNS, TNF-α is secreted from

microglia as well as astrocytes[17] and is involved in microglia-induced astrocyte activation to form a phenotype called A1[28]. TNF-α exists in two forms[17], one is soluble and the other is bound to the membrane[25]. The soluble TNF-α signals through receptor 1 (TNFR1) that is expressed in neurons, whereas the membranous TNF-α signals through receptor 2 (TNFR2) that is expressed in astrocytes[25]. There is a study shows that culturing neurons on hippocampal astrocytes for 16 h stimulate TNF- α production and result in reduction of the neurite outgrowth and branching[26]. However, it is unknown which molecule or molecules mediate this neurotoxic effect. TNF-α also promotes the release of extracellular vesicles, which are known to be increased during neuroinflammation in mouse's astrocytes[27].

Problem statement

Astrocytes are able to release a variety of neurotrophic and neurotoxic factors, which directly or indirectly participate in neuronal function and synapse formation. However, there are few evidences that astrocytes produce and release Wnt ligands in response to physiological or pathological stimuli that may participate in the astrocyte-mediated neuronal homeostasis.

Hypothesis

Activated astrocytes by TNF-α or glutamic acid differentially regulate the expression and secretion of the Wnt agonists WNT5A, WNT7A and the antagonist DKK1molecules.

Objective

Characterize both gene expression and protein levels of WNT5A,WNT7A and DKK1 in astrocytes after treating them with the neurotransmitter glutamic acid and the proinflammatory molecule TNF-α For different time intervals.

Particular Objectives

1. Prepare astrocytes primary culture.

2. Study the effect of stimulating molecules (glutamate and TNF-α) on the astrocytes, after different time intervals by analyzing:

A) the transcription level of *Wnt5a, Wnt7a and Dkk1* genes.

B) the protein level and the location of WNT5A, WNT7A/B and DKK1.

Material and methods

Primary Astrocyte Culture

A primary astrocyte culture of 2 -5 days old rats' cerebral cortex has been prepared as follows. Briefly, postnatal brain cortices were isolated, and the meninges and blood vessels were removed. The dissociated cell suspensions were plated at a density of $210 \times 10^3/cm^2$ in 12-well plates in Basal Eagle's Medium with 10% heat inactivated fetal bovine serum. The medium contained 2mM glutamine, 750 mg glucose, 50U/ml penicillin and 50 µg/ml streptomycin. The culture dishes were incubated at 37°C in 5% CO_2 in humidifier air incubator. After the cells reached 70% confluency, one half of the astrocyte cultures dishes were treated with 5ng/ml TNF-α for 12 and 24 hours. The other half dishes were treated with 100 µM glutamic acid for same time intervals. Control for each time interval has been conducted. Then the cells were collected; RNA was isolated with TRIzol and proteins were extracted in 1% SDS. These samples have been analyzed and quantified for RNA and protein using Nano-drop, gel electrophoresis (1.2% agarose gel) and spectrophotometry before performing RT-PCR (with *Gapdh* as endogenous gene) and Western blot for protein analysis.

Real-time RT-PCR:

To measure the expression levels of the three genes real-time PCR was used. Reverse transcription was performed first using aliquots of total RNA extracted and Oligo (dT) primer. The reverse transcription PCR was carried as following: heat to 70ºC for 5 min, anneal at 25ºC for 5 min, first stand synthesis at 42ºC for 60 min, heat to 70ºC for 15 min for inactivation of PCR. After that, the cDNA samples were diluted to 20 ng/µl. Gene-specific primers for *Wnt5a*, *Wnt7a*, *Dkk1* were used. The forward and reverse primers sequences (respectively) were as follows: *Wnt7a*, (NM_001100473.1, from 472 to 560: 5'- GTGTCAGTTTCAGTTCCGCAAT -3' and 5'- CCGACTCCCCACTTTGAGC -3'); *Wnt5a*, (XM_006252664, from 698 to 795: 5'- CGCACGAGAAAGGGAACGAA -3' and 5'- ATACTGTCCTGCGACCTGCT -3'); *Dkk1*, (NM_001106350, from 685 to 755: 5'- CACCAAAGGGCAAGAAGGCT -3' and 5'- GTCTTGCACAACACAGCCC -3') ; and *Gapdh*, (NM_008084, from 1 to 333: 5'- GCCTGGAGAAACCTGCCAA -3' and 5'- CTTTAGTGGGCCCTCGGC -3'). For each experimental sample, duplicate reactions were performed and negative controls for each gene were included. The reaction was performed using the StepOne Real-Time PCR System (Applied Biosystems) and analyzed with the StepOne v2.3 software (Applied Biosystems). SYBR Green PCR Master Mix was used for the amplification. The thermal cycling conditions were as following: an initial denaturation step at 95ºC for 10 min, 45 cycles for annealing at 95 ºC for 30s, extension 58 ºC for 30s and finally heat to 72 ºC for 30s.

The method used for relative quantification in gene expression was the $2^{-\Delta\Delta Ct}$ method, which was used to normalize the fold changes in gene expression to the internal control gene *Gapdh.*

Western blot:

To examine the changes in WNT7A/B protein with different treatments and timing, western blot was performed. Whole cell extracts were fractionated by SDS-PAGE 12% gel and transferred into nitrocellulose membrane using a transfer apparatus. After that, the membrane was incubated overnight with 5% milk in Tris-saline buffer/Tween 20 (0.01%) (TBST). Later, the membrane was washed three times with TBS-T for 10 min each and incubated with antibodies against Wnt7a/b (5:1000) at 4 °C overnight. After that, the membrane was washed again three times for 10 min each and incubated overnight with a (1:10000) dilution of secondary antibodies. The GAPDH was used as a loading control and the primary antibody was used at a dilution of (1:1000) and the concentration of mouse secondary antibody was (1:10000). Blots were washed with TBST three times and results were detected using chemiluminescence reagents.

Immunofluorescence:

Different sets of astrocyte culture have been prepared for immunofluorescence analysis with both treatments of the same concentration and under the same conditions. However, the time intervals for these treatments have been set to 6 and 24 h. After treatment, the cells were washed twice with 0.1 M PB (Phosphate Buffer without salt, pH7.4), fixed with 4% paraformaldehyde/0.1 M PB for 20 min, then washed with 0.1 M PB/0.3% Triton X-100 three times. Then, they were permeabilized with the same solution for 10 min and left in a blocking buffer of (0.1 M PB/3% Triton X-100 /5% BSA) overnight at 4ºC. After that, the cells were incubated with first antibodies overnight, washed three times and incubated with second antibodies for 2 h at room tempreture. At the end, cells were washed and covered with fluorescent mounting medium (DAKO) and visualized under a confocal microscope. We have used GFAP from rabbit (1:2000, Santa cruz Biotechnology, cat. Sc-365062) as a first antibody

for the astrocyte immunostaining and a secondary antibody was goat anti-rabbit (1:2000, Santa cruz Biotechnology, cat. Sc-2357), and the cells were visualized at 647 nm wavelength (red). For the WNT5A and WNT7A/B protein, we have used WNT5A and WNT7A/B goat antibodies (1:1000, Cell Signaling Technology, cat. 2392 and R&D systems, cat. AF-3008) respectively, and a secondary antibody donkey anti-goat (1:1000, Millipore, cat. AP163P). The protein was visualized at 488 nm wavelength (green). For DKK1, we used a monoclonal antibody (1:1000, Abcam, cat. Ab61275), and the second antibody was goat anti-mouse (1:1000, Invitrogen, cat. 61-6520) and visualized at 488 nm wavelength. Finally, DAPI was used to stain the nucleus.

Negative controls were conducted by incubating them in the same solutions as experimental sections but without primary antibodies.

Image analysis:

For densitometric analysis of immunofluorescence experiments, 2-3 sections from 3 cultures per each experimental condition were used. In order to detect the changes in protein level between different treatments or after different time intervals, computer software Fiji was used. Using a logarithmic option "otsu", the slide picture of the culture was transferred to 1 or 0, where 1 appears black and represents the protein whereas 0 appears white and represent anything else. After that, the area of protein was measured in five different cells of three different cultures. The percent of immunolabeled area was obtained, and the statistical analysis was done. In the case of GFAP protein fluorescence signals were converted to rainbow RGB using ImagJ software for intensity analysis.

Statistics:

Results obtained for mRNA and Protein were quantified using the computer software Prism. One-way ANOVA test followed by post-hoc Tukey was used to determine differences between samples groups, where P value < 0.05 was considered significant.

Results

Morphological changes after incubation with Glutamate or TNF-α

We first analyze the effect of glutamate and TNF-α on astrocyte morphology and GFAP contents in order to analyze astrocyte activation. Control astrocytes exhibited stellate processes and a polygonal morphology typical of monolayer astrocyte cultures. After 24 h of exposure to glutamate and TNF-α, some astrocytes appeared with elongated processes that were more evident in the TNF-α condition (*Figure 7 A, B*).

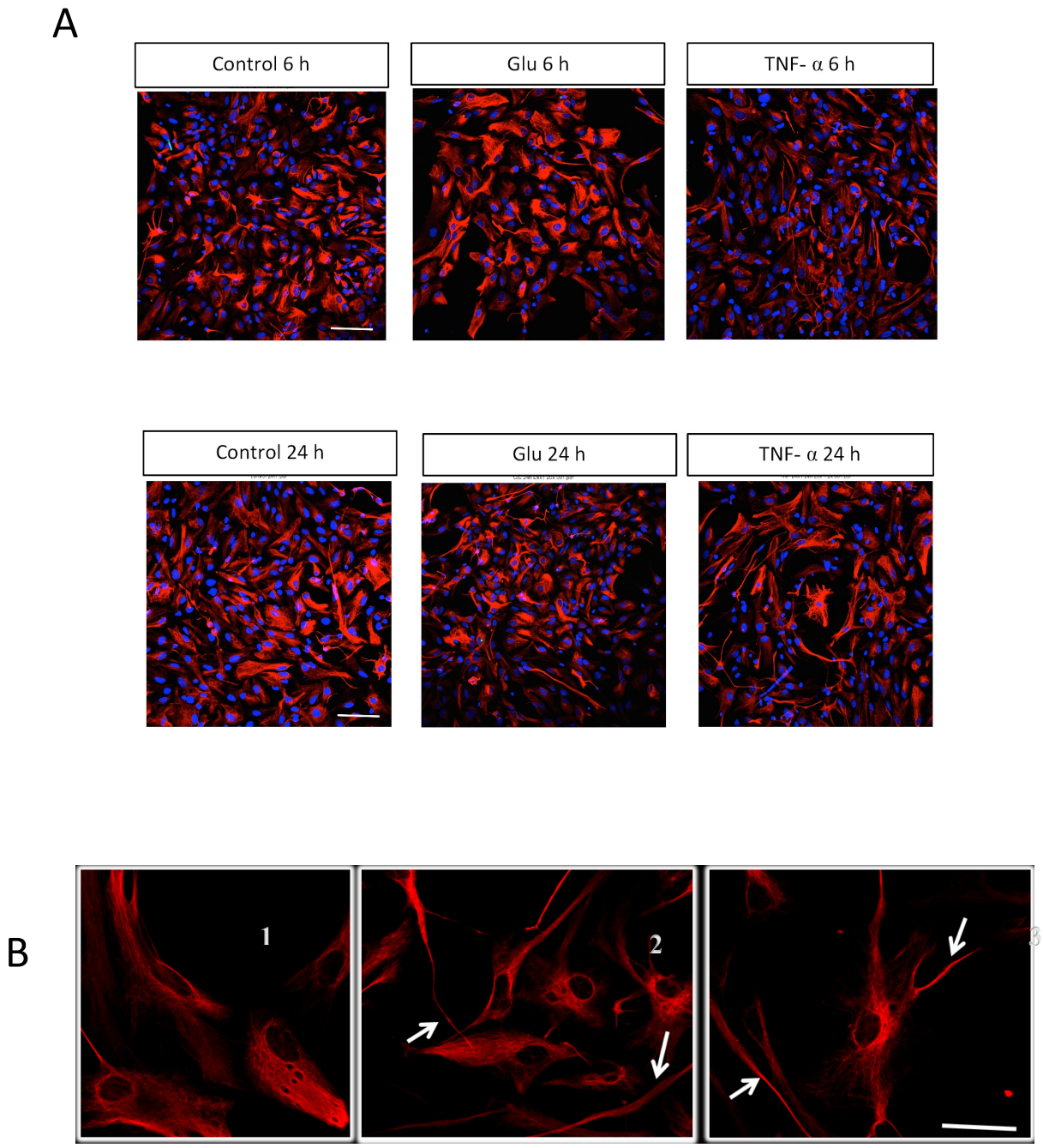

Fig 7. **Changes in astrocyte morphology after TNFα treatment.** Astrocytes were stained with antibody against Glial Fibrillary acidic protein (GFAP) and DAPI to stain the nucleus (A) All images are representative of 2-3 fields from 3 different cell cultures. Scale bar =80 μm; (B) high magnification of astrocytes from two different cultures (2,3) after 24 h of TNFα treatment in compared to control (1), showing the presence of enlarged processes (arrows), scale bar = 80 μm.

Concomitant with the morphological changes observed after 24 h of incubation with glutamate or TNF-α a slight increase in GFAP protein levels was also observed, suggesting the presence of some activated astrocytes (*Figure 8*)

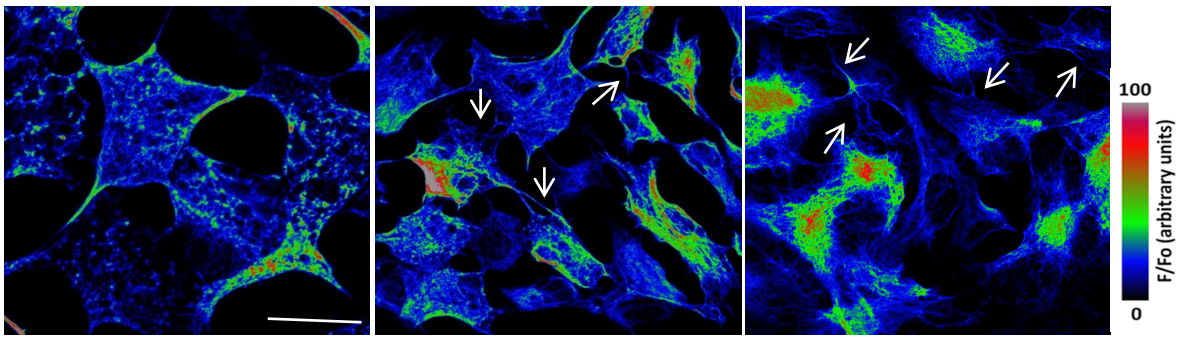

Fig 8. **Astrocyte morphology and GFAP content.** Astrocytes were incubated with glutamate (100 µM) or TNF-α (5 ng/ml) for 24 h. Fluorescence signals were converted to rainbow RGB for intensity analysis. Arrows indicates the presence of filopodia. Images are representative of 3 cultures in duplicate. Scale bar = 80 um.

Wnt ligands expression

Exposure to glutamic acid and TNF-α induced changes in mRNA of *Wnt5a, Wnt7a* and *Dkk1* genes after different time intervals *(Figure 9).*

The expression level of *Wnt5a* was slightly affected by glutamate treatment. In fact, only a significant mRNA upregulation was observed after 24 h compared with the tendency to diminish after 12 h. However, after exposure to TNF-α a significant decrease of *Wnt5a* mRNA was produced at 12 h (*Figure 9).*

The expression pattern of *Wnt7a* mRNA did not change in the presence of glutamate, but after 24 h of treatment with the pro-inflammatory cytokine TNF-α, a small but significant increase near to 25% was observed (*Figure 9*).

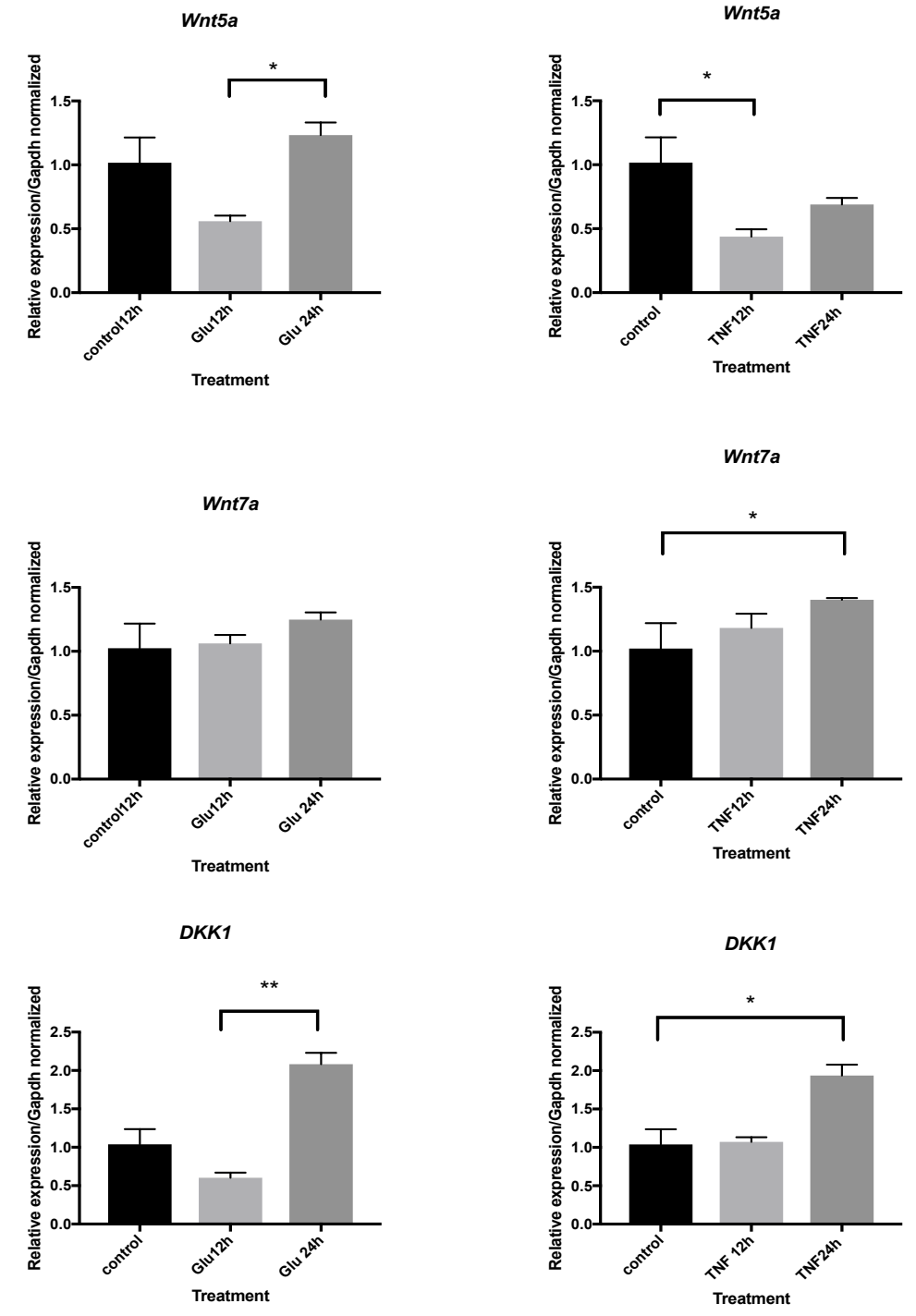

Fig 9 **.Changes in mRNA expression of different Wnt ligands normalized to *Gapdh* after exposure to glutamate or TNF-α.** Astrocytes were treated for 12 or 24 h. Each determination was done in duplicate and represents the mean ± SEM from 3 independent astrocyte cultures.

In case of the antagonist *Dkk1*, the mRNA expression showed a slightly but no significant decrease after 6 h of glutamic acid treatment and then a three-fold up-regulation after 24 h in comparison with the 12 h time point. Similarly, when treated with TNF-α, the level of *Dkk1* mRNA expression showed a two-fold increment after 24 h (Fig. 9).

Protein content and cellular distribution:

In order to detect changes in protein level between different treatments or after different time intervals, we analyzed this content by immunofluorescence labeling as well as a determination of WNT7A/B protein by Western-blot. The immunofluorescence results showed clearly that astrocytes do produce WNT5A, WNT7A/B and DKK1 in control cells without any stimuli *(Figure 10).* We can also easily Observe WNT5A, WNT7A/B and DKK1 in the cytoplasm and in the astrocytes' processes. However, DKK1 appears in a punctate pattern in contrast to WNT5A and WNT7A/B, which seem to follow the astrocyte shape.

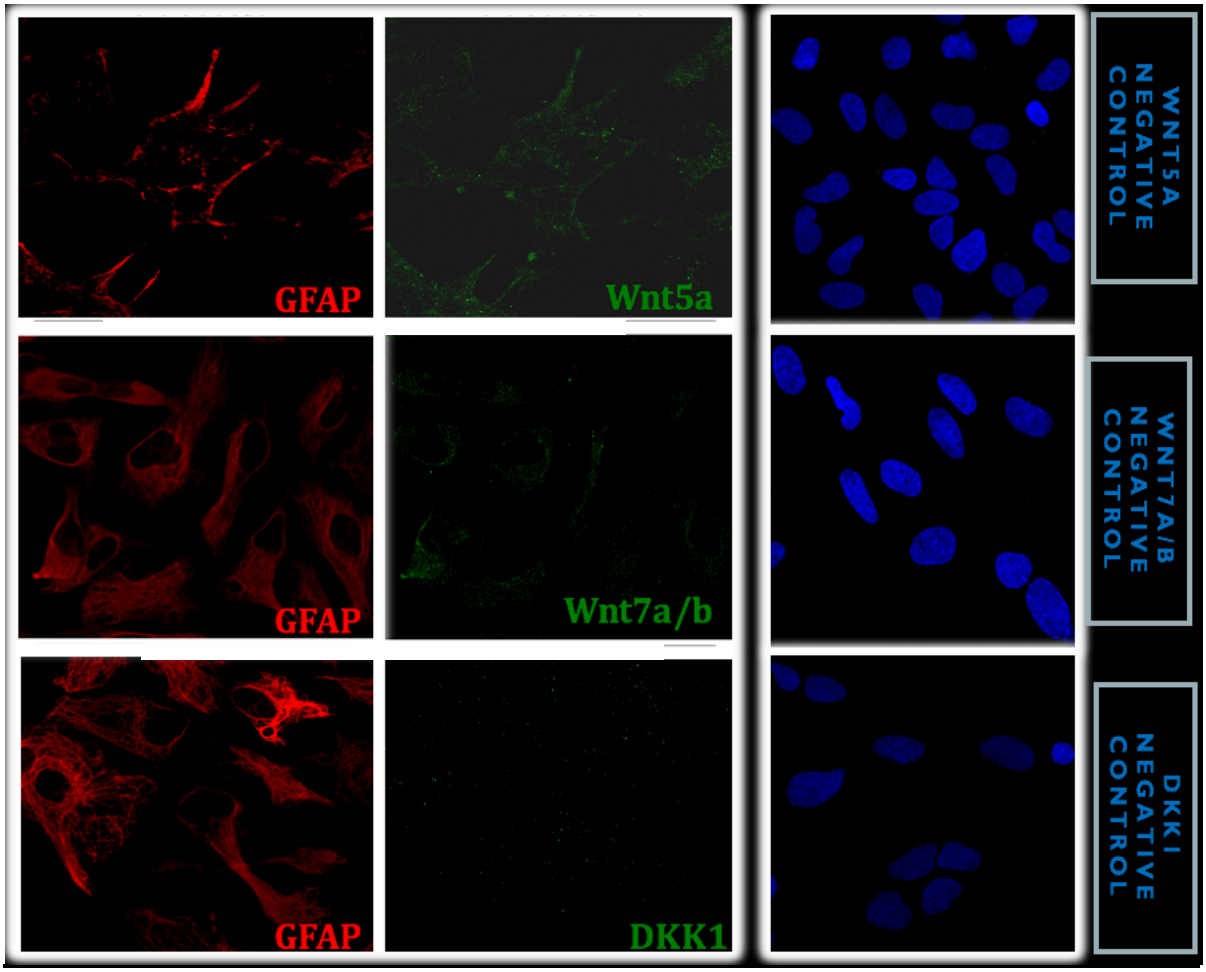

Fig 10. **Immunodetection of Wnt5a, Wnt7a/b and DKK1 in astrocytes**. Images are representative of the immunofluorescence experiments to analyze the location and content of proteins in non-stimulated astrocytes.

We found that the protein level varied between the different treatments and timing for each ligand as shown in Figures 11 and 12. The densitometric analysis showed an elevated production of WNT7A/B after both treatments that is slightly but significant higher after 24 h of TNF-α treatment compared to the other conditions (*Fig. 11*). In contrast, the level of DKK1 protein increases fast in the first 6 h, and then recovers to the control level after 24 h (*Fig. 12*).

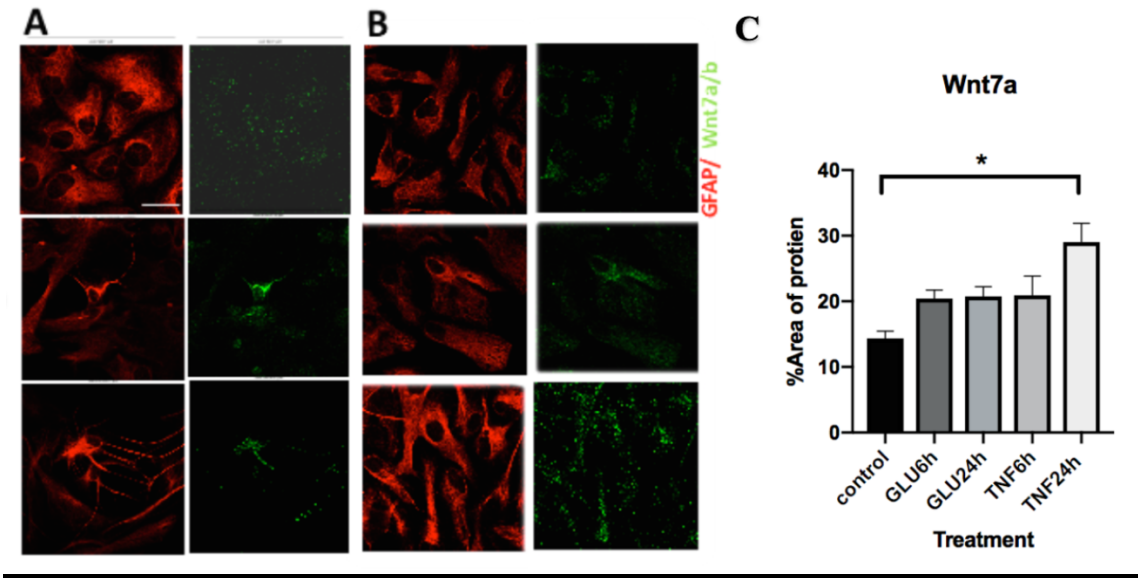

Fig. 11. **Cellular distribution of Wnt7a/b protein** after treatment with glutamic acid (A) or TNF-α (B) in control (upper lines) 6 h (middle lines) or 24 h (bottom lines) and quantification of total protein expressed as % immunofluorescence density. Images are representatives from 3 different cultures.

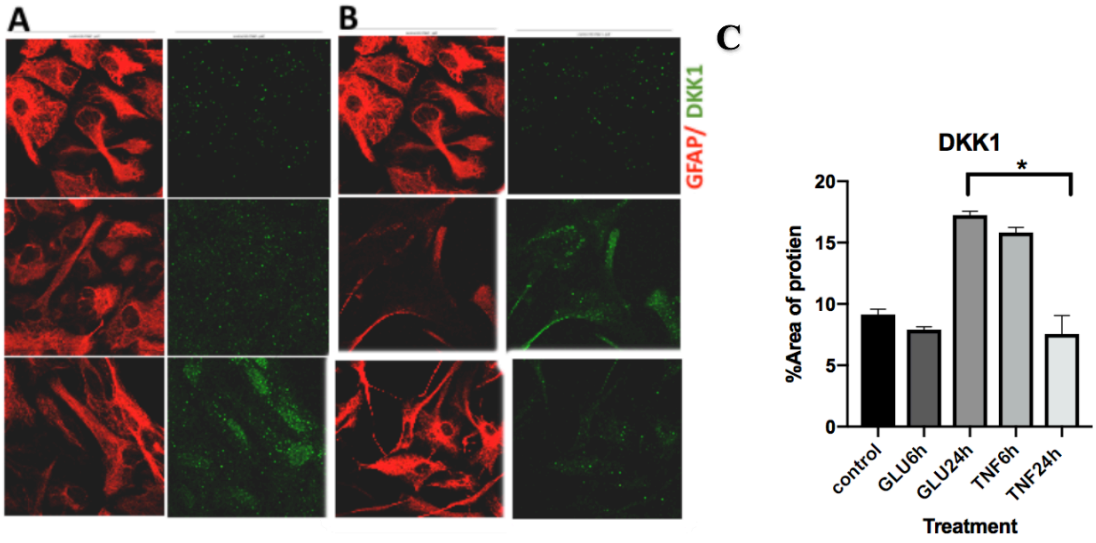

Fig. 12. **Cellular distribution of DKK1 protein** after treatment with glutamic acid (A) or TNFα (B) in control (upper lines) 6 h (middle lines) or 24 h (bottom lines) and quantification of total protein express as % of density of immunofluorescence. Images are representatives from 3 different cultures.

We have obtained some preliminary results of WNT7A/B protein by Western blot shown in Figure 13. It was noteworthy that astrocytes contain significant levels of this protein (line 1) that changes after exposure to glutamate or TNFα. Particularly, after TNFα treatment, WNT7A/B remained elevated at 36 h.

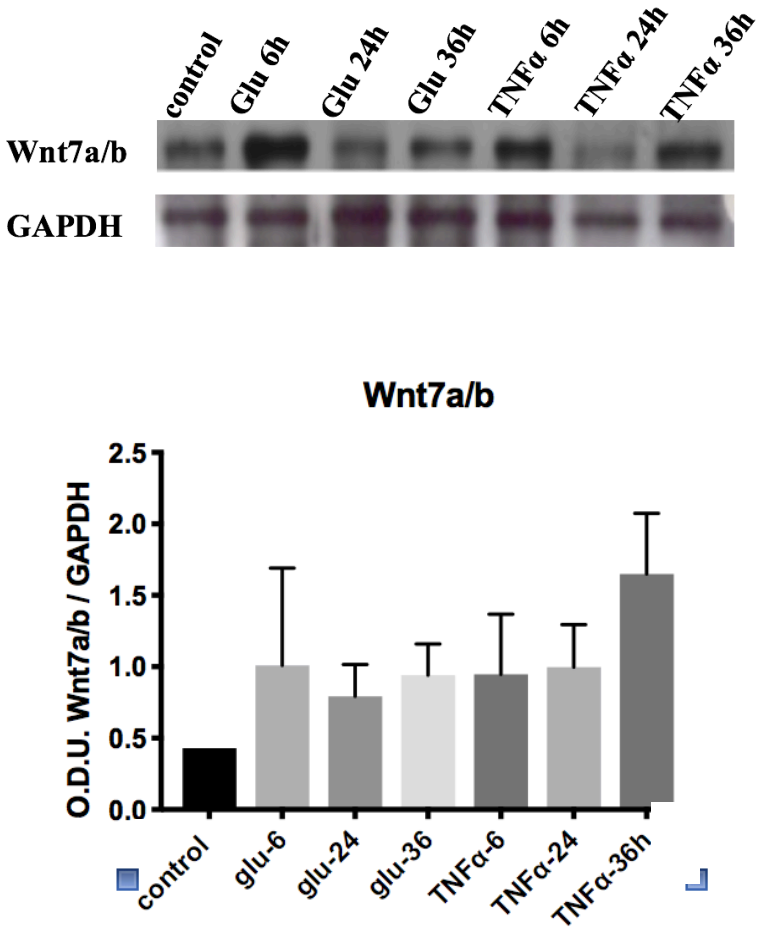

Fig. 13. **Temporal course of WNT7A/B contents in astrocytes after different treatments**. Representative Western blot and densitometric analysis from two experiments.

Discussion

Despite the functional role of the Wnt signaling pathway in adult neural circuits, there is scarce evidence regarding the relationships between Wnt modulation and astrocyte function that can participate in the maintenance of neuronal function in the adult brain. Herein we described specific changes in some Wnt ligands expression in astrocytes under different stimuli that may contribute to neuroplasticity or neurodegeneration.

In the first part of the study, basal mRNA expression of *Wnt5a, Wnt7a* and *Dkk1* was detected. The results showed that astrocytes under basal conditions (inactivated) were able to express two Wnt agonists, *Wnt5a* and *Wnt7a* and the canonical antagonist, *Dkk1*. This finding suggests the possible role of Wnt as a signaling pathway that provides the astrocytes some of their supportive role on neurons.

WNT5A is a non-canonical Wnt agonist whose expression was significantly increased after 24 h of glutamate exposure and reduced after short-term TNF-α treatment. From these results it is plausible to speculate that glutamate-dependent expression of this ligand may have a positive effect on neuronal plasticity in view of some reports that found that WNT5A plays a role in the maintenance dendritic architecture in the adult hippocampus and it is also related to cognitive function in mice[32]. In contrast, the reduce content of WNT5A expression in the presence of TNF-α may be associated with disruption of brain plasticity. In fact, they also demonstrated that deletion of WNT5A, is sufficient to elicit profound disruptions in synaptic plasticity, structural maintenance, and learning and memory processes in the adult mice[32]. This pintpoints the importance of this particular non-canonical Wnt agonist in the later-life functions. It is interesting to note that in other reports incubation of hippocampal slices with TNF-α at the same

dose that we used here (5μg/ml) produced a significant decrease in long term potentiation (LTP)[29]. Our results open the possibility to explore the role of astrocyte-dependent expression and release of WNT5A as a mechanism that participate in neuronal changes under glutamate stimulation and during neuroinflammation.

Although we detected basal levels of *Wnt7a* mRNA and its protein, glutamate was unable to increase the expression of its mRNA during the evaluated times, while TNF-α treatment increased both the mRNA and the protein after 24 h incubation. Interestingly, both glutamate and TNF-α also increased *Dkk1* at 24 h and at 6 h respectively.

In view that DKK1 is a Wnt canonical antagonist, it is not expected that its increase after glutamate treatment could interfere with the WNT5A effects. DKK1 inhibits the canonical β-catenin signaling through its binding to the LRP5/6 co-receptor but lacks inhibitory properties on the non-canonical Wnt pathways. In this sense, DKK1 might block the endogenous canonical Wnt signaling without affecting the non-canonical endogenous stimulation mediated by WNT5A, which was up-regulated by glutamate. However, the observed TNF-α-dependent expression and production of DKK1 might antagonize the Wnt canonical pathway associated to WNT7A activation and in this way diminish some positive effects of the Wnt/β-catenin pathway such as LTP. It even might be associated to neuronal damage, which has been observed in the brain of Alzheimer´s disease patients[13,29].

The DKK1 response to TNF-α was also observed in other experimental models such as the inflammatory conditions associated to arthritis in mouse[2].

The final outcome of the observed changes on neurons or astrocytes that receive Wnt ligands in an autocrine or paracrine way can be the sum of multiple factors, including the metabolic context

of the cells and the presence of modulating molecules rather than a single molecule effect. It is possible that induction of DKK1 is lower than that of WNT7A, which might prevent a complete inhibition of the Wnt canonical signaling in the target cell. It is also possible that the level of translation of DKK1 is slower than that of its transcription. This can be confirmed by measuring its mRNA expression level after longer periods of time.

One important observation from the immunofluorescence images was the induced morphological changes in some astrocytes after 24 h of TNF-α treatment. It seems that the effect of TNF-α on the astrocyte morphology has just started and might increase with time, or with high TNF-α concentration. These morphological changes include cell elongation and a decrease in the astrocytes´ processes. The decrease in astrocyte outgrowth as a result of TNF-α treatment has been published before[3]. The morphological changes can be linked to changes in the contents of the GFAP because exposure to high levels of TNF-α have been associated with the over-expression of GFAP[4]. Glutamate also produced some morphological changes as was the induction of filopodia structures. Astrocytes' α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) are thought to be involved in astrocytic filopodia outgrowth[14]. It has been demonstrated that glutamate at the concentration used in the present work (100 μM) stimulates ionotropic receptors that increase the internal Ca^{2+} concentration[30] and is able to induce morphological changes consisting in the induction of numerous filopodia from the astrocyte surface[14]. Detection of changes in the GFAP protein level can be used for assessing astrocyte reactivity in injury and disease animal models and also after exposure to inflammatory mediators, where indeed, an increase in this protein has been observed. In this regard, we have observed a slight increase in GFAP content and the presence of some astrocytes with long

process after 5ug/ml of TNF-α. These morphological changes suggest the appearance of the "reactive astrocytes"[28].

Looking at the immunofluorescence images, we can locate the three Wnt ligands in the astrocytes. We can also observe the variation in protein levels with different treatment or after different periods of time. Yet, we cannot confirm this variation using this qualitative method, and that was the reason for calculating the protein content in astrocytes as a percentage of the total cell area. However, some changes in the WNT7A/B protein contents after astrocyte incubation with glutamate or TNF-α were also observed by Western blot.

At present, we do not have information about the mechanisms involved in the expression of Wnt-related ligands after different stimuli. Astrocytes have TNF-α receptor 2 (TNFR2) type that transduce through a signaling cascade leading the activation of c-Jun N-terminal kinases (JNKs) and p38 MAPK, depending on the context[25]. Interestingly, there is evidence that the DKK1 promoter is regulated by c-Jun, which could constitute a mechanism for TNF-mediated DKK1 upregulation. Less is known about the transcription factors involved in regulation of *Wnt7a* and *Wnt5a* genes.

In conclusion, in this work we have obtained evidence of the differential regulations of Wnt ligands in astrocytes by glutamate and TNF-α, which suggest that these molecules are part of the molecular repertory of astrocytes involved in neuronal homeostasis.

Conclusions

1.- Under basal conditions, astrocytes were able to express *Wnt5a, Wnt7a* and *Dkk1* genes and this expression varies when astrocytes are activated with glutamic acid or TNF-α over time.

2.- Protein levels of the Wnt agonists, WNT5A and WNT7A/B and the antagonist DKK1 were produced in inactive and active astrocytes and are located in the cell cytoplasm. This production varies as well with different treatments and time interval.

The results obtained in the present work regarding mRNA and protein content of different Wnt ligands are shown in Table 1:

	Wnt5a		Wnt7a		DKK1	
	mRNA	protein	mRNA	protein	mRNA	protein
Glutamate						
6 h				↑		
12 h	----		----		----	
24 h	↑		----	↑		↑↑
TNFa						
6 h				↑		↑↑
12 h	↓		----		----	
24 h	----		↑	↑↑	↑	----

Table 1. Changes in mRNA and protein levels of different Wnts after astrocyte exposure to glutamate or TNF- α at different times

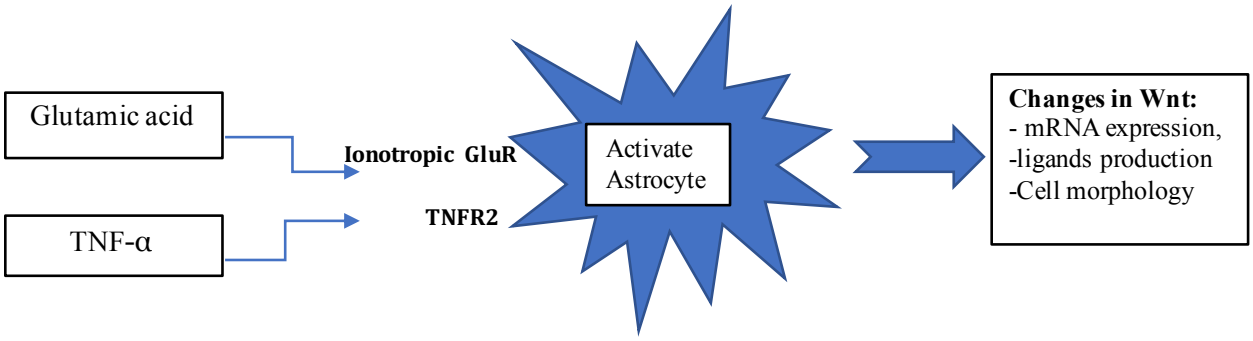

Fig 14. An illustration that Summarizes the activation of astrocytes by glutamic acid and TNF- α, through the illustrated receptors, that leads to different changes as mentioned in *table 1*.

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