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"Mechanical stimulation increases cellular contractility through the mechanosensors $\alpha_V\beta_3$ Integrin/Syndecan-4 in astrocytes"

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TABLE OF CONTENTS

ILLUSTRATION INDEX

RESUMEN

Las lesiones del sistema nervioso central cambian química y físicamente el entorno de las neuronas y las células gliales mediante la liberación de moléculas proinflamatorias y proteínas de la matriz extracelular, generando una matriz más rígida. Estos cambios desencadenan estímulos mecánicos que pueden afectar muchas respuestas celulares, incluida la motilidad, la proliferación y la diferenciación celular. El entorno proinflamatorio induce la reactividad de los astrocitos, un mecanismo por el cual los astrocitos extienden sus procesos y migran al tejido dañado. Los astrocitos reactivos tienen un fenotipo que se caracteriza por la presencia de fibras de estrés y adhesiones focales, estructuras que conducen a la contractilidad celular. La integrina αVβ3 y el Syndecan-4 son proteínas clave expresadas por estos astrocitos reactivos y son receptores de la glicoproteína de superficie neuronal Thy-1, que promueve una mayor contractilidad celular tras la unión del ligando, y un aumento de la motilidad de los astrocitos. Por otro lado, αVβ3 Integrina y Syndecan-4 se han descrito como mecanosensores en fibroblastos cuando éstos son estimulados por fuerzas mecánicas generadas por una mayor rigidez de la matriz extracelular secretada por los mismos fibroblastos. Sin embargo, este fenómeno no ha sido estudiado en astrocitos y el papel de la αVβ3 Integrina y Syndecan-4 en astrocitos estimulados por estresores físicos, junto con los cambios en su fenotipo reactivo no se conocen. Además, en los fibroblastos, Syndecan-4 es una proteína

importante que regula el reciclaje de la integrina αVβ3 a la superficie celular a través de la fosforilación de tirosina 180 mediada por Src en Syndecan-4. Las vías canónicas río debajo de los receptores de integrina incluyen la activación de la quinasa Src por la quinasa de adhesión focal y esta Src activada podría fosforilar la tirosina 180 de Syndecan-4. Por lo tanto, proponemos que el estrés mecánico es detectado por el mecanorreceptor de astrocitos Syndecan-4, aumentando los niveles de superficie de integrina αVβ3, la contractilidad celular y las fuerzas de tracción ejercidas por las integrinas inducidas por Thy-1 a través de la fosforilación de la Y180 de Syndecan-4. En esta tesis, estudiamos la contractilidad celular después de la estimulación mecánica midiendo la fosforilación de la cadena liviana de la miosina por inmuno-transferencia y monitoreando las fibras de estrés y la formación de adhesiones focales con ensayos de inmuno-fluorescencia indirecta. Para estudiar los niveles de integrina después de la estimulación mecánica, medimos los niveles de superficie de la integrina αVβ3 mediante análisis de células activadas por fluorescencia [FACS], utilizando la sobreexpresión y el silenciamiento de Syndecan-4, y los dos mutantes disponibles: formas no-fosforilable y fosfomimética, donde el residuo de tirosina 180 de Syndecan-4 se muta a L o E, respectivamente. Para estudiar la fuerza de tracción, utilizamos microscopía de fuerza de tracción con Thy-1 y estímulos de estrés mecánico. Encontramos un aumento más rápido de los niveles de superficie de integrina αVβ3 en astrocitos estimulados con fuerzas mecánicas en comparación con células no estimuladas o estimuladas con Thy-1

solamente, y que esta respuesta dependía de la fosforilación de Syndecan-4 en tirosina 180. Por otro lado, también encontramos un aumento de las fibras de estrés y formación de adhesiones focales a través de la activación de la vía β3 Integrina / pY180 Syndecan-4. Finalmente, encontramos que las fuerzas de tracción aumentaron en las células estimuladas con Thy-1 y que los valores fueron aún más altos cuando se agregó estrés mecánico. En conjunto, nuestros resultados nos ayudan a comprender los eventos fisiopatológicos que tienen lugar durante un daño al sistema nervioso central, el papel de los cambios en la rigidez de la matriz extracelular y las fuerzas ejercidas por la comunicación célula-célula sobre los mecanosensores integrina αVβ3 y Syndecan-4 en astrocitos. Más importante aún, nuestros hallazgos nos ilustran sobre los mecanismos por los cuales los niveles de integrina αVβ3 aumentan en los astrocitos, un evento que modula la reactividad de estas células.

ABSTRACT

Injuries to the central nervous system chemically and physically change the environment of neurons and glial cells through the release of pro-inflammatory molecules and extracellular matrix proteins, generating a more rigid matrix. These changes create mechanical stimuli that can affect many cellular responses, including motility, proliferation, and differentiation. The pro-inflammatory environment induces astrocyte reactivity, a mechanism by which astrocytes extend their processes and migrate to the wounded tissue. Reactive astrocytes have a phenotype characterized by the presence of actin stress fibers and focal adhesions, structures that lead to cellular contractility. $\alpha_V\beta_3$ Integrin and Syndecan-4 are key proteins expressed by these reactive astrocytes and are reported receptors of the neuronal surface glycoprotein Thy-1, which promotes higher cell contractility upon ligand binding, and astrocyte increased motility. On the other hand, $\alpha_V\beta_3$ Integrin and Syndecan-4 have been described as fibroblast mechanosensors when stimulated with mechanical forces generated by increased stiffness of the extracellular matrix secreted by the same fibroblasts. However, the role of $\alpha_V\beta_3$ Integrin and Syndecan-4 in astrocytes stimulated by physical stressors, together with the changes in their reactive phenotype have not been studied. Additionally in fibroblasts, Syndecan-4 is an important protein that regulates recycling of $\alpha_V\beta_3$ Integrin to the cell surface through Src-mediated phosphorylation of tyrosine 180 in Syndecan-4. Canonical pathways downstream

of integrin receptors include activation of Src kinase by Focal Adhesion Kinase and this activated Src could then phosphorylate the tyrosine 180 of Syndecan-4. Therefore, we propose that mechanical stress is sensed by the astrocyte mechanoreceptor Syndecan-4, increasing $\alpha_V\beta_3$ Integrin surface levels, cell contractility, and traction forces exerted by integrins induced by Thy-1 through the Y180 phosphorylation of Syndecan-4. We studied cell contractility after mechanical stimulation by measuring phosphorylation of myosin light chain by western blot, and by monitoring stress fiber and focal adhesion formation with indirect immunofluorescence assays. To study integrin levels after mechanical stimulation, we measured surface levels of $\alpha_V\beta_3$ Integrin by fluorescent activated cell sorting [FACS], using overexpression and silencing of Syndecan-4, and the two available mutants: non-phosphorylatable and phosphomimetic forms, where the Syndecan-4 tyrosine 180 residue is mutated to L or E, respectively. To study traction force, we used traction force microscopy with Thy-1 and mechanical stress stimuli. We found faster increase of surface levels of $\alpha_V\beta_3$ Integrin in astrocytes stimulated with mechanical forces compared with non-stimulated cells and Thy-1, and that this response depended on Syndecan-4 phosphorylation on tyrosine 180. On the other hand, we also found an increase in the stress fibers and focal adhesion formation through the activation of β_3 Integrin/pY180SDC4 pathway. Finally, we found that traction forces were increased in cells stimulated with Thy-1 and that values were even higher when mechanical stress was added. Taken together, our results help us to understand the pathophysiological events

taking place during insults of the central nervous system, the role of the changes in rigidity of the extracellular matrix, and the forces exerted by cell-cell communication on the $\alpha_V\beta_3$ Integrin and Syndecan-4 mechanosensors in astrocytes. Most importantly, we shed light on the mechanisms by which $\alpha_V\beta_3$ Integrin levels increase in astrocytes, an event that modulates astrocyte reactivity.

BACKGROUND

Central Nervous System injuries and changes in the microenvironment

The **central nervous system [CNS]** harbors multiple neuronal and non-neuronal cell types that interact over time to maintain homeostasis, protect viable cells, clear debris and preserve CNS function. The non-neuronal cells in the CNS include three different types of glia: oligodendrocytes, astrocytes and microglia[1]. **Glia** are more numerous than **neurons** in the brain, outnumbering them by a ratio of 1.6:1[2], and although they do not directly participate in synaptic interactions and electrical signaling, their supportive functions help to define synaptic contacts and maintain the signaling abilities of neurons[3]. Moreover, there is functional integration and physical proximity between the pre- and post-synaptic membranes with the surrounding glia, as well as a combined contribution of these three synaptic components, known as the tripartite synapse[4]. As an example of this supportive role, a single **astrocyte end-feet domain** can contact as many as 2,000,000 neuronal synapses in the human cortex[5].

After a lesion, injury or disease that affects the CNS, **several extracellular signals** produce changes in the microenvironment, and the formation of a **glial scar is** important to close the wounded tissue. However, this scar also acts as a chemical and physical barrier that **prevents axonal regrowth and astrocyte migration**. On the other hand, plasticity-limiting factors that stabilize neuronal networks in the healthy brain, exacerbate reorganization in the case of damage,

changing the composition of the Extracellular Matrix [ECM][6]. These events, among others, change the mechanical properties of the CNS after injury and play a prominent role in the repair and remyelination of the damage tissue. Additionally, the glia also plays an important role during CNS injury. Reports indicate that oligodendrocytes are capable of responding to the mechanical properties of the ECM, since stiffer matrices inhibit the branching and elongation of oligodendrocytes. This inhibition results in significant reduction of oligodendrocyte branching complexity, which correlates strongly with decreased expression of differentiation markers[7]. Thus, chemical and physical changes in ECM after CNS injury clearly affect glial cells, such as oligodendrocytes; however, **the role of these mechanical signals in astrocyte reactivity has not been clearly defined**.

Astrocyte reactivity and the lack of CNS repair

Astrocytes respond to all forms of injury and disease in the CNS through a process known as **astrocyte reactivity[3]**. Studies over the past twenty years have provided clear evidence that astrocyte reactivity is not a simple all-or-none phenomenon, but is a finely graded series of changes that include reversible alterations in gene expression, cell hypertrophy and scar formation, with permanent tissue rearrangement[8]. It has also been demonstrated that the structural and functional changes that characterize astrocyte reactivity occur in a context-dependent manner, and are regulated by many different signaling

events[3, 9] [see Figure 1]. Astrocyte reactivity is induced by pro-inflammatory cytokines such as **Interleukin [IL]-1β, IL-6, and Tumor necrosis factor [TNF][10]**. These cytokines are secreted in part by the astrocytes themselves, microglia, and other cell types[11].

The most prominent changes of reactive astrocytes are hypertrophy of cellular processes and up-regulation of intermediate filament [nanofilament] proteins, in particular **Glial Fibrillary Acidic Protein [GFAP]**, which is a unique component of the intermediate filament system of astrocytes[12]. This is accompanied by the up-regulation of many other proteins, such as Connexin-43, inducible nitric oxide synthase [iNOS][13]. Our own *in vitro* studies performed with astrocytes isolated from neonatal rat cortices have shown that TNF-induced cell reactivity leads to elevated expression of **β3 Integrin, Syndecan-4**, GFAP, iNOS, Pannexin-1, and Connexin-43 proteins[14]. Depending on the type of damage, the time point after the lesion, and the position with respect to the injured zone, reactive astrocytes are also responsible for the production/secretion of several ECM and membrane proteins. These proteins include Tenascin-C [TN-C][15, 16], Brevican, Versican[17], Decorin[18], Laminin, and Fibronectin [FN][19], as well as chondroitin sulfate proteoglycans [CSPGs] such as Neurocan[20] and Phosphacan[21]. Collagen, which is also involved in glial scar formation, has been described in reactive astrocytes; the basement membrane-associated Collagens type IV[22] and type VIII have been detected[23]. In addition, reactive astrocytes express Heparan Sulfate Proteoglycan membrane proteins, such as Glypican,

Syndecan-1 to 4, and Agrin[24-26]. The secretion of the ECM proteins changes the mechanical properties of the microenvironment in the CNS. **However, the cellular responses triggered by such changes remain unclear.** In addition, up-regulated expression of Syndecan-4, which is a known mechanical sensor[27], is likely to play a role in the damaged brain; however, **the role of Syndecan-4 in astrocyte reactivity has not been studied in depth.**

Although astrocyte reactivity is a normal physiological response to an injury that allows reconstruction of the blood-brain barrier by facilitating the remodeling of brain circuits in areas surrounding the lesion, and limiting the damaged zone from the rest of the tissue, detrimental effects on neuronal survival and axonal regeneration have also been described[28]. Multiple *in vivo* and *in vitro* models have demonstrated that the molecular composition of the glial scar formed by reactive astrocytes, oligodendrocytes and myelin produced by the latter, are factors that contribute to the low regenerative capacity observed in CNS neurons. Inhibitory molecules present in the glial scar include CSPGs secreted by astrocytes, myelin-derived molecules such as myelin-associated glycoprotein [MAG] and myelin glycoprotein in oligodendrocytes [OMgp], and Nogo[28-30]. All of these myelin molecules recognize a single neuronal receptor identified as the GPI-anchored protein, NgR1, which induces inhibition of neurite outgrowth[31]. A different GPI-anchored protein that contributes to this non-permissive environment is Thy-1, also known as CD90. As for NgR1, Thy-1 has been suggested to participate in stabilizing neuronal connections and inhibiting neurite

outgrowth[32]. Thus, lack of axonal regeneration in the CNS is due to the presence of many molecules that create a non-permissive environment, which exists in the brain upon injury.

Interestingly, our laboratory has reported that endogenous Thy-1 expressed by neuronal cell lines, directly binds the $\alpha_V\beta_3$ Integrin receptor in astrocytes to trigger downstream signaling events in both neurons and astrocytes. In neurons, Thy-1/Integrin interaction induces axonal retraction[33, 34], whereas in astrocytes, it induces **Focal Adhesion [FA] and Stress Fiber [SF] formation**, which are strong points of adhesion of the cells to the ECM and bundles of microfilaments, respectively[33-38]. Thus, $\alpha_V\beta_3$ Integrin is an astrocyte receptor for Thy-1 that upon binding, induces **morphological changes in neurons and astrocytes, that could likely preclude CNS repair upon injury**.

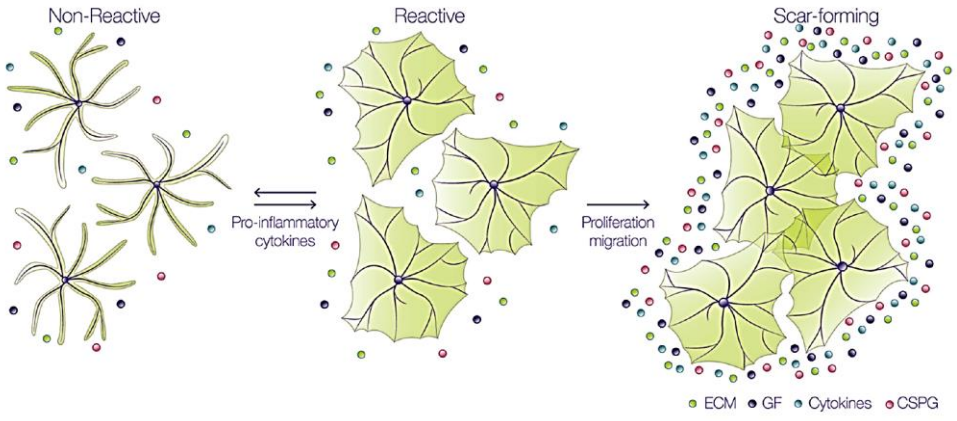

Figure 1. Astrocytes undergo astrogliosis in a pro-inflammatory environment. *Astrocytes change their morphology from a non-reactive into a reactive state when exposed to pro-inflammatory cytokines. They undergo hypertrophy and not only change their shape, but also their protein expression; up to this stage, the process is reversible and reactive astrocytes do not*

overlap their branches. These cells then proliferate and migrate to the lesion site to form the glial scar, where they secrete many factors, such as extracellular matrix (ECM) proteins, cytokines, growth factors (GF), and chondroitin sulfate proteoglycans (CSPG). In this scar-forming stage, astrocytes no longer move, and the process is irreversible[39].

Astrocyte interactions with neurons and the extracellular matrix

Reactive astrocytes cultured *in vitro* have been reported to respond to Thy-1, increasing their adhesion to the underlying substrate [ECM], changing their cellular phenotype, **contractility, and** inducing **cell migration[14].** The neuronal surface glycoprotein **Thy-1 not only binds to $\alpha_V\beta_3$ Integrin, but also recognizes Syndecan-4 receptors in reactive astrocytes** and triggers the recruitment of FA proteins to adhesion points[40]. These interactions activate signaling pathways that includes Focal Adhesion Kinase [FAK], Src, paxillin, vinculin, and p130Cas[33] [Figure 2], which in turn leads to the activation of the conventional PKCα, the small GTPase RhoA and its effector ROCK, producing **changes in the astrocyte actin cytoskeleton and strong cell attachment to the ECM[33-37, 40]**. After a more prolonged stimulation [24 hours], Thy-1/Integrin binding induces the activation of FAK, PI3-kinase and the GTPase Rac1, thus leading to **astrocyte migration[41]**. Thy-1 possesses an **integrin binding domain** [the tripeptide RLD] and a **Heparin binding domain** [HBD] that interact with $\alpha_V\beta_3$ Integrin and Syndecan-4, respectively[41]. Interestingly, **HBD-mutated Thy-1-Fc**, which does **not bind to Syndecan-4**, fails to cover the cell-free area in a wound healing

assay, suggesting the **requirement** of this heparan sulfate proteoglycan in the induction of **astrocyte migration[40, 41]**.

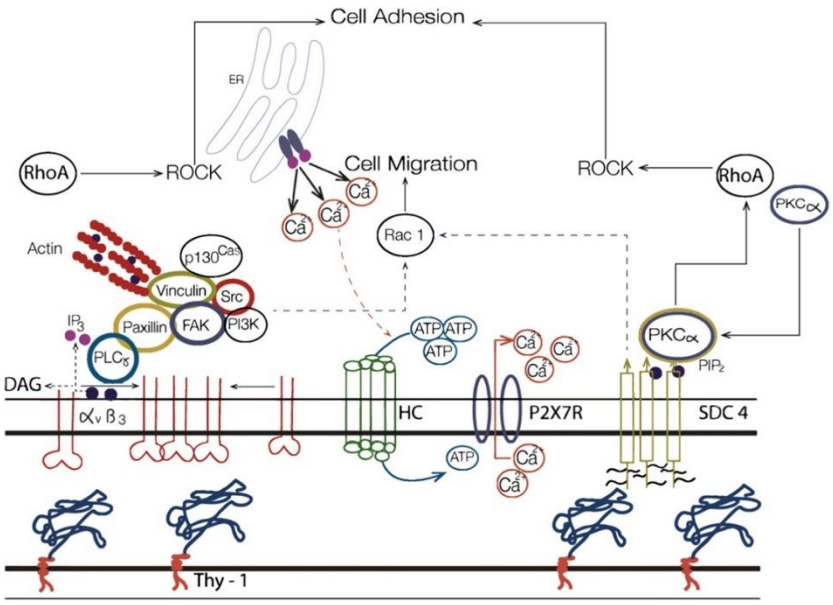

Figure 2. Signal transduction pathways in astrocyte migration activated by Thy-1 receptors αVβ3 Integrin and Syndecan-4. *αVβ3 Integrin [receptor 1] engagement by Thy-1 is followed by intracellular signaling pathways that include activation of PI3K and PLCγ, IP3 production, IP3R activation in the ER, increase in cytosolic Ca2+, opening of hemichannels and subsequent ATP release, ATP-mediated P2X7R [receptor 2] activation, Ca2+ entry, PKCα activation, and Syndecan-4 [receptor 3]-triggered signaling pathways that include activation of the Rho/ROCK axis, with consequent changes in the actin cytoskeleton. Signaling pathways downstream of integrin, which include proteins forming FAs, also control the interplay between RhoA and Rac1 activation and actin remodeling[42].*

On the other hand, regulation of integrin-induced signaling pathways by Syndecan-4 has been well documented in fibroblasts. Syndecan-4 acts as a regulator of α5β1 Integrin rather than as a co-receptor that triggers separate signaling pathways[43]. Binding of Syndecan-4 to FN allows the association of Syndecan-4 with PKCα, which activates RhoG. This GTPase triggers endocytosis

of β1 Integrin and a faster turnover of FAs, promoting migration of mouse embryonic fibroblasts[43]. Moreover, **phosphorylation of Syndecan-4** mediated by the **non-receptor tyrosine kinase Src** acts as a **control point** to coordinate the formation of stable FAs and cell migration[44]. The phosphorylation of **tyrosine 180 of Syndecan-4** inhibits ADP ribosylation factor 6 **[Arf6]** activation — shown to participate in β1 Integrin recycling— and thus, controls the amount of $\alpha_5\beta_1$ Integrin on the cell surface. By decreasing β1 Integrin, phosphorylated Y180 of Syndecan-4 increases the levels of $\alpha_V\beta_3$ Integrin at the plasma membrane [Figure 3], which promotes the stability of FAs and directional/persistent migration[44]. Thus, these studies suggest that **the phosphorylation status of Y180 of Syndecan-4 determines which integrin is recycled to the cell surface**. Considering that changes in the ECM upon injury increase mechanical forces exerted over cells, the questions that arise here are: i) Does **mechanical stimulation increase surface levels of $\alpha_V\beta_3$ Integrin**, thereby allowing astrocytes to bind and respond to Thy-1? and ii) Are **these changes in Integrin levels controlled by Syndecan-4 phosphorylation on Y180**? It is important to highlight that $\alpha_V\beta_3$ Integrin and Syndecan-4 are also considered receptors of mechanical stimuli[27, 45].

Typical **ECM ligands** for β1 or β3 integrins and Syndecan-4 are proteins like **FN** and **Vitronectin**. FN possesses three types of FN repeats containing an integrin-binding site [RGD tripeptide] and HepII [or HBD] that interact with the integrin extracellular domain and the heparan sulfate motifs of Syndecan-4,

respectively. Through these types of contacts, ECM proteins and their receptors control the cyclic variation of FAs, determining the strength and dynamic changes of these structures, which allow the switching from strong cell adhesion to cell migration[46]. Interestingly, the **astrocyte receptors** $\alpha_V\beta_3$ Integrin and Syndecan-4 have **dual activity**, sensing **chemical** [Thy-1] and **mechanoreceptor** [ECM stiffness, mechanical stress][43] signals from the environment after injuries.

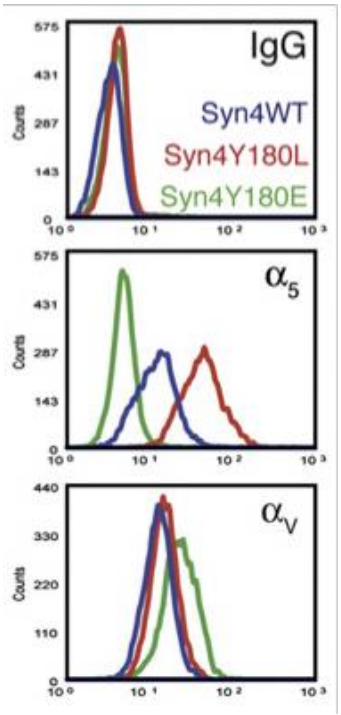

Figure 3. Integrin levels are Syndecan-4 dependent. *Flow cytometric analysis of cell-surface α5 and αV Integrin expression levels in fibroblasts expressing Syn4WT [blue], Syn4Y180L [red], and Syn4Y180E [green][44].*

Cellular mechanotransduction triggered by mechanical signals

Cells in multicellular tissues are subjected to different types of forces, including **compressive, tensile, fluid shear stress and hydrostatic pressure**, each of which plays a complex role in the shaping, development and maintenance of tissues[47]. **Mechanotransduction** occurs in many biological processes, and cells respond with a diverse scope of behaviors[48] such as embryology development or wound repair among others. The **cells are able to mechanosense** different mechanical inputs, and convert them into a **coordinated response**. When external forces are applied, cells actively rearrange the organization and contractility of the cytoskeleton and redistribute their intracellular forces[49]. This **mechano-sensing of the ECM is a process that** involves **integrins** and the **actomyosin cytoskeleton**, and is followed by a **mechanoregulation process**, which includes the deposition, rearrangement or removal of the ECM to maintain overall cellular form and function[50]. At a molecular level, mechanical forces can rearrange proteins laterally within the membrane, regulate their activity by inducing conformational changes and probe the mechanical properties and bond strength of receptor–ligands[51, 52]. Mechanical forces exerted by the ECM for example, can change the expose to hidden ratio of domains of the proteins, alter the concentration and exposure of growth factors within the ECM, or release stored factors from the ECM, thereby influencing cell behavior[53] [Figure 4].

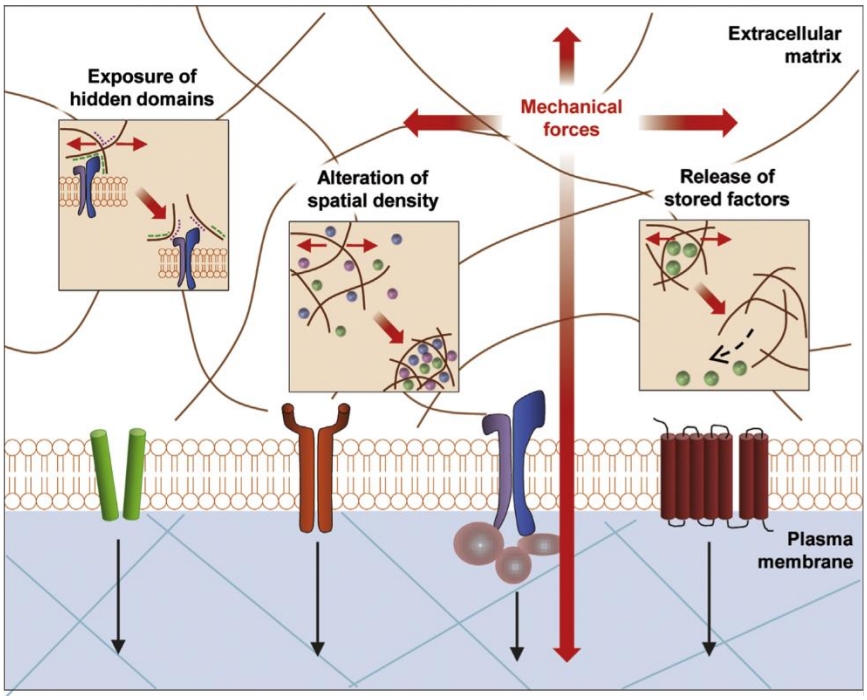

Figure 4. Extracellular Mechanotransduction. *Mechanical forces in the ECM can expose hidden domains and alter spatial density of growth factors within the ECM, or release stored factors from the ECM, thereby influencing cell behavior[53].*

Matrix and tissue rigidity guides many cellular processes, including the differentiation of stem cells and the migration of cells in physiological and pathological conditions[54]. The sensing of rigidity requires that cells first exert force on the substratum and then sense and respond to the resultant strain or stress. Focal adhesions increase their strength on stiff but not soft substrata, which thereby influences cytoskeletal structure and signaling[54, 55]; thus, **cell adhesion is critical to mechanosensivity[56, 57]**. The activation of different pathways by mechanical forces that act as ligands can activate molecular pathways as those mediated by integrins[58], tyrosine kinase receptors, G-protein

coupled receptors or stretch-activated ion channels[59] [Figure 5]. On the other hand, cells are able to respond to mechanical stimulus changing their cytoskeletal tensegrity. **Tensegrity** is a building principle that was first described by the architect R. Buckminster Fuller in 1961. He defined tensegrity systems as structures that stabilize their shape by continuous tension or **"tensional integrity"** rather than by continuous compression [e.g., as used in a stone arch][60]. To extrapolate this concept to a biological context, **cellular tensegrity models** propose that the whole cell is a **pre-stressed tensegrity structure**. In this model, tensional forces generated by cytoskeletal microfilaments and intermediate filaments are balanced by interconnected structural elements that resist compression; most notably, internal microtubule rods and adhesion points that contact the ECM[60]. Thus, in mechanotransduction, all signals point to the cytoskeleton, matrix, and integrins as controllers of the response to mechanical forces[61]. However, the response to changes in the rigidity of the ECM is cell-type specific[62]. For example, physical and chemical microenvironmental signals orthogonally control the degree and duration of fibrosis associated epithelial-to-mesenchymal transitions[63]. Moreover, during cell migration, forces generated by the actin cytoskeleton are transmitted through adhesion complexes to the substrate[64], showing that forces are sensed from and towards the ECM. Importantly, **the response of the damaged brain to changes in ECM rigidity has not been thoroughly studied**. The biomechanical characterization of human brain tissue is a challenging task because of its multiphasic nature, its regulated

mechanical response, its multiple modes of loading, and its regional variation of mechanical properties[65].

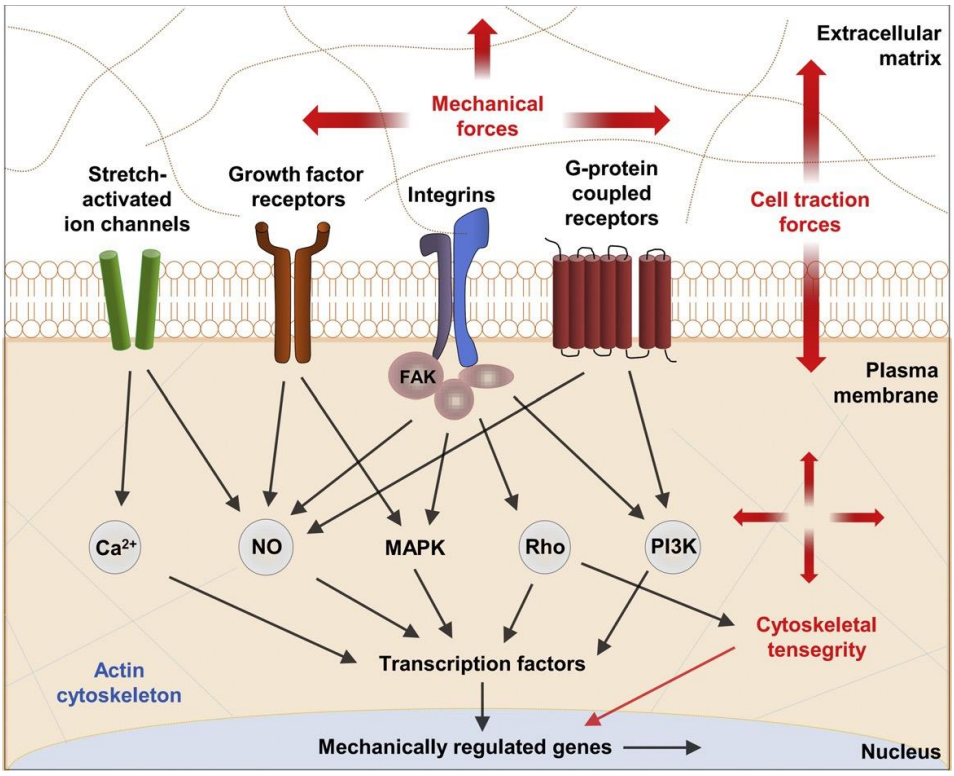

Figure 5. Scheme of cell mechanotransduction pathways. *Mechanical forces [red arrows] act via interrelated biochemical and structural pathways to convert physical cues to biologic responses. The growing field of mechanobiology aims to modulate these complexes signaling networks to promote repair and prevent complications such as chronic scar formation. NO, nitric oxide; MAPK, mitogen-activated protein kinase; Rho, Ras homolog gene family member; PI3K, phosphoinositol-3-kinase; FAK, focal adhesion kinase[59].*

Studies about the viscoelastic properties of the CNS have indicated that in **all CNS cells**, the elastic behavior dominates over the viscous behavior. The mechanical properties differ in distinct cell compartments, such as soma and cell processes, most likely because of the unequal local distribution of cell organelles;

in comparison to most other eukaryotic cells, both neurons and glial cells are very soft [“rubber elastic”], and interestingly, glial cells are even softer than their neighboring neurons[66].

Syndecan-4[27] and I**ntegrin[45]** are mechanosensors of forces exerted by the ECM and are the molecules that initiate mechanotransduction signaling pathways. Previously, our laboratory reported that $\alpha_V\beta_3$ Integrin is activated by neuronal Thy-1, activating the signaling pathway FAK, Src, the small GTPase RhoA and its effector ROCK, thereby producing **changes in the astrocyte actin cytoskeleton and strong cell attachment to the ECM**[35-37, 40, 41]. However**,** the potential role of the phosphorylation of **Syndecan-4 on the regulation of this integrin-triggered molecular pathway has not been studied.**

Therefore, taking all the aforementioned information into consideration, we propose the following hypothesis [Figure 6]:

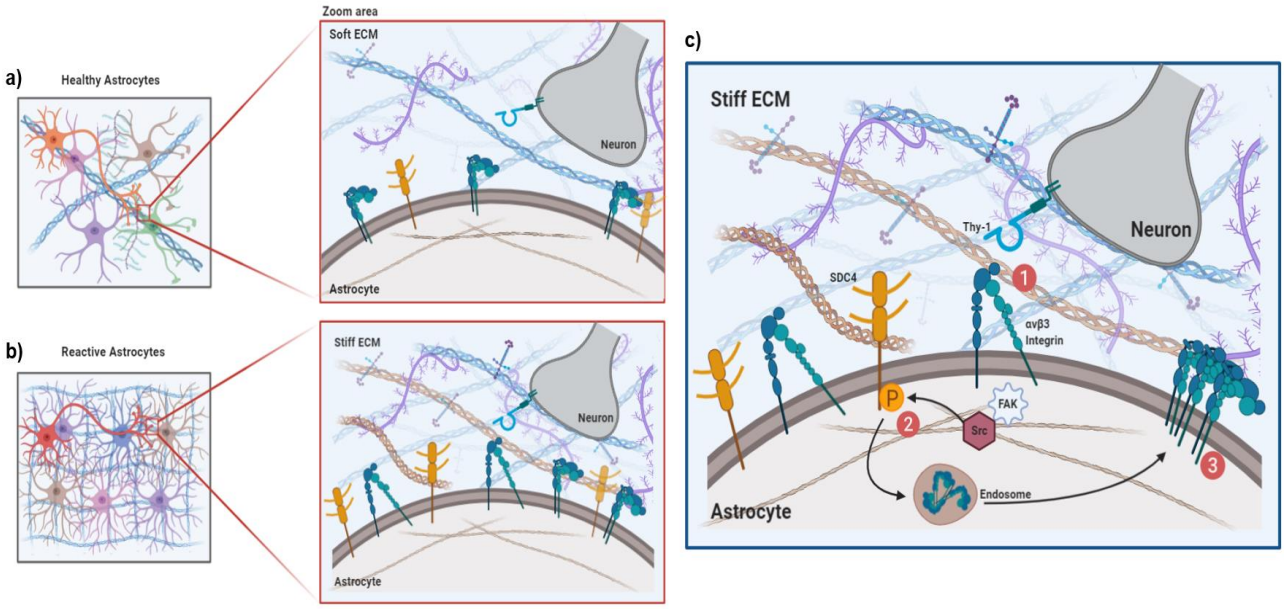

Figure 6. Scheme explaining the Hypothesis. *a) Normal astrocytes are surrounded by a soft ECM in the brain, and express low levels of the mechanoreceptor $\alpha_V\beta_3$ Integrin and Syndecan-4. b) Reactive astrocytes show a denser ECM, which induces mechanical stress sensed by the astrocyte up-regulated mechanoreceptor Syndecan-4, increasing $\alpha_V\beta_3$ Integrin surface levels and cell contractility. Thus, c) after injury, ECM rigidity is enhanced, stimulating mechanoreceptors such as $\alpha_V\beta_3$ Integrin ①, and the effectors downstream of this receptor, such as Focal Adhesion Kinase [FAK]. FAK is activated and recruits non-receptor tyrosine kinase Src, which phosphorylates Syndecan-4 in tyrosine 180 [Y180SDC4] ②. This phosphorylation is key to up-regulate levels of $\alpha_V\beta_3$ Integrin cell surface levels ③.*

HYPOTHESIS

“Mechanical stress sensed by the astrocyte mechanoreceptor Syndecan-4 increases $\alpha_V\beta_3$ Integrin surface levels and cell contractility induced by Thy-1, through the Y180 phosphorylation of Syndecan-4”.

AIMS

General Aim

To study the effect of mechanical forces on the signaling cascades involved in Thy-1-induced astrocyte contractility.

Specific Aims

I. To study whether mechanical forces with or without Thy-1 stimulation increase surface levels of $\alpha_V\beta_3$ Integrin and whether these changes are controlled by Syndecan-4.

II. To determine whether mechanical forces enhance Thy-1-induced cell contractility of astrocytes through $\alpha_V\beta_3$ Integrin engagement regulated by Syndecan-4.

III. To study whether mechanical forces enhance β3 Integrin levels and contractility through the Y180 phosphorylation of Syndecan-4.

METHODS

Cell Line

The rat astrocytic cell line DITNC1 [ATCC CRL-2005] was obtained from P Magistretti [University of Lausanne, Switzerland], maintained in RPMI medium 1640 [GIBCO, Pittsburgh, PA, USA] containing 5% fetal bovine serum [FBS, HyClone, Pittsburgh, PA, USA], 0.1 mM 2-mercaptoethanol [GIBCO] and 100 U/mL penicillin/100 µg/mL streptomycin [PS mixture, GIBCO]. DITNC1 were maintained in culture at 37°C in a 5% CO_2 humidified atmosphere. The medium was changed twice a week and passages were carried out by detaching the cells with 0.1% trypsin [Invitrogen, Grand Island, NY, USA].

Plasmids and Transfections

Astrocytes were transfected with Syndecan-4 mutants: Y180L-Syndecan-4 [non-phosphorylable] and Y180E-Syndecan-4 [phosphomimetic] were kindly donated by Dr. Mark Morgan, UK[44]. We also transfected siRNA Syndecan-4 [Ambion, Austin, TX, USA] and Syndecan-4 wild type to silence or overexpress Syndecan-4, respectively. Transfections were performed using the Amaxa Nucleofector system, following the manufacturer's instructions for the VCA-1003 transfection kit [Lonza, Cologne, Germany].

Fusion Proteins and Magnetic Beads

Thy-1-Fc and TRAIL-R2-Fc fusion proteins were obtained as described previously[36, 37, 40]. These fusion proteins were incubated with magnetic beads coated with Protein A of 4.2 µm [Spherotech Inc. IL, USA] in a 10:1 ratio for 1 h at 4°C prior to their use. Of note, TRAIL-R2-Fc is a fusion protein of the receptor for the soluble apoptosis-inducing ligand, TRAIL, and it is used in these assays as a negative control.

Cell cytometry

Cells were detached using trypsin/EDTA and incubated at 4°C to avoid internalization of surface proteins. After blocking with 5% Bovine Serum Albumin [BSA, Sigma-Aldrich , St. Louis, MO, USA], cells were immune-labeled with anti-$\alpha_V\beta_3$ Integrin/Phycoerythrin [Santa Cruz Biotechnology, Dallas, TX, USA] for 2 h. Cells were analyzed using a FACS Canto [BD Bioscience, Franklin Lakes, NJ, USA] flow cytometer. Data were analyzed and plotted using FlowJo software [version vx].

Western Blot

Protein extracts were prepared in a lysis buffer [150 mM NaCl, 0.1% SDS, 0.25% sodium deoxycholate, 1% Triton-X100, in 50 mM Tris-HCl pH 7.4] supplemented with a protease and phosphatase inhibitor cocktail [Biotool,

Houston, TX, USA]. Extracts were electrophoretically separated on 10% or 12% SDS-PAGE gels and transferred to nitrocellulose membranes [Millipore, Billerica, MA, USA], which were blocked with 5% w/v BSA Fraction V in PBS containing 0.1% Tween-20, and subsequently incubated with anti-Phospho-Myosin Light Chain 2 [Ser19] [Cell Signaling Danvers, MA, USA], total Myosin Light Chain 2 [Cell Signaling Danvers, MA, USA], anti-HSP90 [Santa Cruz Biotech] or anti-Syndecan-4 [Abbexa, Cambridge, UK] primary antibodies. The membrane was then washed and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG [Jackson ImmunoResearch Labs, Inc.,West Grove, PA, USA] or goat anti-mouse IgG polyclonal antibody [Bio-Rad Laboratories, Inc., Hercules, CA, USA] for 1 h at room temperature. Bands were visualized with a chemiluminescence kit [Pierce, Thermo Scientific, Rockford, IL, USA], according to the manufacturer's instructions.

Indirect Immunofluorescence

DITNC1 cells [200.000 cells] or primary astrocytes were seeded on 12-mm coverslips and left to adhere for 24 h. Cells were then washed and fixed, as previously described [5]. After removing the fixative agent, cells were stained with anti-phospho Tyrosine antibody [Abbkine, Australia], followed by secondary antibody conjugated to IF546 [Abbexa, Cambridge, UK]. Astrocytes were stained with phalloidin/FITC [Molecular Probes, Eugene, OR, USA]; and DAPI [diamidino-

2-phenylindole] [Sigma-Aldrich]. Samples were visualized using a confocal Nikon Spectral C2+ Plus microscope [Nikon, Tokyo, Japan].

Focal adhesion and Stress fiber quantification

Quantification of the number and area of FA was performed using ImageJ program with the Analyze particle tool[67]. Number, alignement, and thickness of the SFs were quantified by using Hessian matrix-based analisys as reported[67].

Pull-up Traction Assay

DITNC1 cells [200.000 cells] were seeded over 12 mm glass coverslip overnight for IIF, or over 35 mm cell culture plate [Corning] for FACS or WB. Cells were serum deprived for 30 min prior stimulation. We used magnetic beads [4.2 µm diameter] covered with Protein A and coated with Thy-1-Fc [5.8 µg of Thy-1 in 0.25% beads; Total volume 20µL]. The beads were left to bind to the surface of astrocytes [5 min] and then pulled with a neodymium permanent magnet [N42 grade; K & J Magnets]. After different times of magnetic force application [5, 10, 15, 30 and 45 min], the cells were prepared for FACS, IIF or WB.

Traction Force Microscopy

Tension gauge tether [TGT], a 75 x 25 mm glass slide was rinsed in Ethanol 3 times and sonicated with ethanol for 30 min and then sonicated again in nanopure water [18.2 $M\Omega cm^{-1}$] for 10 min. The cleaned slides were then treated with fresh piranha solution [7:3 v/v = H_2SO_4: H_2O_2], mixed and then used to clean the substrates for 30 min. Afterwards, the substrates were rinsed with plenty of nanopure water and then, rinsed with ethanol to remove excess of water and to further clean the substrates. Subsequently, 3% v/v [3-Aminopropyl] trimethoxysilane [APTMS] solution in ethanol was added to the slides and incubated for 2 h. After that, surfaces were rinsed with ethanol and dried in an oven set at 80°C for 30 min. Dried surfaces were incubated with biotin [2 mg in 1 mL anhydrous DMSO] over parafilm in a Petri dish at room temperature, overnight. The coverslips were then rinsed in ethanol and dried under a stream of N_2. Surface were mounted in a microscope chamber and cleaned with PBS. The surface was incubated with BSA 1% for 30 min, washed with PBS and then functionalized with 40 µg/mL of streptavidin in PBS. After 1 h incubation, the coverslips were rinsed in PBS to remove the unbound streptavidin, which was followed by the final modification with 40 µg/mL of DNA biotinylated ligands [cRGD] in PBS for 1 h. Unbound ligand was washed away with PBS and the surfaces were used within the same day of preparation.

RESULTS

Mechanical stress increases surface levels of αVβ3 Integrin achieving peak expression faster than with Thy-1 alone

Considering that the ECM is altered upon CNS damage increasing mechanical forces exerted on cells, we set out to study whether mechanical stress increases cell surface αvβ3 Integrin levels in astrocytes.

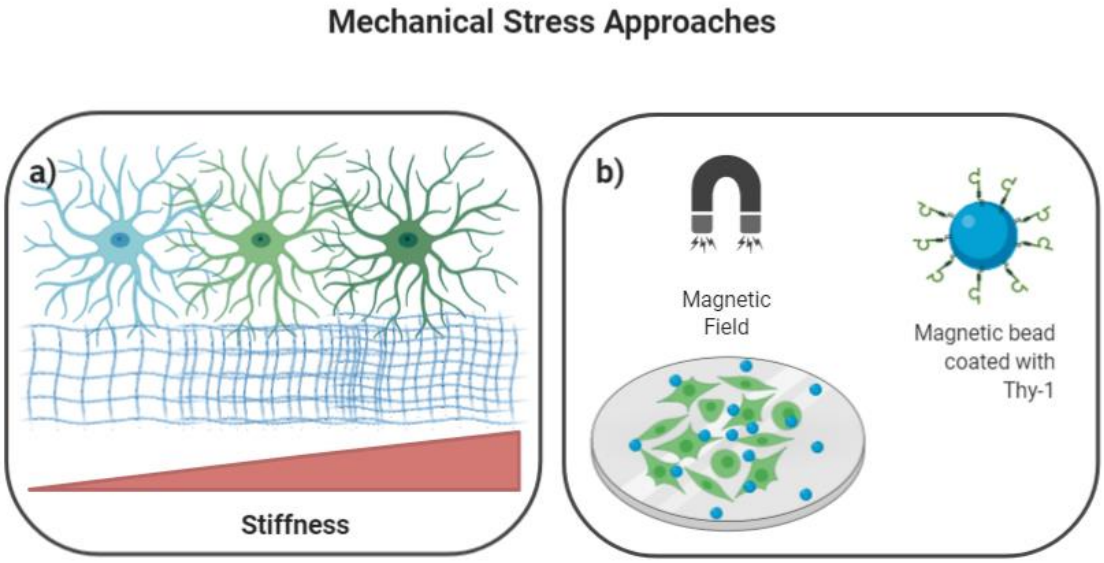

Figure 7. Two Different Mechanical Stress Approaches. *a) Astrocytes plated over different extracellular matrix stiffness, healthy ECM are soft [1 kPa], whereas after injuries, ECM become stiffer [30 kPa]. b) Astrocytes plated over glass are stimulated with Magnetic beads coated with Thy-1, then, mechanical stimulation is achieved applying a Magnetic field over astrocytes to pull the Integrin/Thy-1 interaction.*

As a first approach, DITNC1 cells were stimulated by plating them on rigid [30 kPa] or soft [1.5 kPa] ECM. ECM rigidity is controlled by coating

polyacrylamide hydrogels, containing varying acrylamide/bisacrylamide concentrations on amino-silanized coverslips, with ECM proteins, as previously described by Tse and Engler[68]. The role of Thy-1 stimulation was tested adding soluble Thy-1-Fc coupled to Protein A. Urbanski and coworkers showed that a stiffer ECM has a Joung's Modulus E= 30 kPa, similar to a scar of connective tissue, while soft ECM has Joung's Modulus E= 1.5 kPa, similar to healthy brain tissue[7]. However, after several attempts to obtain polyacrylamide hydrogels, containing varying acrylamide/bisacrylamide concentrations on amino-silanized coverslips or plates, it was impossible to achieve a smooth and even surface. We then obtained commercially available plates through Matrigen, [Brea, CA, USA]; however, after cells were plated over hydrogels with two different stiffness [soft and rigid], cells did not attach to the surface [not shown]. Even when the matrices were previously incubated with conditioned media from astrocytes, cells only poorly attached to the substrate, presented slow growth, and differences in morphology. [Figure 8]

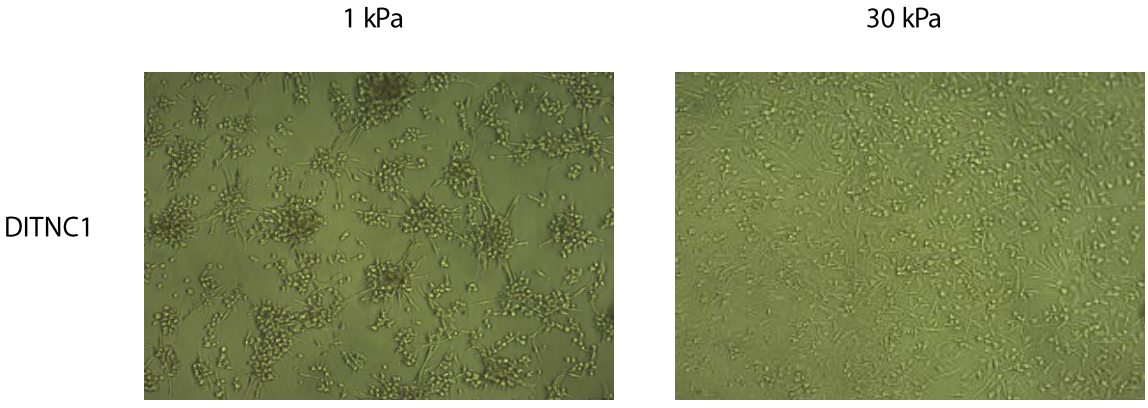

Figure 8. DITNC1 cells plated over matrices with 1 and 30 kPa of stiffness. *Cells [250000] plated in different substrate stiffness needed a long time [at least 96 h] in culture to achieve the*

confluence shown in these pictures. In addition, cells grown in 1 kPa stiffness showed poor attachment to the substrate, formed aggregates due to self-attachment and grew very slow.

Then, as a different mode to apply mechanical stress, we use magnetic beads covered with Protein A and coated with Thy-1-Fc. The beads were left to bind to the surface of astrocytes and then pulled with a neodymium permanent magnet [N42 grade; K & J Magnets]. After different times of magnetic force application [5, 10, 15, 30 and 45 min], the cells were prepared for cell cytometry to measure the surface levels of $\alpha_V\beta_3$ Integrin. In the cell cytometry analysis, we first selected the cell population by volume and rugosity [Figure 9 a]. This selection was the same for all samples. The different labels, which were used to identify $\alpha_V\beta_3$ Integrin and the isotype control were thus monitored [Figure 9 b]. We then tested the response of cells to Thy-1 stimulation alone [Figure 10 a], and to Thy-1 plus mechanical stimulation [Figure 10 b]. TRAIL-R2-Fc coupled to magnetic beads-Protein A were used as a negative control. Upon mechanical stimulation, we found higher surface levels of $\alpha_V\beta_3$ Integrin compared with Thy-1 stimulation alone, and observed that the integrin surface levels peaked with mechanical stimulation at shorter time points than Thy-1 alone [15 and 45 min, respectively] [Figure 10 a and b]. The surface levels of $\alpha_V\beta_3$ Integrin under negative control treatment remained similar to basal levels. These results indicate that mechanical stimulation of Thy-1-engaged receptors makes the $\alpha_V\beta_3$ Integrin to appear faster at the astrocyte surface than when Thy-1 is used alone.

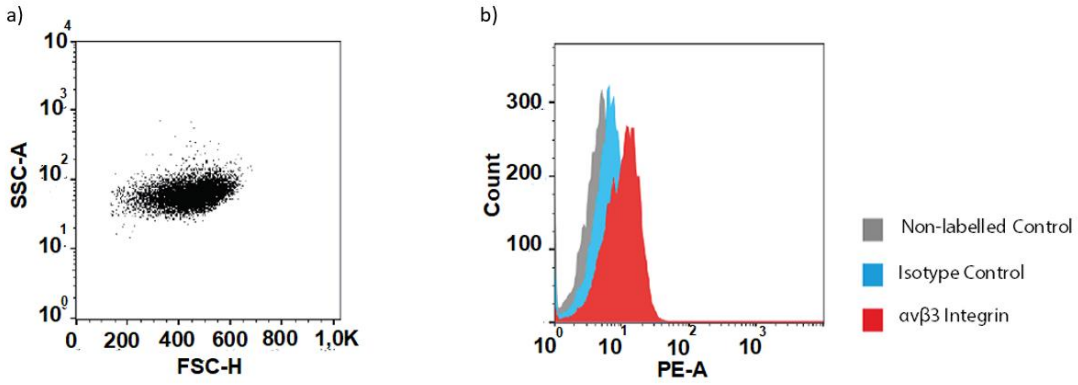

Figure 9. Cell population selection and labelling control. *a) Left panel shows cell population selected for all samples according to volume and rugosity of the cells. Right panel shows 3 populations: non-labelled control [gray], isotype control [cyan] and αVβ3 Integrin/Phycoerythrin [red].*

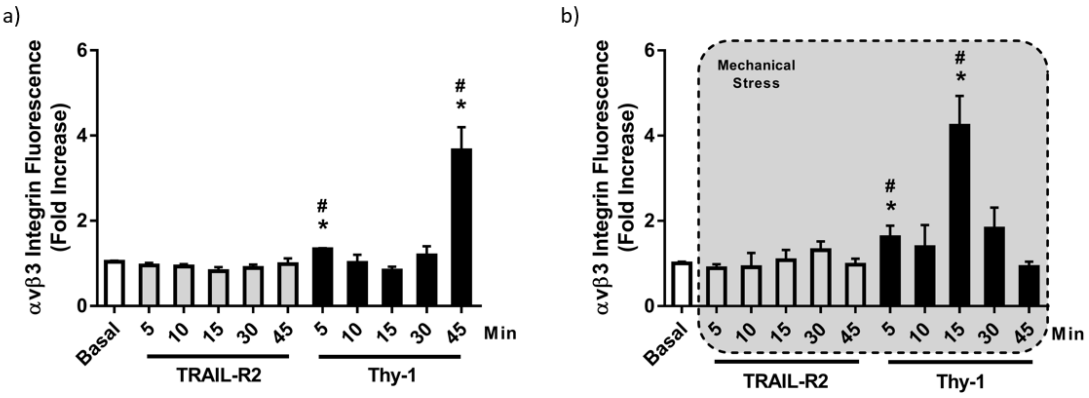

Figure 10. Thy-1 plus mechanical stress increase surface levels of αVβ3 Integrin faster than Thy-1 alone. *DITNC1 cells stimulated with Thy-1 only (a) or with Thy-1 and Mechanical Stress (b). Cells were incubated in serum-free medium for 30 min and then stimulated with magnetic beads coated with Thy-1 or treated with TRAIL-R2-coated magnetic beads without (a) or with (b) mechanical forces exerted with a magnet for 5, 10, 15, 30, and 45 min. Values in the graphs are the mean fluorescence intensities normalized to the control signal [Basal], TRAIL-R2-Fc beads were used as negative controls. Bars represent mean ± s.e.m. [n=3]. MS, Mechanical Stress; **

*and #, p<0,05. * comparison with basal value, # comparison with the same time with or without Mechanical Stress.*

Increased surface levels of αVβ3 Integrin induced by Mechanical stress are regulated by Syndecan-4.

A role for Syndecan-4 phosphorylation on Y180 in regulating β1 or β3 Integrin expression levels has been reported in fibroblasts as a consequence of binding to FN[44]. Considering that both $\alpha_V\beta_3$ Integrin and Syndecan-4 are receptors for Thy-1 in astrocytes, a possible role for Syndecan-4 on the $\alpha_V\beta_3$ Integrin surface levels in astrocytes was studied by silencing Syndecan-4 or overexpressing different Syndecan-4 plasmids. Transfections were performed using nucleofector [AMAXA technology] to silence or overexpress Syndecan-4. The Syndecan-4 mutants: Y180L-Syndecan-4 [non-phosphorylatable] and Y180E-Syndecan-4 [phosphomimetic] were also used [kindly donated by Dr. Mark Morgan, University of Liverpool, UK]. Both lack of expression and overexpression of Syndecan-4 were monitored by Immunoblot. Results obtained show a moderate decrease [44 %] of expression of Syndecan-4 with the siRNA, and a high increase [526 %] with the overexpression of Syndecan-4 wild type [Figure 11 a]. The participation of Syndecan-4 in the translocation of $\alpha_V\beta_3$ Integrin to the cell surface was assessed by altering Syndecan-4 expression and stimulating with Thy-1 or Thy-1+mechanical stimulation for 15 min. Interestingly, the silencing and overexpression of Syndecan-4 showed a decrease and increase, respectively, of $\alpha_V\beta_3$ Integrin surface levels in astrocytes stimulated with Thy-1 alone and Thy-1

plus mechanical stress [Figure 11 b]. The results obtained with Syndecan-4 Y180 mutants show that the non-phosphorylatable Syndecan-4 decreases the $\alpha_V\beta_3$ Integrin surface levels, while the phophomimetic form of Syndecan-4 seems to increase its levels. Although these results suggest that Syndecan-4 plays a role in regulating the surface levels of αVβ3 Integrin in astrocytes, further experimentation is required to determine whether values obtained are significantly different, given the fact that the numbers in the graph represent the average and standard deviation of two independent experiments.

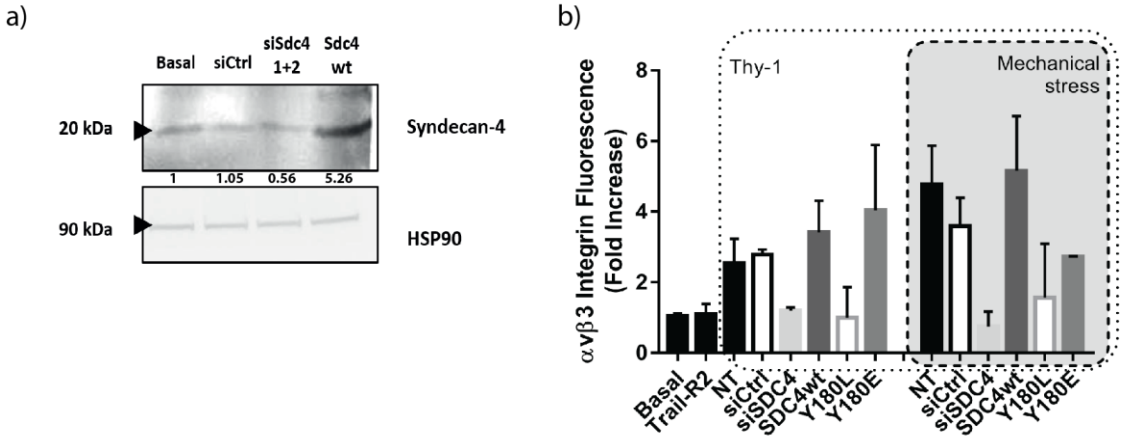

Figure 11. Syndecan-4 regulates αVβ3 Integrin surface levels in astrocytes. *Syndecan-4 protein levels. DITNC1 cells were transfected with a mix of two different siRNAs targeting Syndecan-4 to silence or a plasmid of the wild type Syndecan-4 to overexpress the protein. As a control for silencing, we used siRNA control (siCtrl). Cell extracts were subjected to immunoblot with anti-Syndecan-4 antibody, while HSP90 was used as a loading control. b) DITNC1 cells non-transfected [NT], or transfected with silenced Syndecan-4, overexpressing WT Syndecan-4, or Syndecan-4 mutant proteins [Y180L non-phosphorylatable, Y180E phosphomimetic] were stimulated with Thy-1 with or without mechanical stress for 15 min. Cells were treated as described in b). Values in the graph are the mean fluorescence intensities of $\alpha_V\beta_3$ Integrin/Phycoerythrin normalized to basal levels without stimuli. Bars represent mean ± s.d. [n=2]. MS, Mechanical Stress.*

Phosphorylation of Myosin Light Chain after Thy-1 stimulation and mechanical stress.

Our laboratory has described a role for inflammation and astrocyte reactivity on $\alpha_V\beta_3$ Integrin levels[69]. Particularly, TNF treatment of primary astrocyte cultures increases β3 Integrin expression levels and only TNF-treated cells respond to Thy-1, increasing focal adhesion and stress fiber formation, as well as cell contractility and migration[14]. However, the role of mechanical stress on astrocyte contractility induced by Thy-1 has not been studied.

To test cellular contractility, we measured phosphorylation levels of MLC by immunoblotting. After different times of magnetic force application [5, 10, 15, and 20 min], we found that stimulation with Thy-1 or Thy-1 plus mechanical stimulus increased levels of MLC phosphorylation on serine 19 compared with basal levels found under non-stimulated conditions and with the negative control TRAIL-R2. In addition, mechanical stress accelerated the MLC phosphorylation and the phosphorylation peak was displaced from 10 to 5 min [Figure 12 a and b]. With the idea of testing the role of Syndecan-4 on cellular contractility, we tested the expression levels of total MLC and the phosphorylated MLC when overexpressing or silencing Syndecan-4. Transfections and cell culture were performed as for Aim 1. We found that basal levels of phospho-MLC and total MLC were unaltered with these transfections [Figure 13 a and b]. However, the effect of Thy-1 with or without mechanical stimulation in cells transfected with the

different plasmids has not been performed yet. We expect that astrocytes transfected with Y180E and Syndecan-4 overexpression show higher levels of MLC phosphorylation compared with non-transfected cells; on the other hand, those cells transfected with Y180L or the siRNA of Syndecan-4 are expected to show levels of MLC phosphorylation similar to non-stimulated, non-transfected cells.

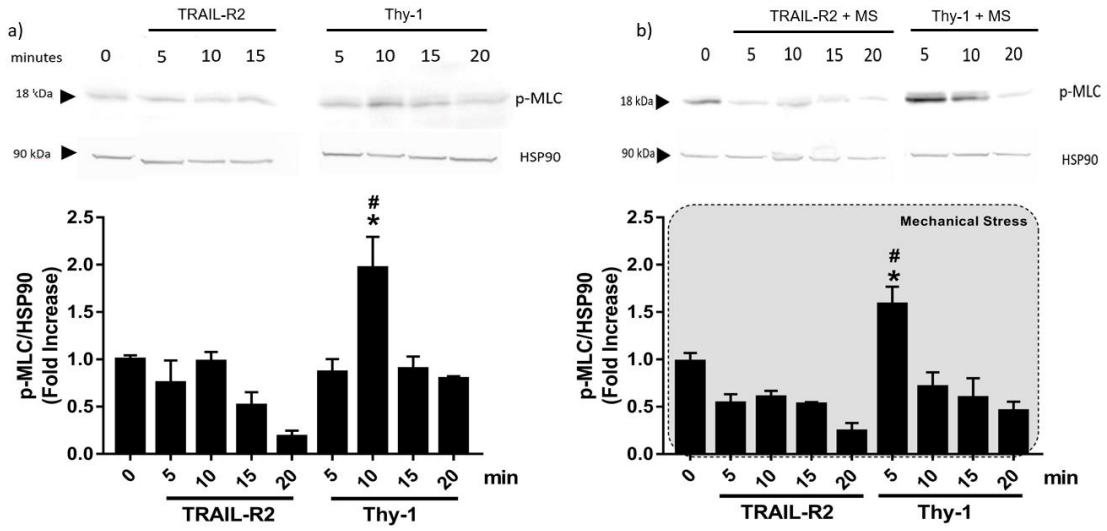

Figure 12. Phosphorylation of Myosin Light Chain after Thy-1 stimulation with or without mechanical stress. *a) DITNC1 cells stimulated with Thy-1. Cells were incubated with serum-free medium for 30 min, and stimulated with magnetic beads coated with Thy-1 or TRAIL-R2 as negative control for 5, 10, 15, and 20 min. Values in the graph correspond to densitometric analysis of band intensity of phospho-Serine 19 Myosin Light Chain. The numbers were normalized to Heat Shock Protein 90 [HSP90] values. b) DITNC1 cells stimulated with Thy-1 plus mechanical stress. Cells were treated under the same conditions as in panel A plus the application of mechanical forces exerted by a magnet using the same time points utilized in (a). * and #, p<0,05. * compared with basal at T=0, # compared with value obtained at the same time with TRAIL-R2.*

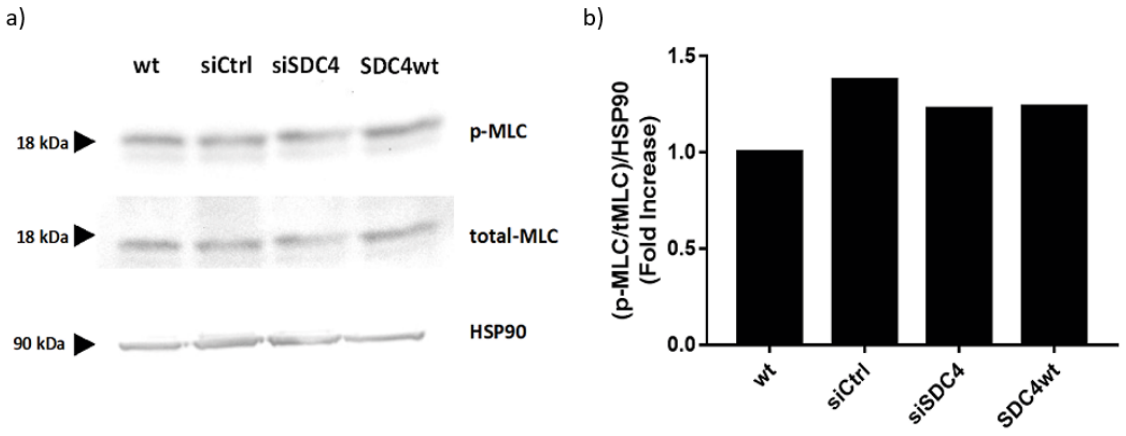

Figure 13. Expression levels of Syndecan-4 does not affect total or phosphorylated levels of Myosin Light Chain. *a) DITNC1 cells transfected with Syndecan-4 wt and siRNA Syndecan-4. Cells were incubated with serum-free medium for 30 min, and lysed with SDS-sample buffer and inhibitors. b) Values in the graph are the densitometric analysis of band intensity of phospho-Serine 19 Myosin Light Chain and total Myosin Light Chain normalized to Heat Shock Protein 90 [HSP90]*

A different mode of measuring global mechanical (e.g. viscoelastic) properties of cells is by using atomic force microscopy [AFM, collaboration with Dr. Nelson Barrera Lab]. AFM offers the advantage that it measures changes in force and space of cells in a tissue culture plate, at a nanoscale resolution[70]. However, AFM assays were performed without CO_2 control of the culture conditions; therefore, to avoid pH changes over time, we changed the regular culture medium to KRH buffer. Thus, as a control for these AFM assays, we tested the cytotoxicity [lactate dehydrogenase, LDH kit] and death [Propidium Iodide] of KRH buffer over time. We found that after 120 minutes, Propidium Iodide staining and LDH levels were <10% and <1%, respectively [Figure 14 a and b], indicating that KRH buffer could be used to incubate cells during AFM assays. However, the

time required to obtain the scan of one single cell takes approximately 20 minutes. Thus, considering that the changes that we describe in cell contractility occur at time points earlier than 20 minutes, AFM seems unsuitable to measure these changes in our settings [Figure 14 c]

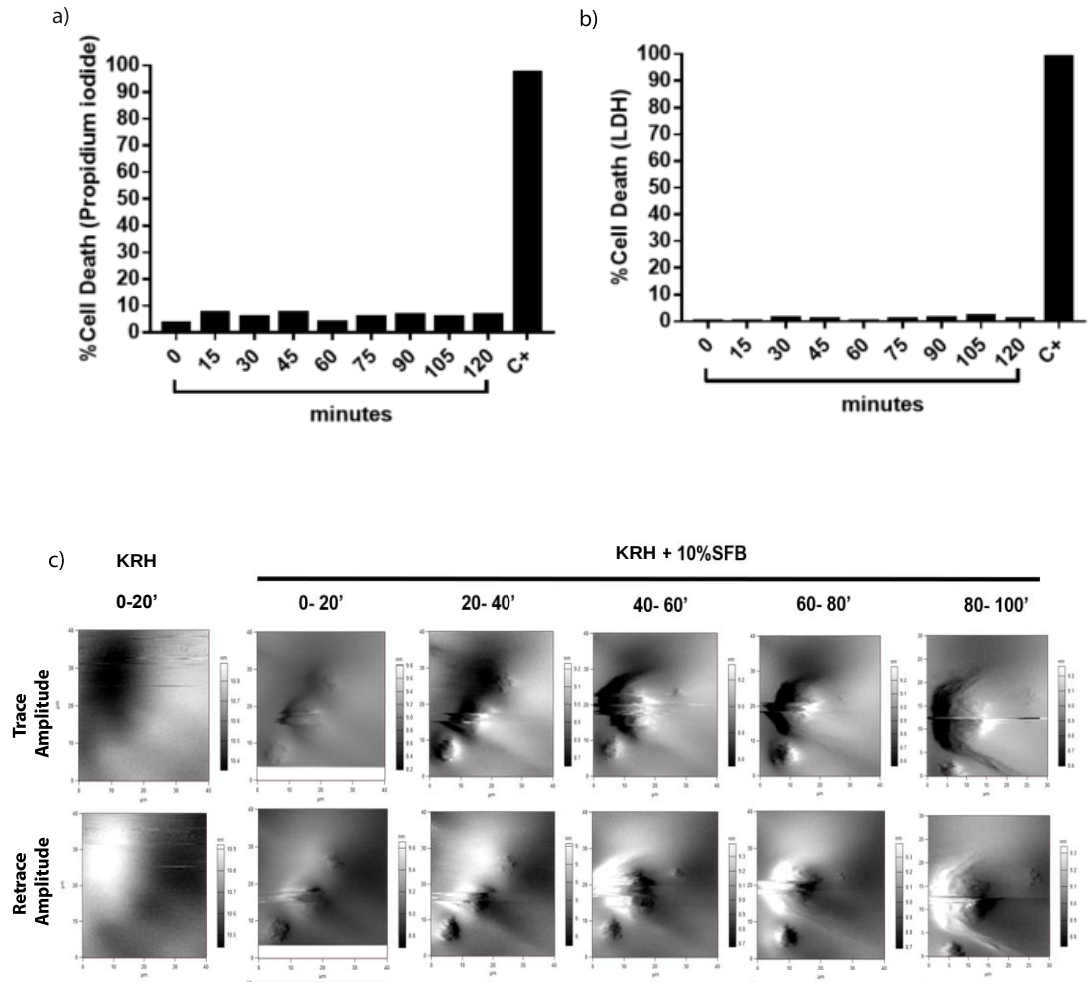

Figure 14. Cell death of cells incubated with KRH buffer and Contractility kinetics with Atomic Force Microscopy. *DITNC1 cells were incubated with KRH buffer for 120 minutes. Cells were trypsinized and incubated with Propidium Iodide and analyzed by FACS and supernatants were collected and analyzed with Lactate Dehydrogenase kit. a) and b) values represent percentage of cell death during 120 minutes. As a positive control, cells underwent a temperature shock, heat and freezing. c) DITNC1 cells in KRH buffer, and scanned for 120 minutes, 10% fetal bovine serum was added after 20 minutes as a positive control. X and Y axis are µm.*

Mechanical Stress increases the number but not the area of Focal Adhesions and are regulated by Syndecan-4

Cell contractility is characterized by changes in cellular shape and morphology. In reactive astrocytes, Thy-1 stimulation triggers cell contractility increasing focal adhesion and stress fiber formation[36] and after Thy-1 stimulation and mechanical stress, the peak of contraction [MLC phosphorylation] is faster [5 min] than with Thy-1 stimulus alone [Figure 12]. Therefore, we evaluated focal adhesions and stress fibers by indirect immunofluorescence [IIF], staining with anti-phospho-tyrosine pAb to label focal adhesions, which are enriched in proteins phosphorylated on tyrosine. We also tested stress fiber formation with FITC-conjugated phalloidin.

As basal levels, we used the number of focal adhesions per cell of astrocytes transfected with siRNA control [siCtrl, black bars], which is a scrambled sequence of Syndecan-4 siRNA. We transfected cells with siRNA of Syndecan-4 [white bars], overexpression plasmid [dark gray bars], or non-phosphorylatable and phosphomimetic mutants of Syndecan-4 [light gray bars]. Basal levels of FA and those after treatment with TRAIL-R2-Fc averaged approximately 40±6.7 FA/cell and no significant differences were found among these values [Figure 16 a]. At 5 min of stimulation, Thy-1 only treatment was insufficient to increase the number of FA in siCtrl, or in Syndecan-4 siRNA transfected conditions; however, upon Syndecan-4 overexpression and phosphomimetic mutant [Y180E], Thy-1 significantly increased the number of FA per cell, compared with siCtrl [under Thy-

1 stimulation] and with Y180E [under basal conditions], respectively [Figure 16 a, and Figure 15]. In addition, a significant increased number of FA was observed with the mechanical pulling of magnetic beads coated with Thy-1 at 5 min of stimulation in siCtrl condition [Figure 16 a] and also in those cells overexpressing Syndecan-4 or the phosphomimetic Y180 Syndecan-4. The significant increase in FA number was observed when compared with the same stimuli under basal conditions; while siRNA of Syndecan-4 and its non-phosphorylable mutant did not show significant changes compared with its respective controls under basal or TRAIL-R2 conditions [Figure 16 a]. Interestingly, as the number of FA increased with mechanical stress stimulation, the size of these FA showed a tendency to decrease in almost all conditions [Figure 16 b]. Focal adhesions are always at the tip of a stress fiber [SF] accounting for its association with cell contractility[71]. SF alignment (coherence), thickness (robustness) and number have been shown to be associated to cell stiffness and they can be quantified through confocal microscopy combined with image processing. Accordingly, SF number increased in a proportion similar to that observed for FA under Thy-1 and Mechanical Stress stimuli. Additionally, this increase was significantly higher than that found at basal levels. On the other hand, Thy-1 alone also increased the SF number, but not as much as with the Thy-1/mechanical stimuli, yet enough to be statistically different compare to basal levels [Figure 17 and 18 b]. Moreover, SF robustness and coherency also showed a statistically significant increase in Thy-1+mechanical stress-stimulated cells compared to controls [Figure 17 and Figure 18 a and c].

Interestingly, although Thy-1 stimulation increase these robustness and coherency parameters, the values were not high enough to be statistically significant [Figure 17 and Figure 18 a and c], suggesting that cell stiffness is achieved better when both Thy-1 and mechanical stress are applied together. SF data was analyzed in collaboration with Dr. Andrés Rodríguez.

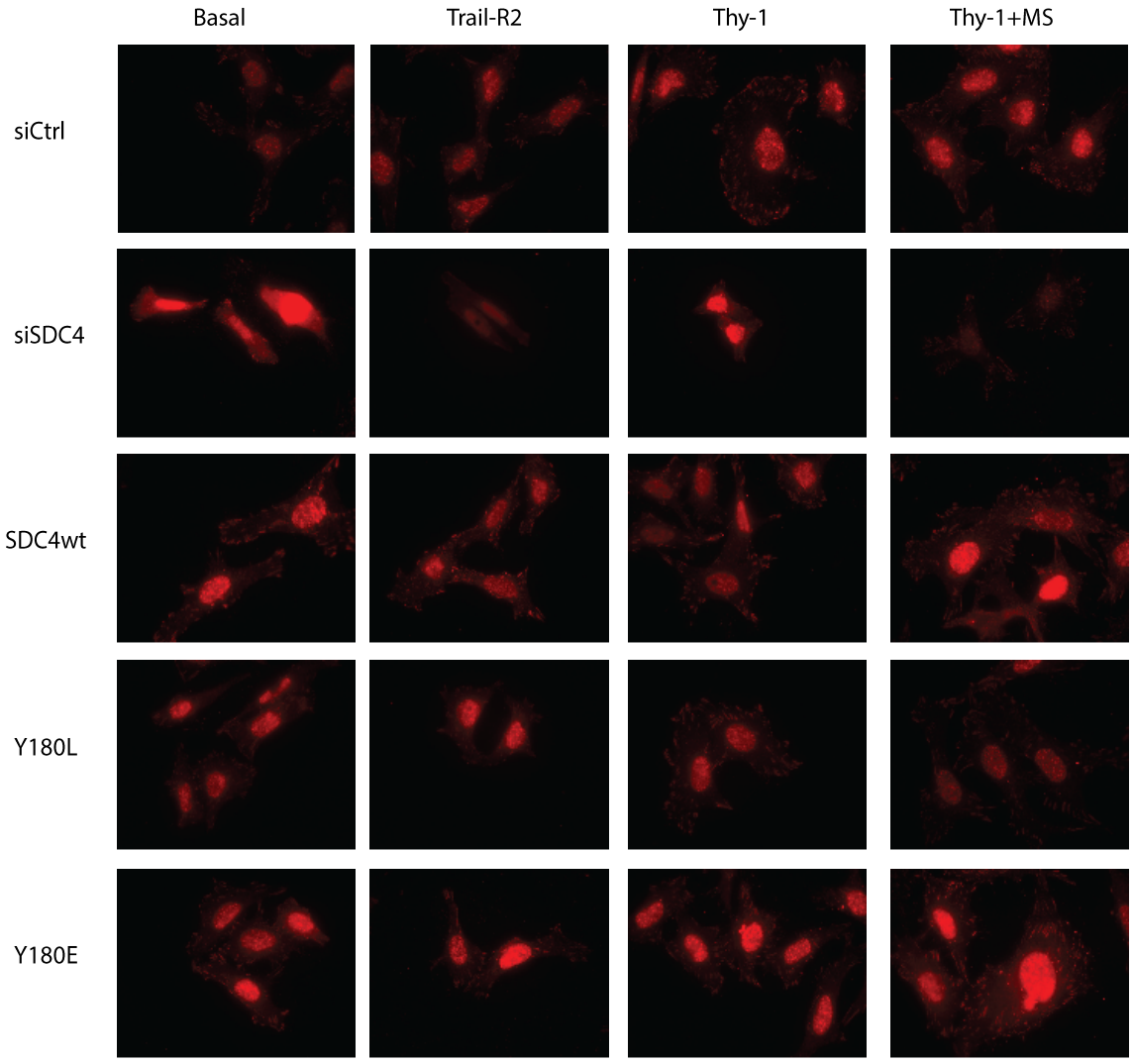

Figure 15. Indirect ImmunoFluorescence of Focal Adhesions in DITNC1 cells. *Cells were plated over coverslips and incubated with serum-free medium for 30 min. DITNC1 cells were stimulated with Thy-1 or mechanically stimulated with magnetic beads coated with Thy-1 or TRAIL-R2 as negative control for 5 minutes. Focal Adhesions [FA] were stained with antibody against phospho-tyrosine [red]. Cells were transfected with siRNA control [siCtrl], siRNA Syndecan-4 [siSDC4], overexpression of Syndecan-4 wild type [SDC4wt], non-phosphorylatable tyrosine 180 of Syndecan-4 [Y180L] or phosphomimetic tyrosine 180 of Syndecan-4 [Y180E].*

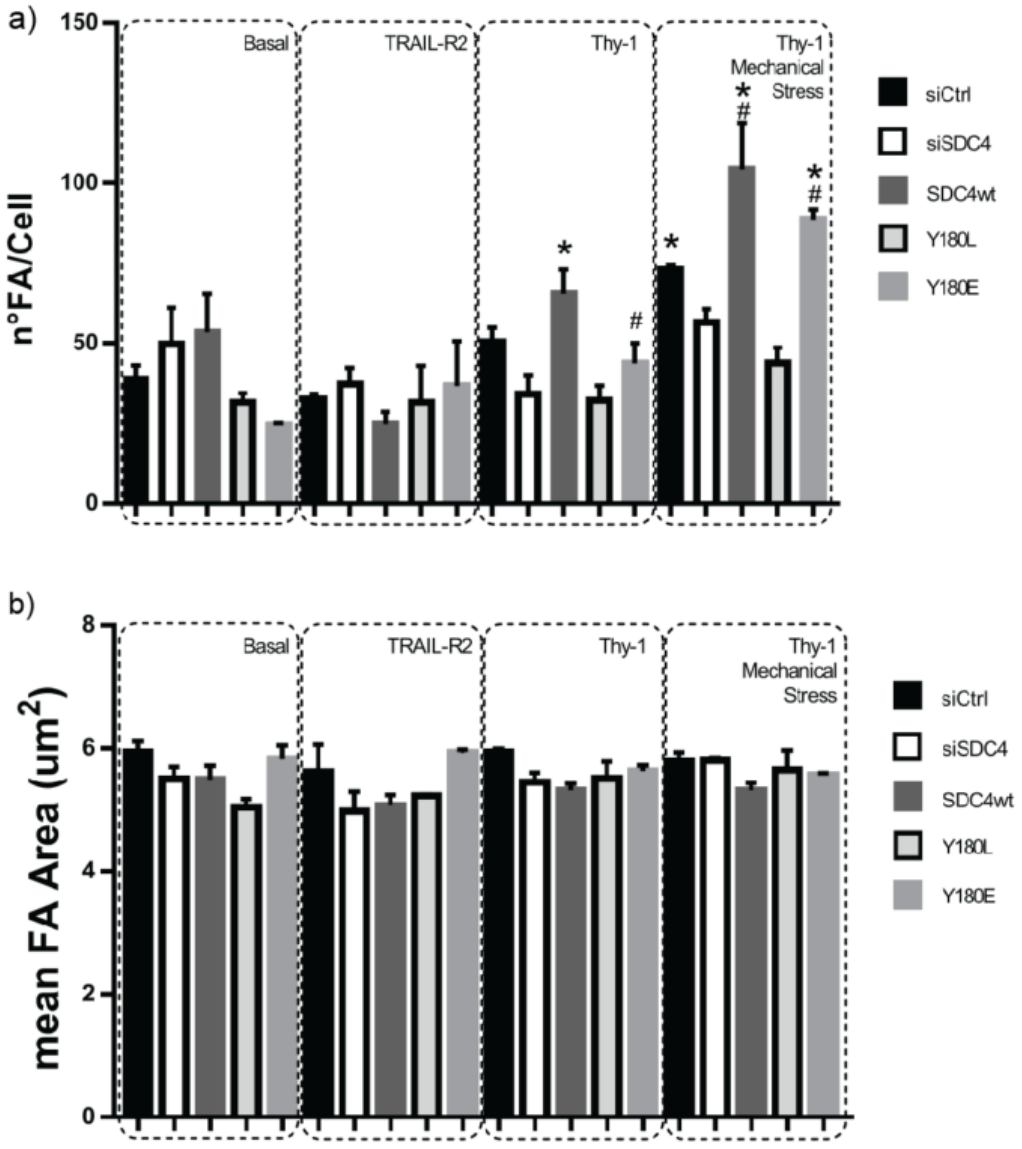

Figure 16. Mechanical Stress increases the number but not the area of Focal Adhesions and is regulated by Syndecan-4. *DITNC1 cells were plated over coverslips and incubated with serum-free medium for 30 min. Cells were then stimulated with magnetic beads coated with Thy-1 or TRAIL-R2 as negative control for 5 minutes with or without mechanical stress. Values in the graph are a) the number of Focal Adhesions [FA] per cell and b) the mean of FA area. Cells were transfected with siRNA control [siCtrl], siRNA targeting Syndecan-4 [siSDC4], a plasmid to overexpress Syndecan-4 wild type [SDC4wt], the non-phosphorylatable tyrosine 180 of Syndecan-4 [Y180L], or the phosphomimetic tyrosine 180 of Syndecan-4 [Y180E]. Bars represent mean ± s.e.m. [n=3]. MS, Mechanical Stress; # and * p<0.05, * compared with siCtrl in basal conditions and # compared with value obtained with cells transfected with the same plasmid, at basal levels.*

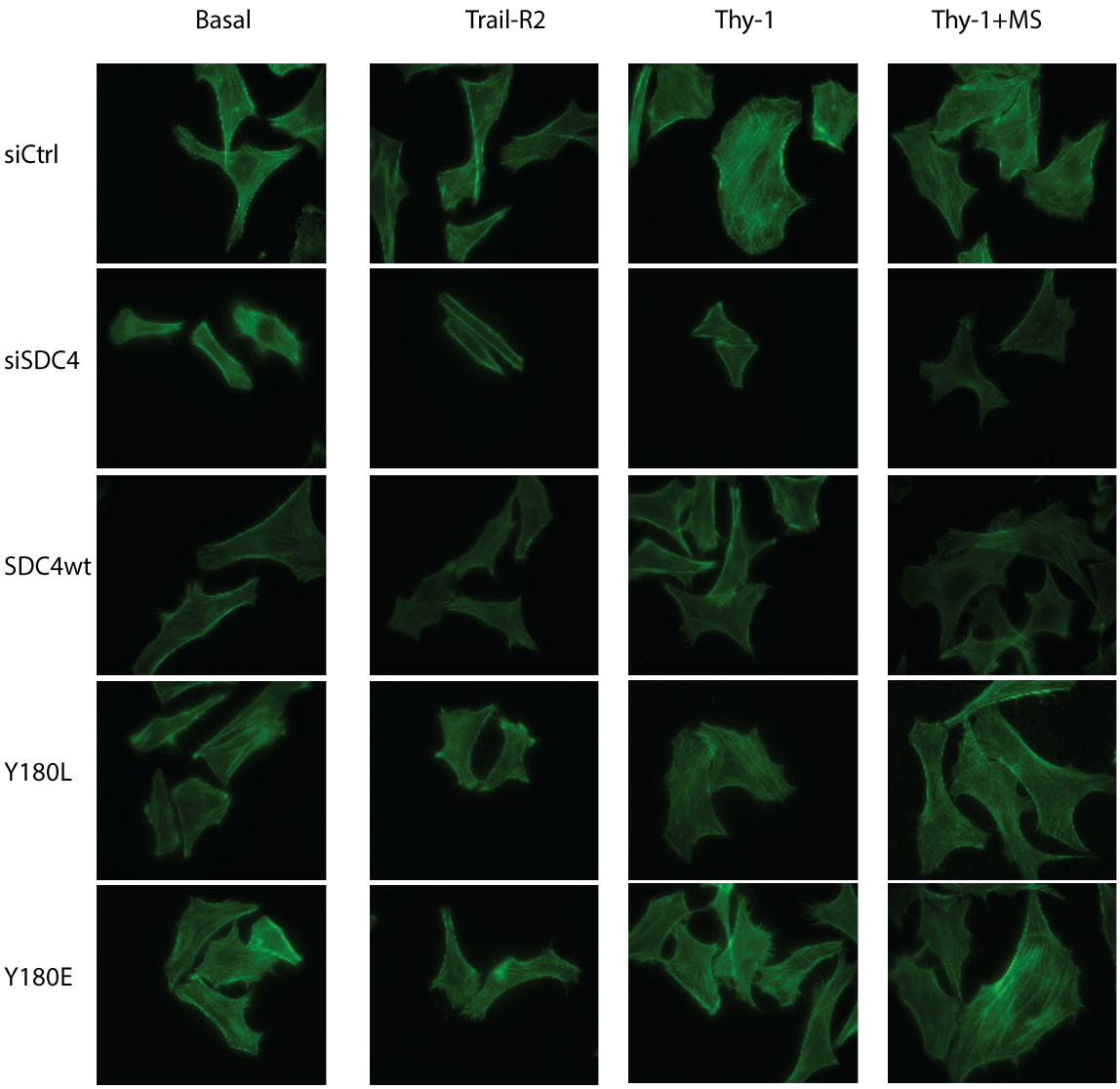

Figure 17. Indirect ImmunoFluorescence of Stress Fibers in DITNC1 cells. *Cells were plated over coverslips and incubated with serum-free medium for 30 min, and then stimulated with Thy-1 or mechanically stimulated with magnetic beads coated with Thy-1 or TRAIL-R2 as negative control for 5 minutes. Stress Fibers were labelled with phalloidin/FITC [green]. Cells were transfected with siRNA control [siCtrl], siRNA Syndecan-4 [siSDC4], overexpression of Syndecan-4 wild type [SDC4wt], non-phosphorylatable tyrosine 180 of Syndecan-4 [Y180L] or phosphomimetic tyrosine 180 of Syndecan-4 [Y180E].*

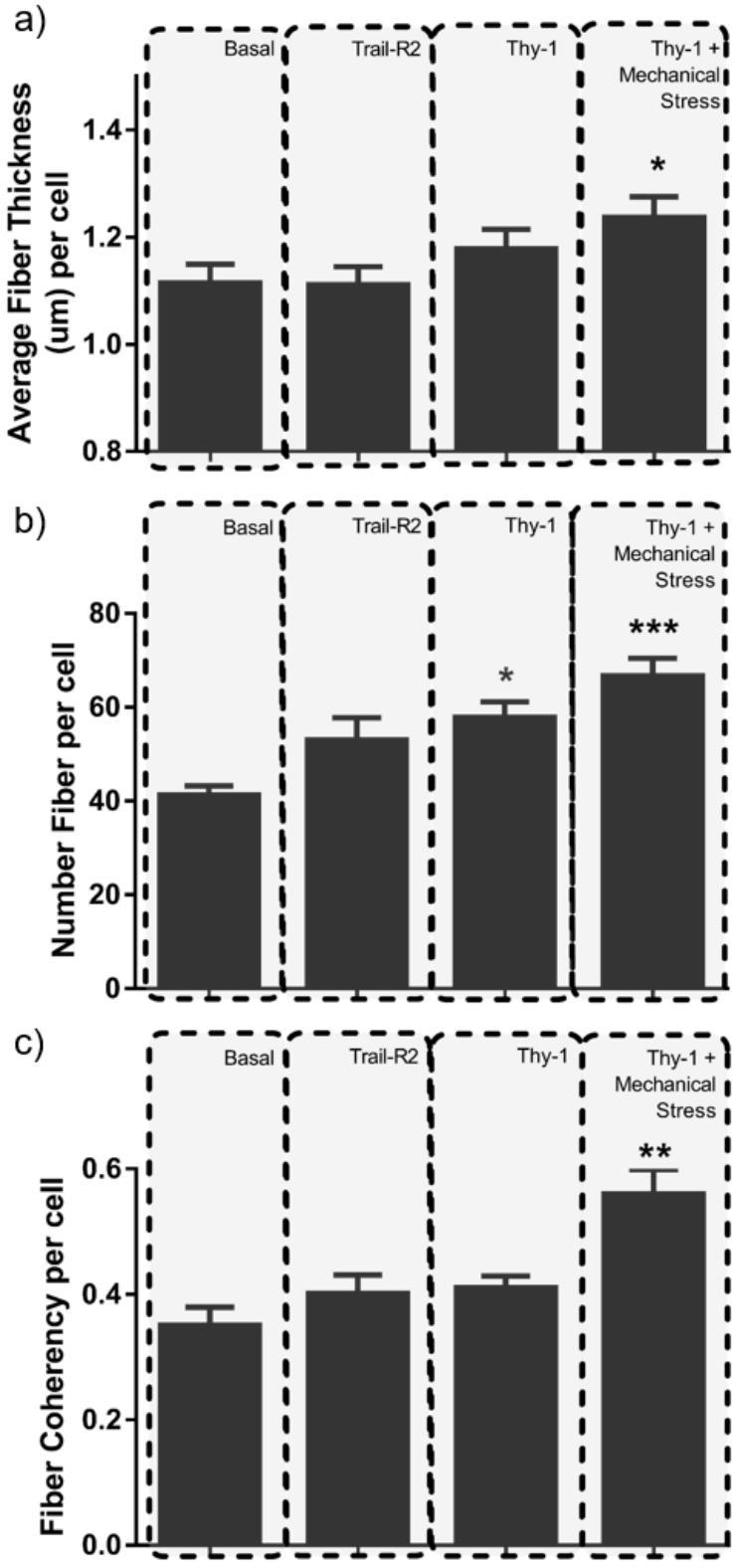
a)
Basal
Trail-R2
Thy-1
Thy-1 + Mechanical Stress
Average Fiber Thickness (um) per cell
1.4
1.2
1.0
0.8
*
b)
Basal
Trail-R2
Thy-1
Thy-1 + Mechanical Stress
Number Fiber per cell
80
60
40
20
0
*
***
c)
Basal
Trail-R2
Thy-1
Thy-1 + Mechanical Stress
Fiber Coherency per cell
0.6
0.4
0.2
0.0
**

Figure 18. Stress Fiber Formation after Thy-1 and Mechanical stress stimulation. *DITNC1 cells were plated over coverslips and incubated with serum-free medium for 30 min. Cells were then stimulated with magnetic beads coated with Thy-1 or TRAIL-R2 as negative control for 5 minutes with or without mechanical stress. Values in the graph are a) Average Thickness of Stress Fibers per cell, b) Number of Stress Fibers per cell and c) Stress Fiber Coherency per cell. Bars represent mean ± s.e.m. [n=3]. MS, Mechanical Stress; * p<0.05, ** p<0.01, *** p<0.0001; * compared with basal conditions.*

Taken together, these results indicate that enhanced mechanical stress leads to increased and faster cell contractility when Thy-1 is added. Also, that Syndecan-4 and its phosphorylation in tyrosine 180 are important for FA formation induced by Thy-1 and mechanical stress.

Astrocytes can sense and exert mechanical stress through Integrins in an outside-in and inside-out manner

Integrins are receptors that span the plasma membrane and anchor cells to the external environment. The glycocalyx, a pericellular sugar coat that surrounds the cell membrane and is attached to proteoglycans, glycolipids, and glycoproteins, is another important player in the cell/microenvironment interface that influences integrin properties[68,69,70,[72]. Its compression in the vicinity of integrin/substrate binding sites leads to mechanical loading of the integrins through force application towards the cell membrane. Furthermore. the compressed glycocalyx acts as a steric kinetic trap that impacts on lateral integrin diffusion and promotes integrin clustering[73].

In the experiments presented above, we report that mechanical stress applied through $\alpha_V\beta_3$ Integrin triggers an outside-in response in astrocytes that

lead to integrin availability and cellular contraction. However, the rearrangement of the cytoskeleton could trigger traction forces through integrins in an inside-out manner. To test this premise, we used Traction Force Microscopy, in collaboration with Dr. Khalid Salaita Lab, Emory University, Atlanta, USA. This technique allows us to measure traction forces exerted by astrocytes on the ECM, through integrins. As a first approach, we used the Hairpin probes, which measure the traction force exerted by integrins in real time and with piconewton resolution [Figure 19]. The probe also acts in a reversible manner [74, 75]. We used 4.7 pN Hairpin Probes to test the traction forces exerted by DITNC1 cells stimulated with magnetic beads coated with Thy-1 and mechanically stressed. Despite the advantages of using these probes, the tension signal obtained was weak. In addition, dark spots appear in the potential point of traction, which might be indicative of probe degradation [Figure 20].

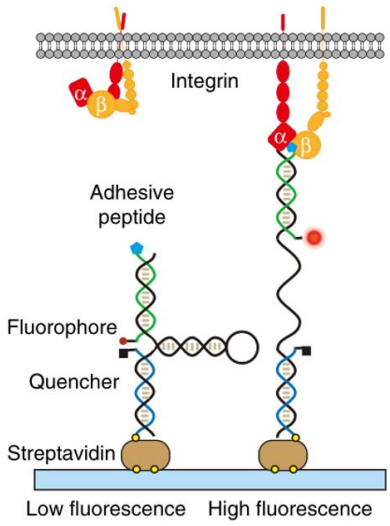

Figure 19. Hairpin Probe. *Schematic representation of the integrin tension sensor, which is comprised of an anchor strand immobilized onto a streptavidin-coated surface (light blue), a*

hairpin strand that unfolds under sufficient tension (black) and a ligand strand presenting an adhesive peptide (green). At the apposing termini of the ligand and anchoring strands, a fluorophore and quencher were coupled to report the force-induced by unfolding of the hairpin[75].

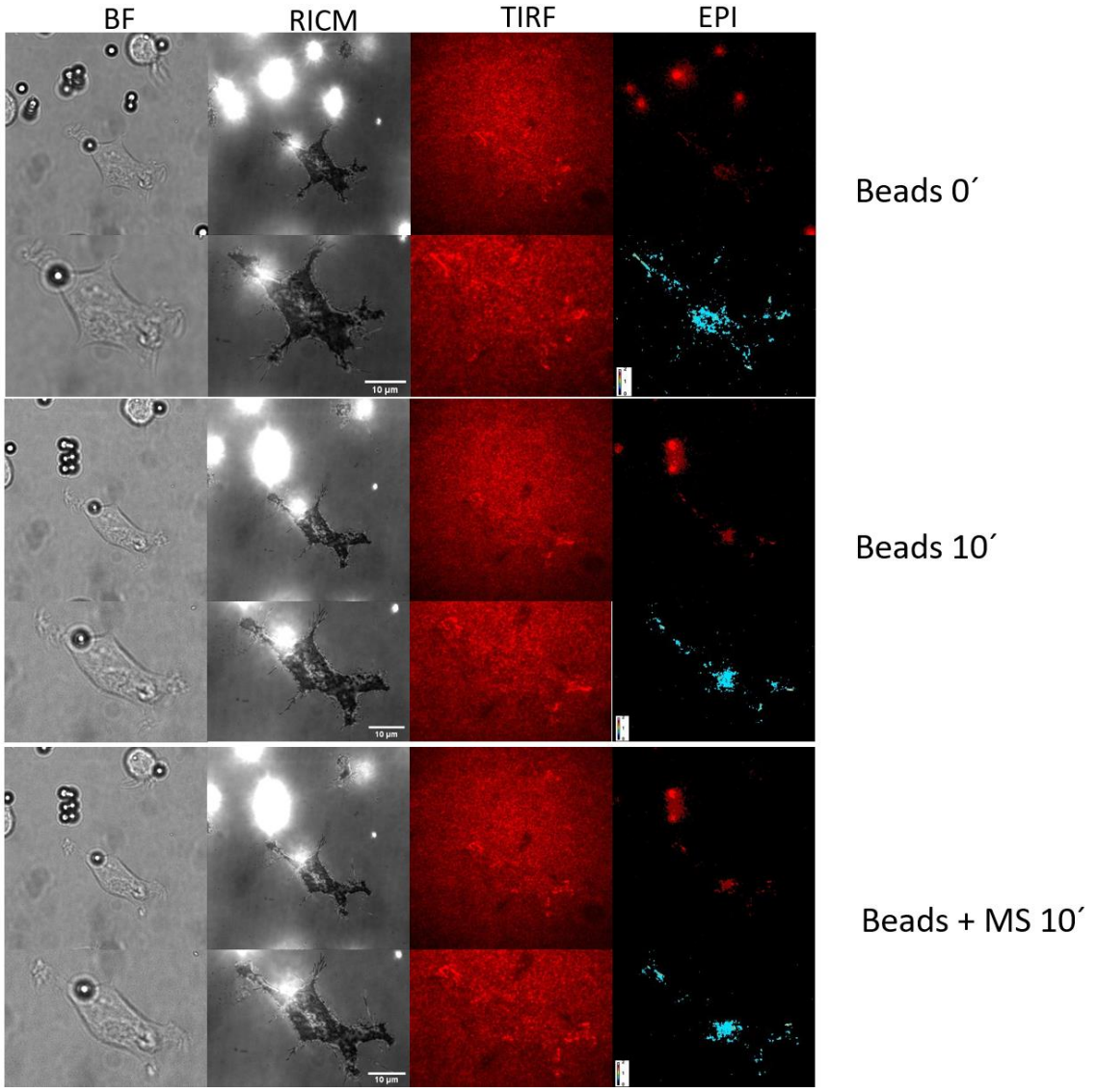

Figure 20. Traction Force Microscopy with 4.7 pN Hairpin probe of DITNC1 cells. *Representative images of DITNC1 cells over hairpin probes and stimulated with magnetic beads coated with Thy-1 and mechanical stress. Bright Field [BF], Reflection Interference Contrast Microscopy [RICM], Total Internal Reflection Fluorescence [TIRF] and Epifluorescence [EPI] channels. Normal [top row] and Magnification [bottom row] of each condition show dark spots in TIRF channels and a loose of tension signal in EPI channel that could correspond to DNA degradation by nucleases or disengagement of the probe.*

Thus, we decided to change the strategy and test a different probe, Tension Gauge Tether [TGT], which is an irreversible probe that senses traction force with an aperture threshold of 12 pN or 47 pN depending on the opening mode [unzipping or shearing mode, respectively]. To this end, we plated DITNC1 cells over both probes [12 and 47 pN]. Astrocytes adhered better [spread area and morphology] over 47 pN TGT probe [Figure 21]. Thus, we selected this probe for the subsequent experiments.

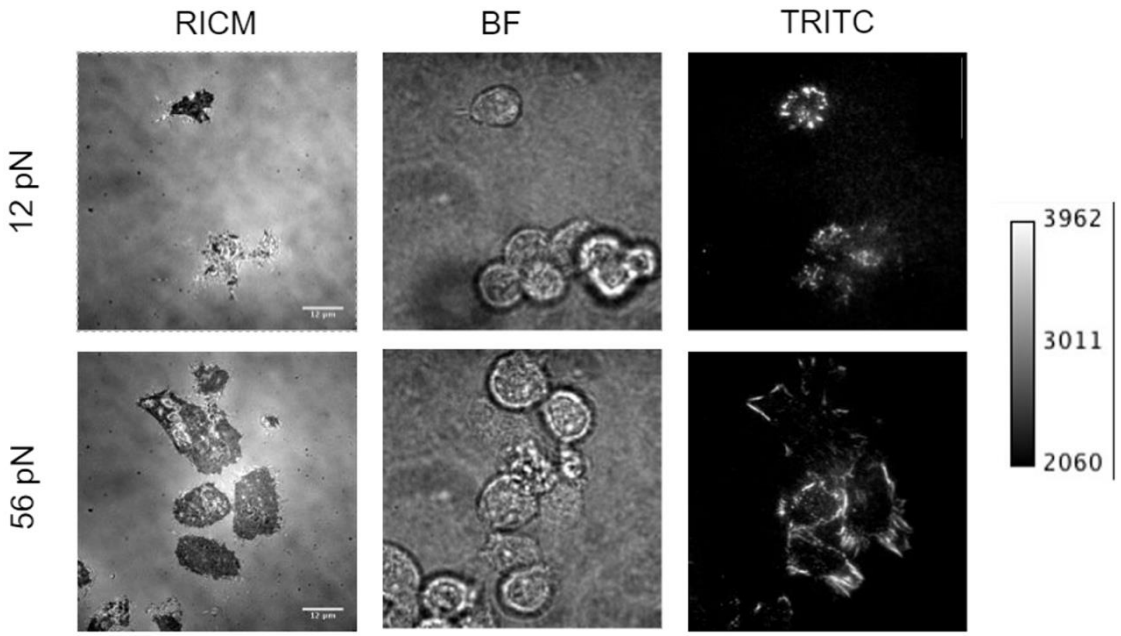

Figure 21. Traction Force Microscopy with 12 pN and 47 pN TGT probe of DITNC1 cells. *Representative images of DITNC1 cells over TGT probes. Bright Field [BF], Reflection Interference Contrast Microscopy [RICM] and Total Internal Reflection Fluorescence [TIRC] channels. The 47 pN TGT probe shows better cell spread, shape and tension signal.*

We then measured the traction force exerted by astrocytes through integrins after Thy-1 and mechanical stress stimuli. DITNC1 cells were plated over 47 pN TGT probes and after 30 min in cell culture media supplemented with 1% of FBS we measured the basal tension levels. Then, we added magnetic beads coated with Thy-1 and after 5 min of stimulation, tension levels were measured. Only cells that had engaged beads were selected. Then, we applied a magnetic field with neodymium magnets for 5 min and measure tension forces in the same cells [Figure 22].

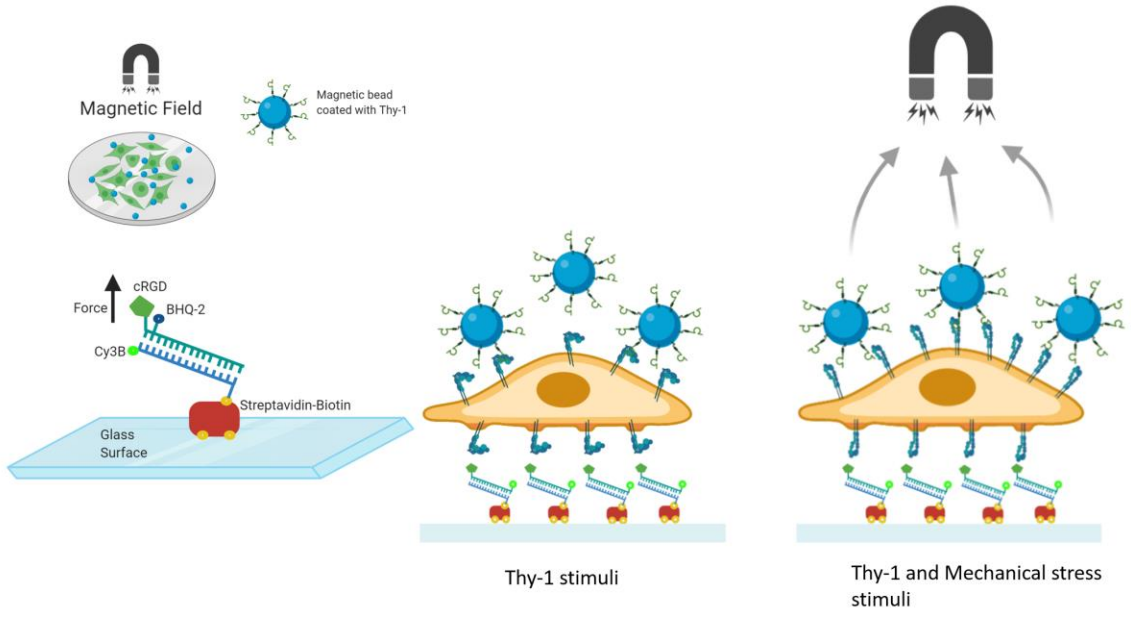

Figure 22. Traction Force Microscopy Scheme. *Upper left illustration shows DITNC1 cells plated over a coverslip and stimulated with magnetic beads coated with Thy-1 and under a magnetic field. The lower left illustration shows a magnification of a coverslip (light blue) that is functionalized with 47 pN TGT probe, with a biotin-streptavidin chemistry that links the lower DNA strand to the glass surface. The lower DNA strand contains a Cy3B fluorophore and the upper DNA strand, a Black Hole Quencher 2 and cyclic RGD peptide as a ligand in a shearing geometry. In the middle illustration, DITNC1 cell is shown stimulated only with Thy-1 beads on the top and the 47 pN TGT probe at the bottom. Right illustration shows the same diagram aforementioned with the addition of a magnetic field that pulls the Thy-1-beads.*

Astrocytes plated over TGT probes [Figure 23 a] showed a dark shape in Reflection Interference Contrast Microscopy [RICM] channel, while the bright spots correspond to magnetics beads [left column]. In Bright Field [BF], we selected only cells that were in contact with beads [middle column] and the TRITC channel corresponds to tension forces exerted by cells through integrins. Traction force measures indicate that 5 min of Thy-1 stimulation increases the force exerted by the cells through the integrins on the ECM substrate compared with basal levels [Figure 23 b]. When Thy-1 was applied for the same period of time but with the addition of mechanical stress, the increased traction force was even higher [Figure 23 b]. These results indicate that **integrins are able to transduce chemical and mechanical signals** in a **outside-in** through the interaction with Thy-1 and mechanical stressors, and **inside-out manner** exerting force from the cytoskeleton to the ECM.

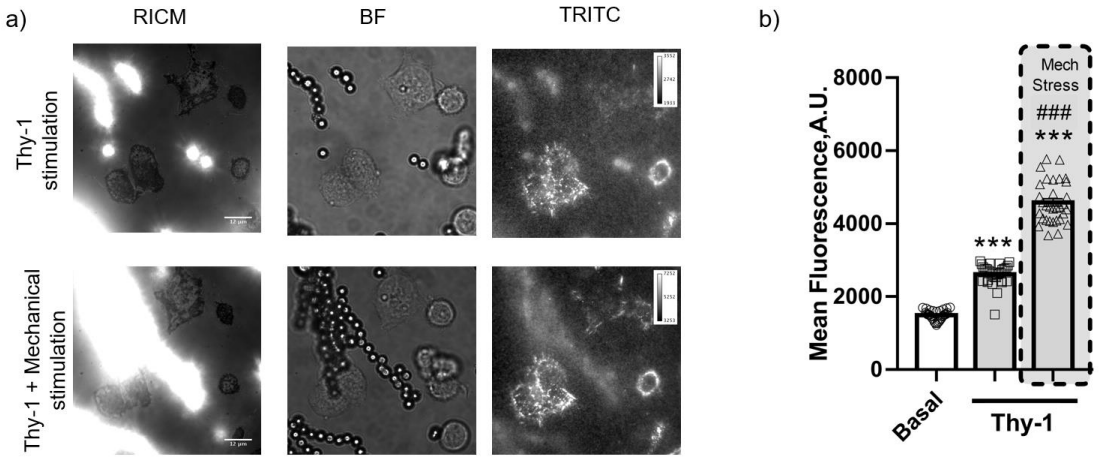

Figure 23. Traction Forces are increased after Thy-1 and Mechanical Stress stimulation in Astrocytes. *a) Representative images of DITNC1 cells stimulated with magnetic beads coated*

*with Thy-1 only [top row] or with Thy-1 plus Mechanical stress [bottom row]. Left column is the Reflection Interference Contrast Microscopy (RICM), Middle column shows Bright Field and right column, Total Reflection Internal Fluorescence of Traction Forces. b) quantification of mean fluorescence ± s.e.m. of three independent experiments of traction force for basal tension, Thy-1 alone and Thy-1 + Mechanical stimulation. ***p<0.001 compared with basal tension; ###p<0.001 compared with Thy-1 alone tension.*

In the present study, we have shown that Syndecan-4 plays an important role regulating integrin surface levels and also in the formation of the stress fibers and focal adhesions in astrocytes stimulated with Thy-1 and mechanical stress. However, the role of Syndecan-4 on cellular traction forces remain unclear. To test this, we used DITNC1 transfected cells with siRNA of Syndecan-4 and after 48 h, we repeated the aforementioned protocol and measure traction forces. Preliminary observations indicate that Thy-1 alone and with mechanical stress stimulation does not increase tension levels in astrocytes when compare with basal levels [Figure 24 a and b]. However, basal levels were high compared with previous results [Figure 23]. This experiment was performed only once, and the condition of basal levels in non-transfected cells was not included. Thus, although these results are promising and could indicate that Syndecan-4 is necessary for inside-out astrocyte response, more experimentation is required to prove this Syndecan-4 function.

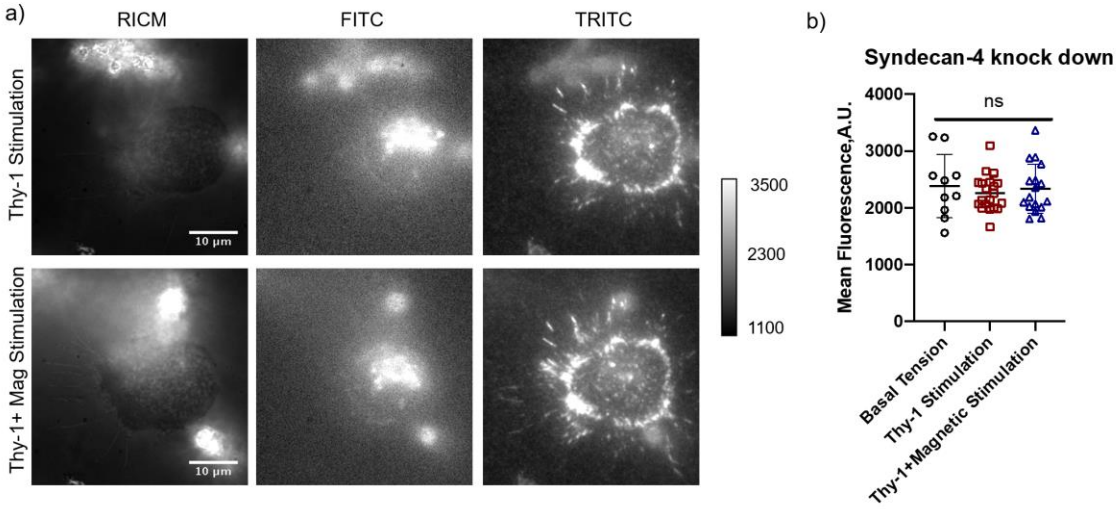

Figure 24. Traction forces in Syndecan-4 knockdown DITNC1 cells. *a) DITNC1 Syndecan-4 knockdown cells co-transfected with cell-glow. Left column shows Reflection Interference Contrast Microscopy (RICM), Middle column shows FITC channel to identify transfected cells, and Total Reflection Internal Fluorescence of Traction Forces. b) Quantification of mean fluorescence intensity of traction forces for basal tension, Thy-1 alone and Thy-1 + Mechanical stimulation.*

Taken all data together, our results show that astrocytes sense, process, and respond to mechanical cues through integrins in a Syndecan-4-dependent manner. These findings are important because both proteins play an important role as chemo and mechanoreceptors in different types of cells.

DISCUSSION

Astrocytes become reactive after injury and in an inflammatory environment; however, signals reported to promote astrocyte reactivity are still scarce to understand how is this process regulated.

Our laboratory has previously reported that $\alpha_V\beta_3$ Integrin directly binds to Thy-1 in neurons[37] and triggers the formation of focal adhesions[36] and stress fibers in astrocytes[35, 40]. The treatment with the pro-inflammatory cytokine TNF for 48 h increases the levels of $\alpha_V\beta_3$ Integrin in the cell surface, and promotes the recruitment and clustering of $\alpha_V\beta_3$ Integrin[69]. All these events are required to induce astrocyte reactivity and the ability of astrocytes to respond to Thy-1 [Figure 25] [69] suggesting a key role for this integrin in the process of astrogliosis. Accordingly, when $\alpha_V\beta_3$ Integrin is overexpressed in astrocytes, they show a reactive phenotype even without TNF treatment, indicating that the sole overexpression of $\alpha_V\beta_3$ Integrin is enough to trigger a reactive gliosis in primary neonatal astrocytes in a TNF-independent manner[14]. In addition, silencing $\alpha_V\beta_3$ Integrin prior to TNF-treatment, completely prevents the expression of reactivity markers and the migratory events induced by Thy-1 in astrocytes, Thus, $\alpha_V\beta_3$ Integrin also regulates astrocyte reactivity[14] and induces cell migration when stimulated by Thy-1[41].

In the present study, we explored a novel response of astrocytes to physical sensing of their environment, and particularly, the role of the mechanotransducing proteins, $\alpha_V\beta_3$ Integrin and Syndecan-4. Here, we verified that Thy-1, as a ligand for $\alpha_V\beta_3$ Integrin receptor is an important promoter of astrocyte contraction, and introduced the novel concept that the addition of **mechanical stress accelerates the appearance of $\alpha_V\beta_3$ Integrin at the cell surface**. As a consequence, astrocytes contract the cytoskeleton exerting traction forces on the ECM in an inside-out manner.

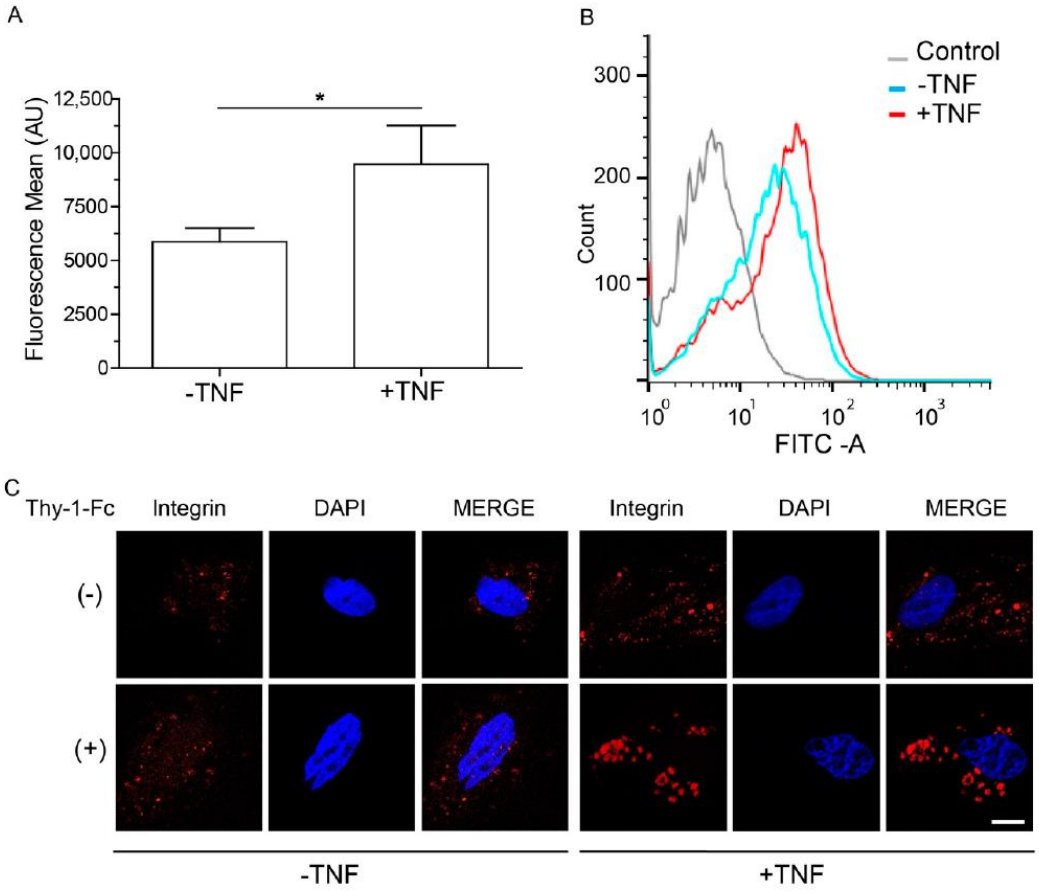

Figure 25. Expression levels of β3 Integrin increase at the cell surface and form β3 Integrin receptor clusters. *A) Analysis of β3 Integrin abundance at cell surface by flow cytometry [FACS] analysis performed in non-permeabilized primary astrocytes. The graph depicts the mean fluorescence intensity in astrocytes in the absence and the presence on TNF. Values in the graph are mean ± s.e.m. of five independent experiments per condition. * p < 0.05; B) The histogram shows a comparison of β3 Integrin expression levels in cells that were within the gates of the control cells, cells without TNF or with TNF, from one representative experiment. Control condition was carried out in the absence of Thy-1 [5 min]. Non-permeabilized cells were stained for αVβ3 Integrin [red] and DAPI [blue], which was used to stain the nuclei. The merge of both colors is also shown. Scale bar = 5 µm (Figure taken from [69]).*

Clustering of integrins is a phenomenon that requires the lateral assembly of integrins at the plane of the plasma membrane as a prior step to focal adhesion formation; however, integrin activation is a prerequisite to integrin clustering[76].

Considering that mechanical stress from stiffer ECM drives clustering of integrins[77], it is possible that mechanical stress, product of structural ECM remodeling by proteins secreted from astrocytes and other cells after brain damage[18-28], could prepare the cells to respond to Thy-1 in a similar manner than TNF. The latter is supported by the fact that cells, which do not contain a threshold level of surface integrins, do not respond to Thy-1[14]. The effect of mechanical stress over astrocytes could be additional or parallel to the effect of pro-inflammatory cytokines, which are released upon brain damage. Thus, in astrocytes, integrin clustering would be promoted by Thy-1 in an inflammatory environment and facilitated by mechanical stress.

Surface levels of $\alpha_V\beta_3$ Integrin, cellular contraction, and phosphorylation of MLC are elevated under Thy-1 plus mechanical stress treatment. These responses are faster than those induced by Thy-1 stimulation alone. Perhaps the force application prolongs the bond lifetime of the interacting molecules, a phenomenon known as “catch-bond” behavior; however, our reported data indicate that Thy-1-$\alpha_V\beta_3$ Integrin interaction shows a bond lifetime that behaves as a “slip-bond”, namely, force accelerates the dissociation between these two molecules[78]. Because these results on bond properties were obtained using purified proteins in a single molecule scheme, it is possible that in a cell-cell interaction, other molecules are involved. Indeed, we have reported that not only integrin, but also Syndecan-4 are required for Thy-1-induced astrocyte adhesion

and migration[40]. Additionally, cell surface levels of $\alpha_V\beta_3$ Integrin are regulated by Syndecan-4 [Figure 10]. Furthermore, using a different cellular model, Barker and co-workers described the Thy-1-$\alpha_5\beta_1$ Integrin interaction as a slip-bond behavior, which turns into a catch-bond behavior when Syndecan-4 is introduced into their cellular system[79]. Thus, it is possible that force applied to a tri-molecular complex, rather than to a bimolecular interaction accounts for a faster response when mechanical force is applied. However, our unpublished data indicate that the slip-bond Thy-1-$\alpha_V\beta_3$ Integrin interaction stays as a slip-bond behavior when Syndecan-4 is introduced into the Optical miniTweezers system. Here, under *in vitro*-generated pro-inflammatory conditions, Syndecan-4 accelerates the effect of integrin-engaged Thy-1 by forming a ternary complex, leading to faster neurite retraction and to the inhibition of neurite outgrowth[80]. Similar outcomes of cellular contraction could also occur in astrocytes upon formation of the trimolecular complex; however, this requires further investigation.

Our previous reports showing that integrin surface availability is increased after pro-inflammatory stimuli [TNF] [Figure 25] in primary astrocytes, also showed a crowding phenomenon of integrins after TNF and Thy-1 stimulation [69]. In view of this possibility, mechanical stimulation could also increase integrin aggregation by crowding, caused by Thy-1-coated magnetic beads. In this context, we reported that higher RhoA activation is achieved when using Thy-1 conjugated to Protein-A beads than when using soluble Thy-1, because higher integrin clustering is attained when presenting Thy-1 in a multivalent fashion[35].

The RhoA GTPase is an upstream activator of the Rho-associated kinase [ROCK]. ROCK phosphorylates myosin phosphatase-targeting subunit 1, MYPT1, resulting in a decreased MLC phosphatase activity and increased in phosphorylated MLC. Rho-kinase also phosphorylate MLC thereby leading to increase cellular contraction[81]. Therefore, since RhoA activation is upstream of MLC phosphorylation[35], the possibility that mechanical stimulation induces integrin crowding is also feasible. If we recapitulate the events triggered by mechanical stress in addition to Thy-1 stimulation, and compare them with Thy-1 alone over time, integrin levels and MLC phosphorylation increase in an accelerated manner when the two stimuli are applied together [Figure 26]. Despite the fact that, cellular responses have a short time window of occurrence, the peak for cellular contraction is faster than the one observed for the raise in integrin surface levels. A possible explanation is that integrin activation due to Thy-1 binding is enhanced by mechanical stress. Because integrin is a mechano-receptor, the mechanical stimulus would induce more molecules of $\alpha_V\beta_3$ Integrin to get activated and allow them to maintain an active conformation [extended and open] faster than without mechanical stress[82]. However, details about this process and the actual mechanism that explains it, remains elusive.

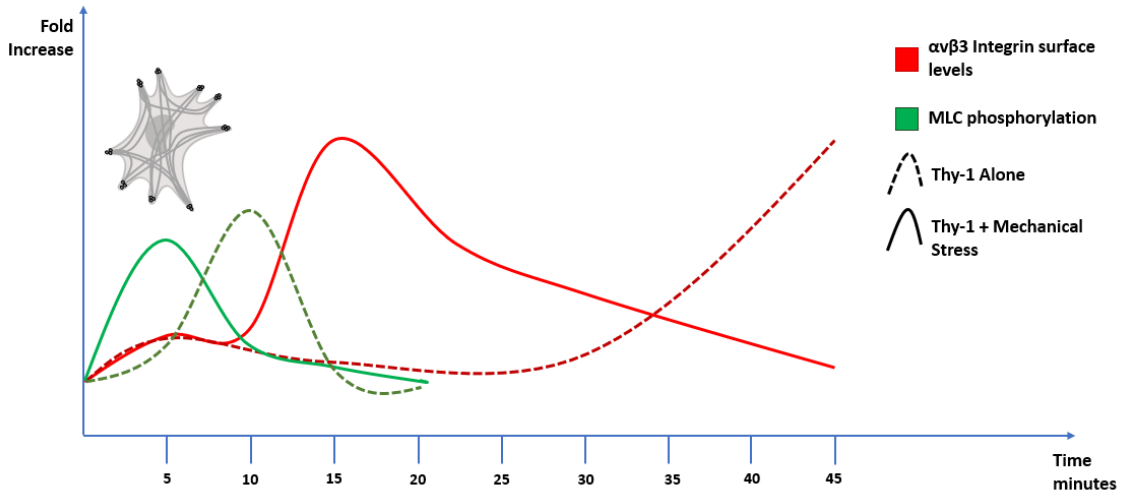

Figure 26. Kinetics of astrocyte cellular response summary. *Solid lines show the curve of Thy-1 plus Mechanical Stress stimulation, dashed lines show Thy-1 alone stimulation over time. Red lines represent the kinetics of αVβ3 Integrin surface levels, while green lines show kinetics followed by the Myosin Light Chain phosphorylation. Gray cell, with gray lines and black spots, represents the increased number of Focal adhesions and Stress Fibers at 5 minutes of stimulation.*

Astrocytes interact with neurons, blood vessels, and ECM proteins, and in an inflammatory environment, these cells respond and adapt to the physicochemical modifications. The adaptative response involves many changes in gene expression[83], and integrins are one of the key surface proteins which mRNA is up-regulated in inflammatory-mediated astrocyte reactivity[14]. Importantly, integrins mediate cell-ECM interface playing an important role in outside-in and inside-out signaling[84]. Force-induced mechanotransduction in an outside-in manner changes cellular stiffness, spreading, adhesion or migration, triggering cellular responses that thereby lead to inside-out signaling [cell-to-matrix] changing the topography, rigidity or confinement of a cell [Figure 27]. However, these responses in astrocytes have thus far, not been investigated.

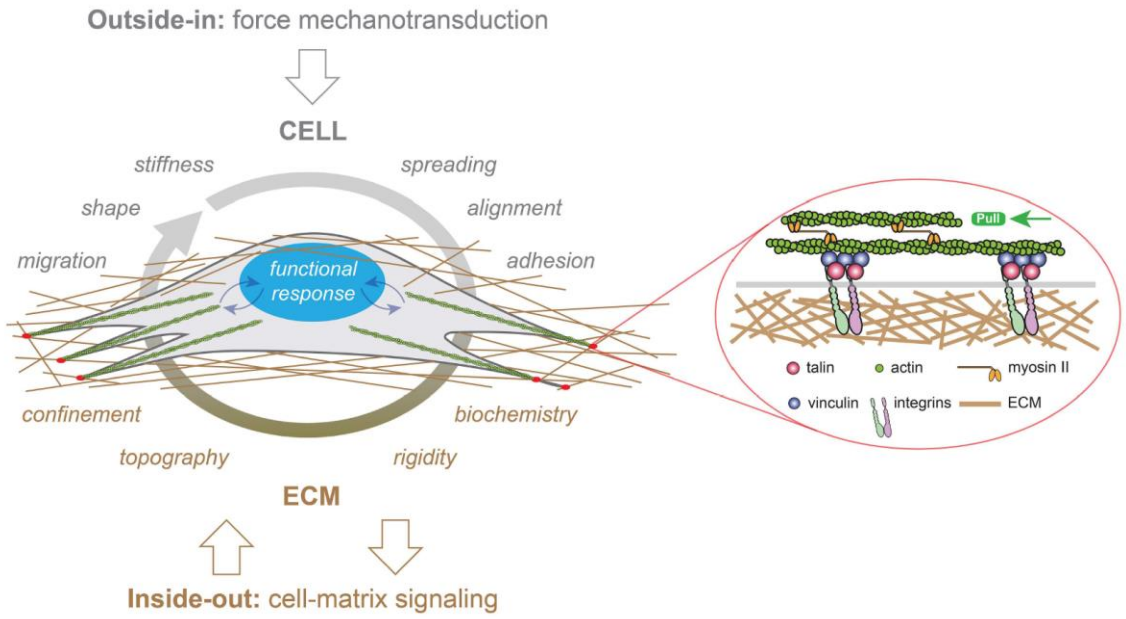

Figure 27. Representation of "outside-in" (in gray) and "inside-out" (in brown) mechanotransduction signals in a cell growing in a three-dimensional (3D) fibrous matrix. *External forces applied to eukaryotic cells modulate their migration, shape, stiffness, spreading, alignment, and adhesion behavior; whereas the ECM provides multiple cues to cells such as confinement, topography, rigidity and biochemical signals. Focal adhesions (FAs, in red) serve as crucial sites for both outside-in and inside-out mechanotransduction through the aggregation of transmembrane integrins. Mechanical signals (curved blue arrows) are converted in biological responses by the nucleus, which is depicted in blue[85].*

Here, we addressed the response of the traction force exerted by astrocytes through integrins on the substrate, these data reinforce the idea of cellular tensegrity proposed by Ingber[60], where the cells not only sense the mechanical cues from its microenvironment, but also generate mechanical cues to ECM that could remodel its surrounding area [Figure 28]. We show that the magnitude of traction forces exerted by astrocytes on the ECM depends on the mechanical and chemical stimuli received by the cells. Specifically, the stimuli

from neurons [Thy-1] in a cell-to-cell communication and those produced by a proinflammatory environment after injury, which generate a stiffer ECM. Thus, the mechanotransduction pathway works bidirectionally through integrins in a outside-in and inside-out signaling.

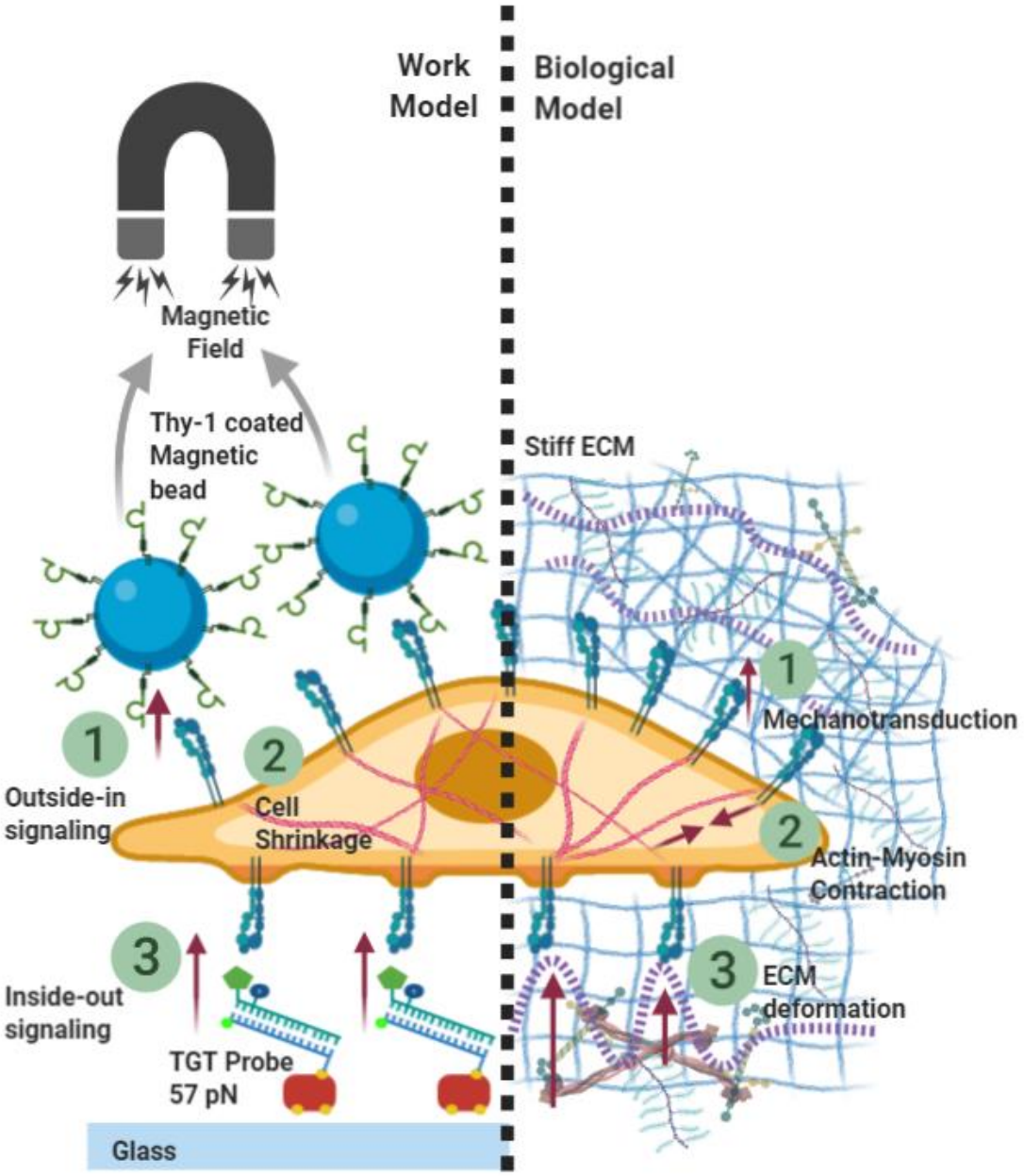

Figure 28. Outside-in to Inside-out signaling scheme. *Work Model: ①Outside-in signaling through αvβ3 Integrin stimulated chemically by Thy-1 and mechanically by magnetic field. ②Cellular shrinkage as a response to integrin activation. ③Inside-out signaling through integrins, the astrocyte exerts tension force to the TGT probe-coated glass. Biological Model: ①Mechanotransduction though αvβ3 Integrin triggered by stiff ECM (cell-matrix) and neuronal Thy-1 (cell-cell) interaction lead to ②Actin-Myosin contraction and traction force exerted by the astrocyte through Integrins generate ③ECM distortion.*

In the context of a proinflammatory environment in the CNS, our data support an important role of integrins as a receptor and effector of signaling pathways. In this context, perhaps integrins represent a target to develop therapeutic tools that could help people experiencing CNS injuries. However, integrins play various and important roles in cellular function and behavior, which makes it difficult to use them as therapeutic targets.

In addition, in this thesis project, we also studied the role of Syndecan-4 in the regulation of $\alpha_V\beta_3$ Integrin levels at the cell surface. Similar to the mechanism reported by Morgan[44] in the fibroblast model, the presence or absence of Syndecan-4 regulates the increase or decrease of surface integrin levels, respectively, and the number of FA and SF formation. Preliminary experiments performed with mutants of Syndecan-4 could support our theory that the phosphorylation of tyrosine 180 of Syndecan-4 regulates the surface levels of $\alpha_V\beta_3$ Integrin; however, these results require confirmation by further experimentation. Moreover, the absence of Syndecan-4 impairs the traction force exerted by integrins exposed to Thy-1 and mechanical stimulation, indicating a role for Syndecan-4 in the regulation of the outside-in and inside-out signals.

In summary, our results suggest that chemical and mechanical cues play a synergistic effect, where mechanical signals ameliorate the response of astrocytes to Thy-1 stimulus [chemical cue]. Mechanical stress could originate from a stiffer ECM, product of structural remodeling by astrocyte secreted proteins

after brain damage[18-28], and it could contribute to the response of astrocytes to inflammatory conditions. Therefore, the integration of combined signals will help us to understand the complex mechanisms that underlie astrogliosis. By learning about the regulation of this process, we might help achieving more successful functional regeneration after brain injury or neurodegeneration.

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