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# Extracellular UTP signaling in Schwann cell migration: A novel role of the P2Y2 receptors.

Memoria de la tesis doctoral presentada por Aloa Lamarca Dams, para optar al grado de Doctora por la Universidad Internacional de Catalunya.

Trabajo realizado en el Departamento de Biología Molecular y Celular de la Universidad Internacional de Catalunya, bajo la dirección de los doctores Àlex Gella Conscustell y Núria Casals Farré.

Proyecto subvencionado por la compañía farmacéutica Ferrer S.A.

St Cugat del Vallés, 2013;

| Doctoranda | Director | Co-Directora |
|---|---|---|
| Aloa Lamarca | Dr. Alejandro Gella | Dra. Núria Casals |

Per tú Jordi! Al final ho hem aconseguit!!!!

Me lo contaron y lo olvidé,

Lo ví y lo entendí,

Lo hice y lo aprendí

"Confucio"

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L'agraïment és la memòria del cor,

Així que gràcies a tots per acompanyar-me en aquest camí!

# II. AGRAIMENTS/ AGRADECIMIENTOS/ DANKWOORD

Crec que una tesis doctoral és un camí completament impossible de fer sol. Des dels primers inicis fins als durs finals, sempre estàs acompanyat de tot un conjunt de persones que fa d'aquesta, una experiència única i irrepetible en la vida d'una persona. M'atreviria a dir que ningú sap massa bé on es posa quan pren la decisió d' iniciar una tesis, però per altra banda, ara que arriba el final, si miro enrere, m'adono que ha sigut probablement el millor camí de la meva vida. És cert que hi ha moments que són molt durs, moments en què voldries tirar la tovallola i dir que tot això no serveix per a res! Però ara, després d'aquest llarg camí recorregut veig que ha sigut una experiència única, preciosa i que guardaré sempre amb mi com un gran i preciat tresor. Després de tot això és evident que haig d'agrair a moltíssimes persones el seu suport, el seu carinyo, la seva amistat i la seva paciència, perquè crec que darrere el manuscrit d'una tesis hi va una filera inacabable de persones.... així que espero no deixar-me a ningú!

No podria començar els agraïments d'una altra manera que no fos agraint a la Dra. Àngels Fabra haver-me permès començar en el seu grup de recerca el meu treball de pràctiques i després la meva tesina, aquell estiu d'ara fa 5 anys... Àngels, gràcies a tu vaig endinsar-me en aquest món, vas despertar en mi la curiositat científica, i em vas ensenyar la importància de la rigorositat i meticulositat. Moltes gràcies de debò per donar-me l'oportunitat de conèixer aquest món que ara tant m'apassiona.

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conèixer-me. Crec que siguis on siguis pots estar contenta amb el que hem aconseguit. No vam tenir gaire temps per fer tot el que haguéssim volgut, però des d'allà on siguis GRÀCIES. Gràcies Núria Casals, per seguir amb el projecte i arriscar-te a dirigir les nostres tesis tot i la feina que ja tenies amb el teu propi projecte. Al final entre tots hem aconseguit tirar-lo endavant i n'hem tret bons fruits i molt bones vivències. Gràcies Pep, per les paraules i les bromes. Al final hauré aconseguit la tesis i l'anell sense necessitat d'arribar a Montserrat caminant! Gràcies Agustí per animar els berenars i els pica-piques.

Dins del petit gran món que es genera en un laboratori sempre hi ha les persones imprescindibles, totes aquelles que estan amb tu dia a dia, lluitant per aconseguir la figura, perquè surti el resultat o simplement generant el caliu d'una petita nova família. Dins del laboratori de la UIC jo he trobat la meva petita gran família, així que ara us toca a vosaltres!

No puc començar d'una altra manera que no sigui donant-te les gracies a TU... sí, sí Taniota a TU! Has sigut un exemple a seguir i una gran amiga, juntes hem treballat com un engranatge molt ben engreixat, però no només hem treballat, sinó que hem après l'una de l'altra i l'una amb l'altra. He après amb tu moltíssimes coses, i per totes elles et vull donar les gràcies, per ajudar-me, per cuidar-me i per ensenyar-me tot el que saps sense dubtar-ho! Necessitaria uns quants fulls per poder-t'ho agrair tot, però sé que tu saps de què t'estic parlant, només que sàpigues que encara que el camí ara ens ha separat sempre formaràs una part especial de mi. A l'altre membre del Durani i Pix grup, a tu Moniqueta (i ja sé que odies que et digui així.... però aquí, ara sí que ho puc deixar plasmat!), tal com em vas dir tu un dia juntes vam començar l'era de les Real Times, tot i que jo després em vaig desviar una mica del tema. Gràcies per la teva alegria, i per aconseguir treure importància a les coses, gràcies per les rialles, les bromes, les cerveses i les atmetlletes.... Gràcies a les dues, noies, per fer del nostre petit grup un grup unit i fort, sí nenes sí... **YES WE CAN**!

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las mañanas y tardes en el coche, por los teatros y por estar siempre dispuesta a organizar alguna fiesta que otra!

Creia que seria més fàcil això d'escriure els agraïments... però encara vaig per el principi i ja tinc els ulls plens de llàgrimes... així que seguim...

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# III. ABREVIATIONS

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**aa**: aminoácid

**ADP:** adenosine 5' diphoshpate

**AKT**: protein kinase B (=PKB)

**AMPc**: adenosine monophosphate cíclic

**ATP**: adenosine 5' trifophosphate

**BPB**: ***b****romo****p****henol* ***b****lue*

**cDNA**:desoxiribonucled acid

**CMP**: citidine monophosphate

**CNS:** Central nervous system

**DBS:** Donor bovine serum

**DEPC**: dietil pirocarbonate

**DMSO**: dimetil sulfóxide

**DNA**: desoxiribonucleid acid

**DRG**: ***d****orsal* ***r****oot* ***g****anglion*

**DTT**: ditiotretiol

**ECM:** Extracellular matrix

**EDTA**: ácid etilendiaminotetraacétic

**ERB:** Epidermal growth factor receptor

**ERK:** Extracellular signal-regulated kinase

**FGF:** Fibroblast growth factor

**GAPDH**: ***g****lycer****a****ldehyd 3'****p****hosphate* ***deh****ydrogenase*

**GFAP:** Glial fibrillary acidic protein

**IGF**: ***i****nsulin like* ***g****rowth* ***f****actor*

**IP3**: inositol-1,4,5- triphosphate

**JNK:** stress-activated protein kinase

**KDa**: kilodalton

**mA**: miliampers

**MAPK**: mitogenic protein kinases

**MMP:** Metalloproteinases

**NCSCs:** Neural crest Schwann cells

**NGF**: ***n****eural* ***g****rowth* ***f****actor*

**NRG1**: neureguline 1 receptor

**NGFR**: ***n****erve* ***g****rowth* ***f****actor* ***r****eceptor*

**NP40**: nonidet P-40

**NT-3:** Neurotrophine 3

**Pb**: **p**airs of **b**ases

**PI3K**: phosphoinositol 3 kinase

**PLC**: phospholipase C

**PBS**: ***p****hosphate* ***b****uffer* ***s****aline*

**P2R**: purinergic receptors

**P2X**: P2R ionotrópics

**P2Y**: P2R metabotrópics

**PDGF:** Platelet derived growth factor

**PBS:** ***p****hosphate* ***b****uffer* ***s****aline*

**PNS:** Peripheral nervous system

**RNA**: ribonucleic acid

**RE**: endoplasmatic reticulum

**SDS-Page**: ***s****odium* ***d****odecylsulfate* ***p****olyacrilamide* ***ge****l* ***e****lectrophoresis*

**SCs:** Schwann cells

**PCR**: polymerase chain reaction

**SCPs:** Schwann cell precursors

**SNC**: central nervous system

**SNP**: Peripheral nervous system

**TBS**: ***T****ris* ***b****uffer* ***s****aline*

**TEMED**: tetrametiletilendiamide

**TM:** Transmembrane

**UDP**: uridine diphosphate

**UMP**: uridine monophosphate

**uPA:** Plasminogen activator system

**UTP**: uridine triphosphate

**V**: **v**oltios

**WB**: ***w****estern* ***b****lot*

# IV. SUMMARY

# IV. SUMMARY

Peripheral neuropaties are one of the major complications of the Peripheral Nervous System. For the moments no drugs are effective for their treatment, and most of the prescribed drugs are based on the inhibition of pain.

Nucleo CMP forte is a drug mainly composed of nucleotides (UMP, UDP, UTP and CMP), prescribed to patients with Peripheral Nervous System disorders, such as inflammatory processes and neuropathies of diferent origins. But their exact mechanism of action is still unknown. We focus our project to understand which are the effects of this drug and the triphosphate nucleotides in Schwann cells, one of the most important population of cells involved in the regeneration of the Peripheral Nervous System. In response to peripheral nerve injury, Schwann cells adopt a migratory phenotype and modify the extracellular matrix to make it permissive for cell migration and axonal regrowth. UTP and other nucleotides are released during nerve injury and can activate purinergic receptors expressed on the Schwann cell surface, but little is known on the involvement of purine signalling in wound healing.

Our results demonstrated that UTP treatment induces Schwann cell migration and wound healing, through the activation of the P2Y2 receptor. P2Y activation induces a biphasic MAPK activation (Early and Late) and also induces the activation of an extracellular metalloproteinase (MMP-2). Knockdown of the $P2Y_2$ receptor or of MMP-2, using specific shRNAs, highly reduces cell the migration and wound closure induced by UTP. MMP-2 activation evoked by injury or UTP is also mediated by the phosphorylation of all three major mitogen-activated protein kinases (MAPKs: ERK ½, p38 and JNK). Inhibition of these MAPKs decreased both MMP-2 activation and cell migration. Interestingly inhibition of MMP-2 activity selectively blocked the late, but not the early MAPKs activation.

These results suggest that MMP-2 activation and the late MAPKs phosphorylation are part of a positive feedback mechanism to maintain the migratory phenotype for wound healing. Moreover, treatment with UTP stimulates Schwann cell migration and wound repair through an MMP-2 dependent mechanism via P2Y receptors and MAPKs pathway activation.

# V. JUSTIFICATION

# V. JUSTIFICATION

Peripheral neuropathies are known to mainly involve alterations in the peripheral nervous system (PNS). At present there are more than 100 peripheral neuropathies described in the literature. The exact causes of these types of pathologies are not well understood, but we do know that they can be a response to nerve injury, tumors, toxins, autoimmune response, nutritional deficits, vascular or metabolic disorders and even alcoholism. Although there is a high incidence of these pathologies in today's society (up to 6% of the worldwide population), no effective treatment for these types of diseases has been found to date.

Learning to understand and control the causes of different neuropathies is the basis for the successful regeneration of damaged axons. In terms of pharmacological treatment for peripheral neuropathies, at present most prescription drugs are used to manage pain. In this framework, Nucleo CMP Forte, a medication comprised of cytidine monophosphate (CMP), uridine triphosphate (UTP), uridine diphosphate (UDP), and uridine monophosphate (UMP), is one of very few drugs prescribed for PNS pathologies (neuritis and neuropathies with osteoarticular, metabolic, or infectious origins). It is believed that its beneficial effects lie in the regenerative and protective capacity of the drug to treat axonal damage. Various clinical works have reported the beneficial physiological effects of this drug, but the exact molecular mechanisms such as the molecular pathways involved in the neurons and myelinating cells of the PNS have remained a mystery. Previous findings of our research group indicate that Nucleo NúcleoCMP Forte, and specifically UTP, induces a broad range of molecular changes in Schwann cells, leading to cytoskeletal reorganization and an increase in cell adhesion mainly due to an overexpression of N-cadherin. The present work aims to elucidate both the molecular and the cellular changes induced by Nucleo CMP Forte and UTP in Schwann cells by focusing primarily on the migration properties of these cells after treatment. The ultimate goal of this project is to gain an understanding of the mechanisms of action of the drug in order to seek out new pharmacological indications for it.

This project has been funded by the pharmaceutical company Ferrer International S.A. and carried out through an agreement between the Universitat Internacional de Catalunya and said pharmaceutical company.

# VI. HYPOTHESIS

# VI. HYPOTHESIS

Nucleo CMP Forte is prescribed to patients suffering from different neuropathies. It is believed that the beneficial effects observed after the administration of this drug lie in its regenerative and protective capacity to treat axonal damage, although the exact molecular mechanisms are still unknown.

The binding of extracellular nucleotides, which are secreted by damaged cells, to the purinergic receptors leads to the activation of the mitogen-activated protein kinase (MAPK) intracellular pathway. This pathway is extensively involved in the proliferation, migration, and survival of different cell types. In addition, the secretion of metalloproteinases is necessary for extracellular matrix remodelling as well as cell migration.

We postulate that the treatment of Schwann glial cells with the nucleotides present in Nucleo Nucleo CMP Forte will activate their purinergic receptors and produce metalloproteases secretion and cell migration, which are critical steps for nerve regeneration.

# VII. OBJECTIVES

# VII. OBJECTIVES

The main objective of this work is to identify the molecular mechanisms induced by the drug Nucleo Núcleo CMP Forte and UTP on myelinating cells (Schwann cells) of the peripheral nervous system.

Specific objectives:

- ✓ To analyze the roles of Nucleo Núcleo CMP Forte and UTP in Schwann migration.
- ✓ To study the effects of UTP on MMP-2 transcription and protein activation.
- ✓ To elucidate the molecular pathway induced by UTP that mediates MMP-2 activation and migration in Schwann cells.

# *VIII. INTRODUCTION*

# VIII. INTRODUCTION

## 1. MYELINATING CELLS IN THE PERIPHERAL NERVOUS SYSTEM

Of all glial cells, Schwann cells are the most important population in the peripheral nervous system. Their main function is to give mechanical and metabolic support to the neuronal system. Schwann cells, differentiate into the myelin sheath of the peripheral nervous system and can proliferate and migrate into the distal end in the injured nerve area to support axonal regrowth (Fawcett, J.W.; 1990).

Schwann cell precursors are originated in the neural crest, and directed by axonal guidance, they develop to immature SCs (**Figure i1**). Immature SCs develop a basal lamina, acquire an autocrine survival loop, surround axon bundles and extend processes inside these bundles in a finely tuned multistep process, termed radial sorting. The final step of Schwann cell development is the formation of mature Schwann cells, which can be myelinating or non-myelinating. This transition mainly depends on the expression of several transcription factors (Birchmeier, Nave 2008). Neuregulin-1 (NRG1) is one of the key regulators involved in nearly all aspects of Schwann cell development (Jessen, Mirsky 2005, Woodhoo, Sommer 2008, Pereira, Lebrun-Julien et al. 2012).

Schwann cell precursors (SCPs) are distinct from their progenitors in the neural crest and also from immature Schwann cells, in diverse phenotypic features such as some molecular markers. Schwann cells at the different stages, also present distinct responses to growth factors (**Figure i2**).

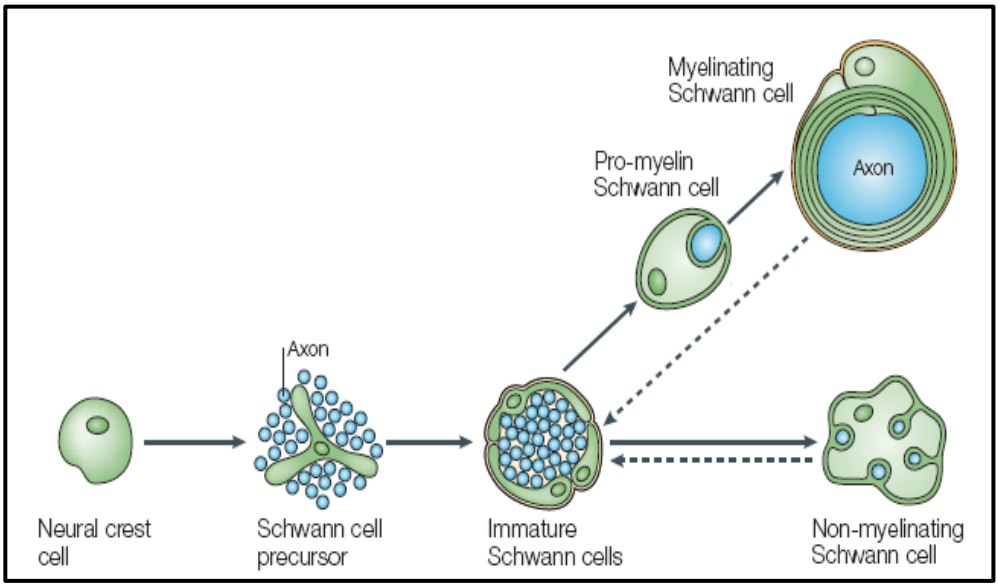


**Figure i1. Scheme of Schwann cell development and transition.** Picking arrows indicate that the phase is reversible. It is divided in three different cell populations: first, migrating cells of the neural crest; second Schwann precursor cells; and finally immature Schwann cells. Depending on the axonal guide this third population can develop to myelinating or non-myelinating Schwann cells (Jessen, Mirsky 2005).

SCP-Schwann cell transition is known to be an important step in the organogenesis of peripheral nerves. SCPs can be distinguished from neural crest Schwann cells (NCSCs) because they express in vivo BFABP, some integrins and N-cadherin (**Figure i2A**). In contrast, both SCPs and immature Schwann cells differ from the NCSCs clearly in their molecular profile. SCPs and immature Schwann cells are closely related to axons, in contrast, NCSCs are related to extracellular matrix, so the survival of NCSCs requires of the extracellular matrix neuregulin-1 (NRG1). In contrast, SCPs and immature cells need external survival factors such as PDGF, IGF or FGF among others (**Figure i2B**). Substantial differences are also revealed by the gene expression profile of the glial cells present in peripheral nerves at the different stages (**Figure i2C**). The differentiation of SCPs to immature Schwann cells result in an up regulation of several markers (GFAP or S100β), and down regulation of others (Cadherin-19, N-Cadherin or AP2α). Moreover, survival mechanism differs a lot between SCPs and Schwann cells *in vitro*. Schwann cells can support their own survival if they are plated at high densities, by secreting some factors such us IGF-2 or NT-3 among others, whereas SCPs do not have such autocrine survival circuits (Downsing, Morrison et al. 1999, Meier, Parmantier et al. 1999, Woodhoo, Sommer 2008).

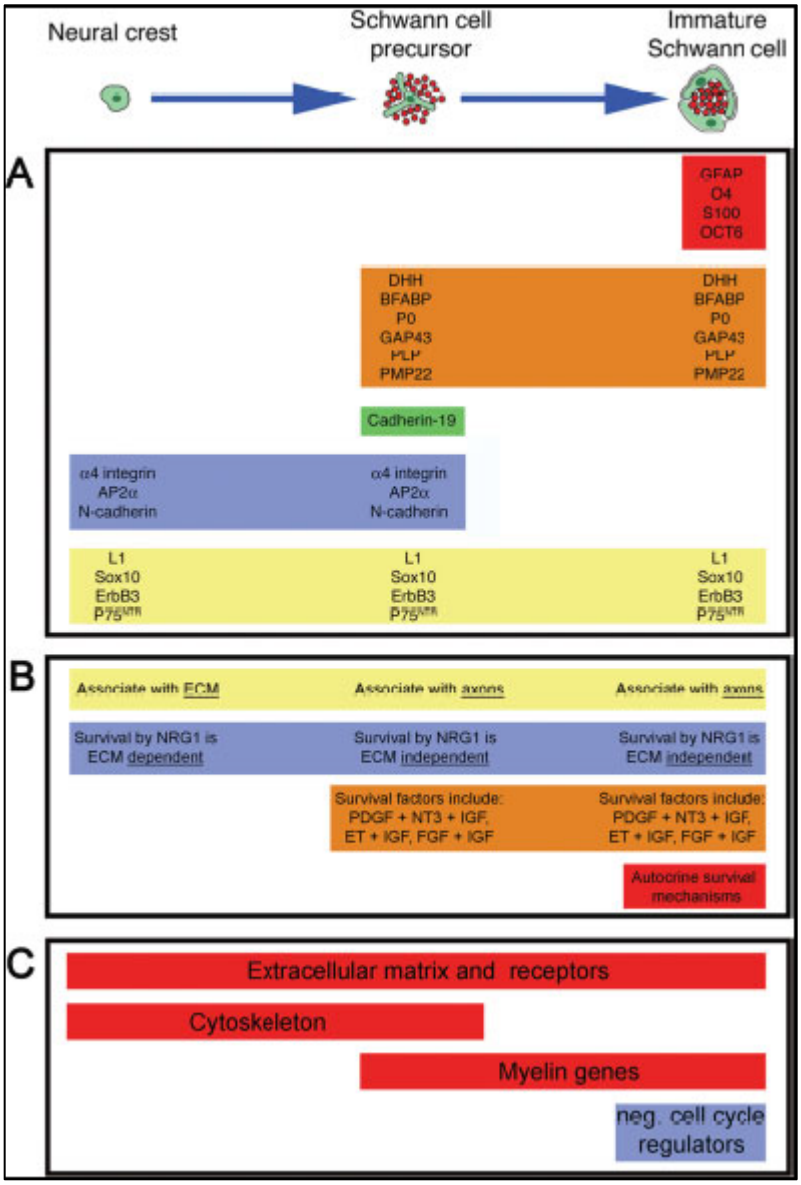


**Figure i2: Stages in embryonic Schwann cell development.** Molecular profile (A); signaling responses (B); and gene expression (C). **A)** Moleculars markers used to distinguish Schwann cell stages, classified into five major groups: present in the whole of the early Schwann cell linage (yellow box), presents only in crest cells and SCPs (blue box), presents only in SCPs (green box) and present only in immature Schwann cells (Red box). **B)** Survival mechanisms are different between stages. NRG-1 mediates survival in SCPs and immature Schwann cells, and is related to ECM association (blue and yellow box). Some growth factors such us PDGF, NT3 or IGF promotes SCPs and immature Schwann cell survival, but are not present in neural crest (orange box). Finally, autocrine survival mechanisms are present only in immature Schwann cells (red box). C) Gene expression profile is extremely different between cell stages. Genes are grouped into functional categories and are indicated in red if they are up regulated genes or in blue if they are down regulated. Adapted from (Woodhoo, Sommer 2008).

## 1.1. Schwann cell migration

Cell migration and wound repair are tightly regulated events critical for a successful wound closure after an injury. During development stages, cells move as a unit to specific regions, in order to repair superficial wounds. Also, migration during wound repair ensures reformation of the cell-cell barrier.

Schwann cells derived from the neural crest and their precursors are able to proliferate until they differentiate between mature myelinating or non-myelinating Schwann cells. Although Schwann cells play critical roles in peripheral nerves repair, due principally to their ability of differentiation, migration, proliferation, and myelination of regenerating axons (Nave, Salzer 2006), little is known about the exact clues that guide migrating Schwann cells. These cells migrate from the periphery to the injury site, where they apparently participate in endogenous repair processes (Oudega, Xu 2006). It has also been reported that Schwann cell migration, which occurs at the proximal end of the injury area, provides a guide for regenerating axons by interacting with nerve fibers or basal lamina (Anton, Sandrock et al. 1994). So, Schwann cell migration is crucial for a successful axonal elongation (Anton, Sandrock et al. 1994).

However, the factors that regulate Schwann cell migration and their signaling mechanisms, remain unclear nowadays. The balance among survival, proliferation and migration of Schwann cells is tightly regulated by neurons to achieve the 1:1 relationship between myelinating Schwann cells and their associated axons segments (Sherman, Brophy 2005). Some studies have defined the pathways that start Schwann cell differentiation, although the molecular nature of the axonal signals that coordinate Schwann cell migration, both *in vitro* and *in vivo*, is not well understood yet (Britsch, Goerich et al. 2001, Dong, Brennan et al. 1995).

Schwann cell migration is crucial for a successful axonal elongation (Torigoe, Tanaka et al. 1996). From all the known molecular factors, responsible of Schwann cell migration, NGF and its receptor were reported to mediate Schwann cell migration on enervated sciatic nerves (Anton, Weskamp et al. 1994). Also, some extracellular matrix components such as laminin and other products of chondroitin 6-sulphotransferase activities have been seen to promote axonal regeneration through Schwann cell migration (Anton, Sandrock et al. 1994, Liu J., Chau et al. 2006).

In the other hand, recent studies made in zebrafish demonstrated that signalling through ErbB (Epidermal growth factor receptor tyrosine kinases) is required to direct the migration of Schwann cells along the ganglias. If ErbB signaling is inhibited, Schwann cells retain their motility. These results suggest that ErbB signalling has a specific role in Schwann cell development, including proliferation, migration and myelination (Lyons, Pogoda et al. 2005, Perlin, Lush et al. 2011). Schwann cells also produce some growth factors crucial for peripheral nerve repair, such as fibroblast growth factor-2 (FGF-2) among others, which exhibit neurotrophic effects on peripheral nerve regeneration, through maintaining cell survival, proliferation and migration (Kushima, Nishio et al. 1992, Grinspan, Stern et al. 1993).

## 1.2. Peripheral neuropathies

A peripheral neuropathy is a damage of one or more nerves in the peripheral nervous system. It is not always easy to pinpoint the exact cause of the peripheral neuropathy because their origins are too broad. Some of the origin factors of these neuropathies are, alcoholism, autoimmune diseases, and diabetes, some infections such as varicella zoster, or trauma or pressure on the nerve. The type and symptoms of the neuropathy depend on the type of the affected nerve (motor, sensory, or autonomous).

### 1.2.1. Treatments

Nowadays, no drugs are capable of curing neuropathies and all of the treatments are only symptomatic. It is important to note that a proper control of the causes of the neuropathy is the base for a correct treatment of the damaged axons. In the diabetic neuropathies, a correct control of the hyperglycemia would be of utmost importance. In this direction, new studied therapies are focused in directing the drug to the metabolic affected pathway, for example Reservatrol, which acts in the mitochondrial transport chain.

### 1.2.1.1. Nucleo CMP forte.

Nucleo CMP forte (**CMPF**) is a drug currently used for the treatment of neuropathic pain and it is a therapeutic support in the treatment of neuritis, neuralgias and myopathy. It is a supplementary drug for the treatment of symptomatic pain. As shown in **Figure i3**, Nucleo CMP forte drug (**CMPF**) is formed by cytidine monophosphate (**CMP**), uridine monophosphate (**UMP**), uridine diphosphate (**UDP**) and uridine triphosphate (**UTP**).

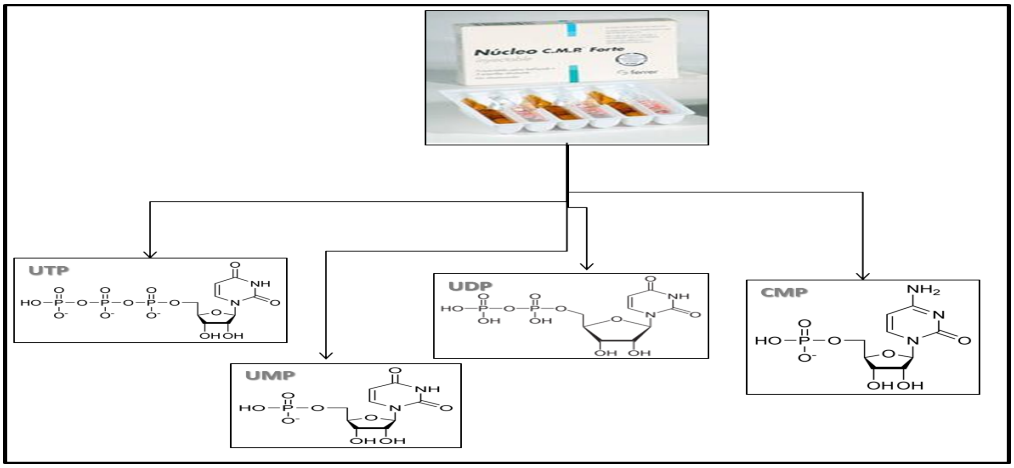


**Figure i3: Components of the Nucleo CMP drug.** This drug is composed by uridine triphosphate (UTP), uridine diphosphate (UDP), uridine monophosphate (UMP) and cytidine monophosphate (CMP).

The useful effects of extracellular nucleotides for the treatment of neuropathies have been known for many years. The first studies are from 1972, when Perez and collaborators reported the possible beneficial roles of the association of CMP/UTP in a broad range of peripheral neuropathies. Nucleotides were administered together with B12 vitamin and treated patients displayed a quickly relief in the pain of some peripheral neuropathies (diabetic neuropathies, acute neuritis and radiculopathies). So, CMPF together with B12 accelerated the recovery of some neuropathies, improving the pain and the motor alterations of the patients. Some clinical trials also reported that a daily treatment with Keltican (previous name of CMPF) for three months in 40 patients with diabetic polyneuropathy, was enough to improve the conduction velocity of electric stimulus in the damaged nerves of these patients, thereby reducing the pain sensations (Muller 2002). Many studies validate the regenerative capacity of nucleotides in the treatment of pain in patients treated with nucleotides. Nonetheless, the molecular mechanisms of this drug has not been analyzed yet.

# 2. NUCLEOTIDES AS CELLULAR COMMUNICATION SIGNALS.

Nucleotides have many intracellular functions such as RNA and DNA synthesis, but they can also act as second messengers rolling effects in intracellular communication. Nucleotides are hydrophilic, so they can hardly enter into the cell, for that reason, this communication is due basically through purinergic receptor activation. Nucleotides are powerful signals and they have highly specific functions as short-term co-transmitters in the nervous system (Abbracchio, Burnstock 1994, Burnstock 1996).

## 2.1. Purinoreceptors classification

Purine and pyrimidine nucleotides/nucleosides, once released to the extracellular fluid, can activate the family of purinoreceptors, classified as metabotropic P1 adenosine receptors, ionotropic P2XR receptors and finally P2Y receptors, as shown in **Figure i4**. The P1 and P2Y receptors are metabotropic receptors coupled to G proteins, adversely, P2X are fast ion channel receptors (Jacobson, Boeynaems 2010). All these receptors are broadly distributed in both neurons and glial cells and its activation mediates a remarkable variety of physiological and pathophysiological reactions (Franke, Verkhratsky et al. 2012). Actually, these receptors have been implicated in many pathologies of the nervous system and may become potential therapeutic targets.

### 2.1.1. P2 receptors

In 1978, Burnstock proposed the first classification of purinergic receptors. This classification was made depending on the kind of ligand that activates the receptors and two major families were described: P1 family (receptors for nucleosides) and P2 family (receptors for nucleotides, **Figure i4**).

Another classification was made several years after, in which two types of P2-purinoreceptors where defined as P2X and P2Y (Burnstock, Kennedy 1985). This classification was consistent with P2X as an ion channel receptor and P2Y with a G protein-coupled receptor. Currently, seven subtypes of P2X and eight subtypes of P2Y are recognized nowadays.

If we take a look into the physiology of purinergic signaling, we see that early studies were largely focused on short-term signaling in such events as neurotransmission, neuromodulation, secretion, chemo attraction and acute inflammation (Burnstock 2009). After those first studies, there began an increasing interest in long-term signalling that involves cell proliferation, migration, differentiation and death during development, regeneration, wound healing, cancer and ageing (Abbracchio, Burnstock 1998).

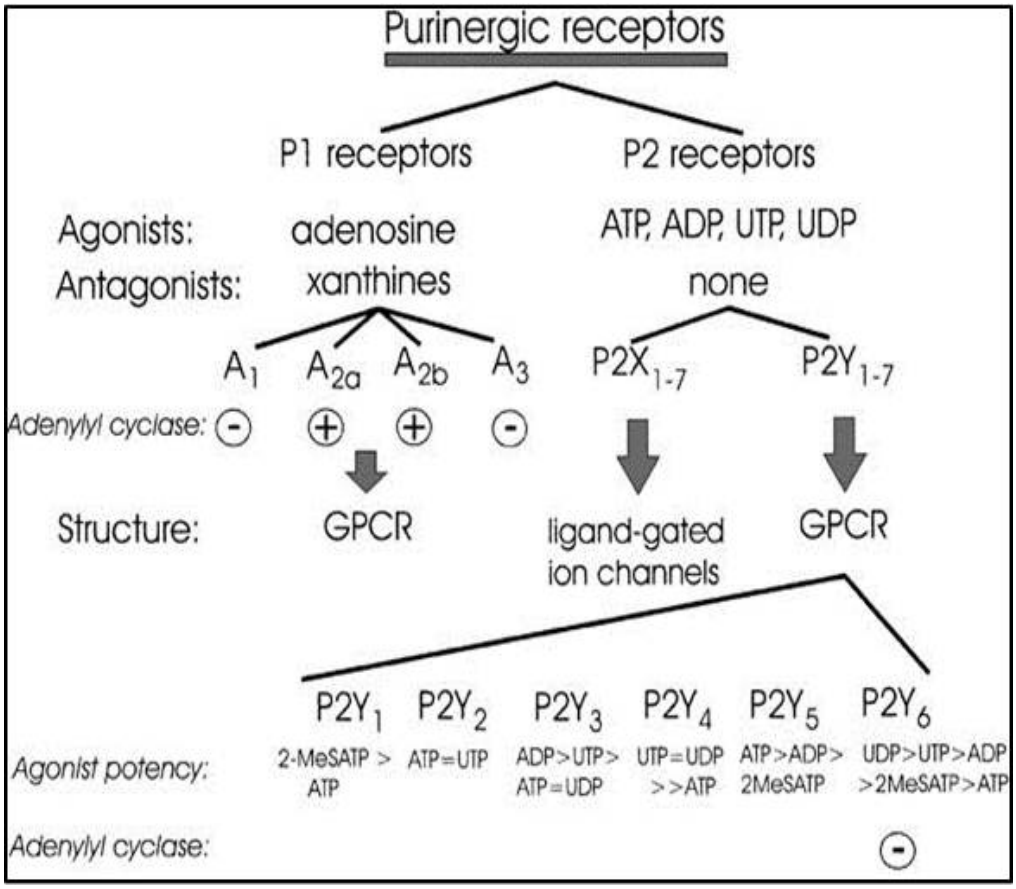


**Figure i4: Purinergic receptor family classification.** There are two principal families of purienrgic receptors, P1 receptors sensitive to adenosine nucleotides, and P2 receptors, sensitive to both adenine and uracil nucleotides. P1 receptor family is divided into four different types ($A_1$, $A_{2a}$, $A_{2b}$ and $A_3$) all of them activated GPCR. P2 family is subdivided into two subfamilies: P2X which are ionic channels sensitive to ATP; and P2Y which are metabotropic receptors sensitive to both adenine and uracil nucleotides. P2Y family is in turn sub-classified in six different families in function of the agonist potency ($P2Y_{1,2,3,4,5,6}$). Adapted from (Kaczmarek-Hájek, Lörinczi et al. 2012)

As shown in the figure above (**Figure i4**), the different purinergic receptors are activated by different nucleotides, and have different cellular responses. Roughly, P1 receptors are specific for adenosine nucleotides, and modulate the activity of adenylyl cyclase. In return, P2 receptors react both to adenosine or uracil nucleotides, and modulate the activity of phospholipase C (PLC) and intracellular calcium concentration. Different previous studies have demonstrated the different responses of each subtype of P2Y receptor in response to different nucleotides (Kaczmarek-Hájek, Lörinczi et al. 2012).

### 2.1.2. Agonists and antagonists of P2 receptors

In 1978, Burnstock G proposed the basis for distinguishing two types of purinergic receptors, one selective to adenosine (P1), which was antagonized by methylxanthines and the other selective for ATP/Adenosine diphosphate (ADP), P2. Several years later, in 1985, Burnstock G made a new subclassification for distinguishing two types of P2 purinoreceptors P2X and P2Y.

Whereas the P2X receptors are all specific receptors for ATP, the various P2Y receptors differ by their selectivity for distinct nucleotides. On the other hand, a good knowledge, not only of the agonists, but also of the antagonists of these receptors is very important to elucidate their physiological roles. Once they are released to the extracellular fluids, nucleotides are rapidly degraded by various ectonucleotidases, and they produce their cellular responses by acting through specific receptors. Nowadays it is known that these specific receptors belongs to two super-families as we had mentioned before in this manuscript: ionotropic P2X receptors and G-protein-coupled P2Y receptors. The release of nucleotides in extracellular fluids can result from several different processes, such as cell death, exocytosis or vesicular trafficking between others, as shown in **Figure i5** (Boeynaems, Communi et al. 2012).

| Cellular Mechanisms | Context | Proteins Tentatively Involved |
|---|---|---|
| Cell lysis | Cell death (necrosis) | |
| Exocytosis | Platelet aggregation | |
| | Neurotransmission | |
| Plasma membrane permeability | Mechanical stimulation | CFTR, P-glycoprotein |
| | Hypoxia | Stretch-activated ion channels |
| | Bacterial invasion | Hemiconnexins |
| | | CD39 |
| Vesicular trafficking | Constitutive | Mcd4P |
| | Mechanical stimulation | |

**Figure i5. Nucleotide release to the extracellular fluids.** There are several cellular mechanisms that can induce nucleotide release to extracellular fluids such us cell lysis, exocytosis, or vesicular trafficking. Adapted from (Boeynaems, Communi et al. 2012).

### 2.1.3. P2X receptors

P2X receptors are non-selective cation channels, permeable to small cations. The major physiological mechanism by which activated P2X receptors control cellular functions, is the elevation in the intracellular calcium concentration (Kaczmarek-Hájek, Lörinczi et al. 2012, Stojilkovic, Tomic et al. 2012). This increase in the calcium concentration leads to the activation of a broad range of signalling cascades that trigger manifold of short and long-term cellular events. From all this range of cellular events, the principal ones are cytokines release, cytoskeletal rearrangements, cell proliferation and differentiation (Monif, Burnstock et al. 2010). These include activation of phospholipase, PKC, JNK (stress-activated protein kinase), ERK and p38 MAP kinases, as well as the Rho-associated protein kinase (ROCK), (Kettenmann, Hanisch et al. 2011).

There are seven cloned human and rat P2X subunits, and they share 35-54% sequence identity between them. All of them have a common topology with two trans-membrane domains (TM), a large extracellular ligand binding loop, and an intracellular N and C terminus domain, as we can see in **Figure i6**. The extracellular domain connects the two TMs and constitutes the largest part of the polypeptide (Kaczmarek-Hájek, Lörinczi et al. 2012).

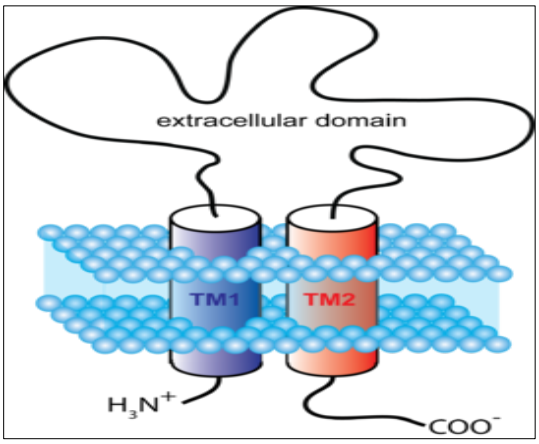


**Figure i6: Schematic representation of a P2X receptor.** We can observe two transmembrane domains (TM1 and TM2), a large extracellular loop and intracellular N terminus and C terminus regions.

### 2.1.3.1. Agonsits and antagonists for P2X receptors

All homomeric and heteromeric P2X receptors are activated by ATP, but in a receptor-specific manner. The $EC_{50}$ for ATP is in the one to ten uM range, for all recombinant P2X receptors except for P2X7, which has an $EC_{50}$ of approximately 100 µM (Coddou, Yan et al. 2011). In contrast, ADP, AMP, UTP, UDP and UMP activate these receptors either weakly or not at all (Ralevic, Burnstock 1998). We can also find other agonists for P2X receptors, which have been recently developed. They are analogs of ATP and act at several P2XR receptors with different potency and efficacy. For example, 2MeSATP is good agonist for all known P2X receptors, and BzATP is a good agonist for $P2X_1$, $P2X_5$ and $P2X_7$ (Jacobson, Jarvis et al. 2002).

As we can see in the **Figure i7** (Li, Liang et al. 2008), there are few selective and competitive P2X receptor antagonists described nowadays. NF449 that is highly potent, but less selective for $P2X_1$ receptor, in contrast A-317491 is very potent, and it is dual for $P2X_3$ and $P2X_2$ receptors (Kaczmarek-Hájek, Lörinczi et al. 2012). Although they are described, the exact molecular action of these antagonists is not well known. On the other hand, in basic research, a variety of more or less selective compounds have been used, such as Suramin, ANAPP3, DIDS or PPADS. Of them, Suramin is the most widely used competitive P2R antagonist. However, this drug is not specific for P2 receptors and also antagonizes for G coupled proteins (Hui, Nayak 2002).

TABLE 1. SUMMARY OF DISTRIBUTION AND PHARMACOLOGY OF P2X RECEPTORS

| *Receptor* | *Main distribution* | *ATP $EC_{50}$* | *Desensitization* | *Modulation* | *Agonist* | *Antagonist* |
|---|---|---|---|---|---|---|
| $P2X_1$ | Smooth muscle, platelets, cerebellum, dorsal horn spinal neurons | 1 | Fast | $Zn^{2+}\downarrow$ | ATP, 2-MeSATP, $\alpha,\beta$-MeATP | TNP-ATP, IP5I, RO-1, NF023, NF449 |
| $P2X_2$ | Smooth muscle, CNS, retina, chromaffin cells, autonomic and sensory ganglia | 6 | Slow | $Ca^{2+}\downarrow$, pH$\downarrow$, $Zn^{2+}\uparrow$ | ATP, BzATP, 2-MeSATP | Suramin, PPADS, RB2, NF770, NF279 |
| $P2X_3$ | Sensory neurons, nucleus tractus solarius, sympathetic neurons | 1 | Fast | $Zn^{2+}\uparrow$ | 2-MeSATP, ATP, $\alpha,\beta$-MeATP | TNP-ATP, PPADS, RO-3, A317491, NF110 |
| $P2X_4$ | CNS, testis, colon | 16 | Medium | $Zn^{2+}\uparrow$ | ATP | TNP-ATP |
| $P2X_5$ | Proliferating cells in skin, gut, bladder, thymus, spinal cord | 10 | Slow | $Ca^{2+}\downarrow$, $Zn^{2+}\uparrow$ | ATP, 2-MeSATP | Suramin, PPADS, BBG |
| $P2X_6$ | CNS, motor neurons, spinal cord | 0.5 | Slow | — | — | — |
| $P2X_7$ | Apoptotic cells, immune cells, pancreas, skin | >100 | Slow | $Ca^{2+}\downarrow$, $Zn^{2+}\downarrow$ | BzATP, 2-MeSATP, ATP | KN62, KN04, RN6189, MRS2427, Az1645373, A-740003 |

**Figure i7: Distribution, agonists and antagonists more described for P2X receptors.** (Li, Liang et al. 2008)

## 2.1.4. P2Y receptors

Metabotropic G protein-coupled P2Y receptors show the characteristic seven transmembrane hydrophobic domains, with short extracellular N- and intracellular C- terminals (Abbracchio, Burnstock 1998), as we can see in **Figure i9**.

Nowadays, P2Y family is composed of eight members, encoded by distinct genes that can be subdivided into two groups based on their coupling to specific G protein as well as structural features. In consecuence P2Y receptors are sorted as P2Y receptors Group A, which activates Gq or Gs proteins, and group B which activates Gi proteins, as we can see in the Table below (**Figure i8**). Also, $P2Y_{1, 2, 4, 6 \text{ and } 11}$ receptors coupled to Gq activate PLC-beta, and $P2Y_{12, 13 \text{ and } 14}$ receptors couple to Gi inhibit adenylyl cyclase (Abbracchio, Burnstock 1998).

| GROUP | RECEPTOR | G protein |
|---|---|---|
| A | $P2Y_1$ | $G_q$ |
| | $P2Y_{`2}$ | $G_q$ (+ $G_i$) |
| | $P2Y_4$ | $G_q$ (+ $G_i$) |
| | $P2Y_6$ | $G_q$ |
| | $P2Y_{11}$ | $G_q$ + Gs |
| B | $P2Y_{12}$ | $G_i$ |
| | $P2Y_{13}$ | $G_i$ |
| | $P2Y_{14}$ | $G_i$ |

**Figure i8. P2Y receptors classification in function of the activated G coupled protein.** Nowadays we know eight subtypes of P2Y receptors. Five of them activates Gq protein ($P2Y_{1,2,4,6,11}$), and the other three activates Gi protein ($P2Y_{12,13,14}$). Adapted from (Abbracchio, Burnstock 1998).

Whereas the P2X receptors are all receptors for ATP, the various P2Y receptors differ by their selectivity for distinct nucleotides (**Figure i6**). (Boeynaems, Communi et al. 2012, Abbracchio, Burnstock 1998). If we focuse in the functions and therapeutic applications of P2Y receptors, we can see that this kind of receptors have a broad range of functions.

Nucleotide actions mediated by P2Y receptors are so ubiquitous and numerous that here we will provide a tentative list of some of their known actions. $P2Y_1$ and $P2Y_{12}$ cooperate together in platelet aggregation, it has been studied that KO mice for one or both of these receptors ($P2Y_1$ and $P2Y_{12}$) show defective platelet aggregation, increased bleeding time and resistance to thrombosis (Andre, Delaney et al. 2003, Leon, Hechler et al. 1999). Also, $P2Y_1$ has been reported to promote astrocytic reaction to traumatic injury *in vivo* through the PI3K/Akt signaling pathway (Lecca, Ceruti et al. 2012). $P2Y_{11}$ has important roles in granulocytic differentiation of promyekocytes and in the maturation of dendritic cells (Boeynaems, Communi et al. 2012). Moreover, $P2Y_2$ receptor plays important roles in inflammatory processes, and also promotes cytoskeletal changes and astrocyte migration through direct interactions with integrins (Weisman, Wang et al. 2005).

Considering P2Y receptors in microglia, some evidence suggests that P2Y receptors play important roles in long-term control of cell proliferation, differentiation, migration and death, development and ageing (Burnstock 2007, Burnstock 2012). Interestingly, $P2Y_2$ receptors can also directly trans-activate growth factor receptors, providing an additional possible explanation for the nucleotide-growth factor interaction in controlling astrocyte functions (Peterson, Camden et al. 2010).

No crystal structure of P2Y receptor is available yet, but it is known that these types of receptors are formed by seven transmembrane domains, with the N-terminal domain in the extracellular space and the C-terminal domain in the cytoplasm, as we can see in **Figure i9**. Different studies based on sequence comparison and mutagenesis revealed that cationic residues, which interact with the negatively charged phosphate groups, are present in transmembrane domains 3, 6 and 7. Otherwise, the eight P2Y receptors have an H-X-X-R\K motif in TM 6 (Boeynaems, Communi et al. 2012).

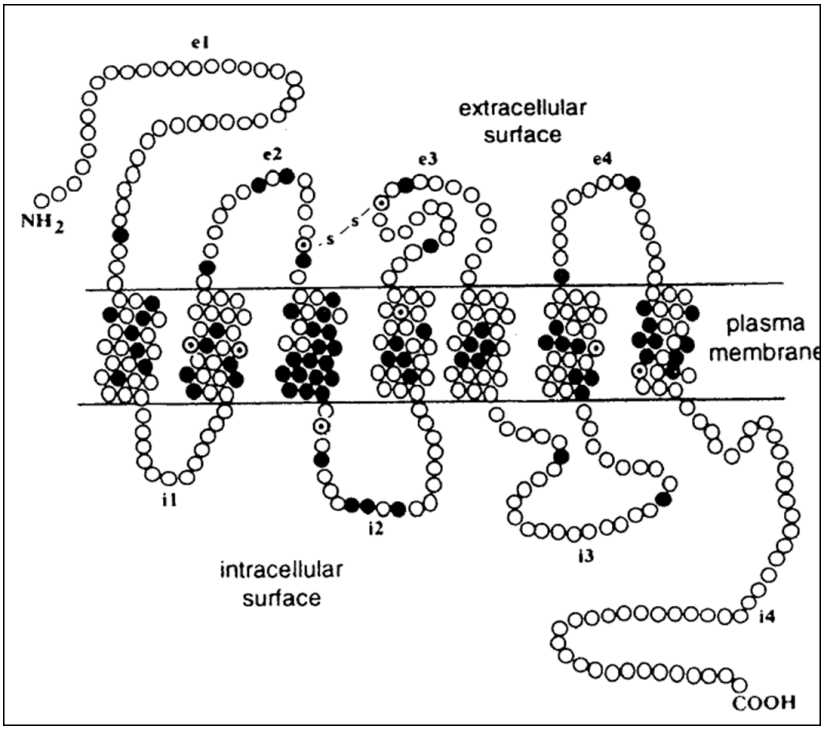


**Figure i9: Schematic representation of a P2Y receptor.** We can observe seven transmembrane domains, a large extracellular loop (N terminus) and a large intracellular C terminus regions (Boeynaems, Communi et al. 2012)

#### 2.1.4.1. Agonists and antagonists for P2Y receptors

It has been explained before in this manuscript that P2X receptors are all receptors for ATP, in return, P2Y receptors differ by their selectivity for distinct nucleotides. The specificity of nucleotides for the different P2Y receptors is presented in **Figure i10**. Dinucleotide phosphates activate various P2Y receptors such as $P2Y_{1,4,6,12,13 \text{ and } 14}$ (Shaver, et al 2055). $P2Y_{11}$ is principally an ATP receptor such as P2X, whereas $P2Y_1$, $P2Y_{12}$ and $P2Y_{13}$ are receptors for ADP. Also, $P2Y_4$ and $P2Y_6$ are pyrimidinergic receptors activated both by UTP or UDP interchangeably (Boeynaems, Communi et al. 2012).

| GROUP | RECEPTOR | AGONIST | G protein |
|---|---|---|---|
| A | $P2Y_1$ | ADP>ATP | Gq |
| | $P2Y_2$ | ATP = UTP | Gq (+Gi) |
| | $P2Y_4$ | UTP> ATP, UDP | Gq (+Gi) |
| | $P2Y_6$ | UDP>UTP | Gq |
| | $P2Y_{11}$ | ATP | Gq +Gs |
| B | $P2Y_{12}$ | ADP | Gi |
| | $P2Y_{13}$ | ADP | Gi |
| | $P2Y_{14}$ | UDP | Gi |

**Figure i10: Types of P2Y receptors, described agonists and action on G coupled proteins.** Adapted from (Boeynaems, Communi et al. 2012)

Purinergic receptors are shown to be potential clinical targets, there is an intense ongoing to design selective agonist and antagonist ligands, especially to generate therapeutic targets. Many selective ligand probes, both with agonists and antagonists of the P2Y receptors are now available. Nevertheless, much more work is needed since some subtypes of P2Y receptors such as $P2Y_4$ are entirely lacking of selective ligands.

Several antagonists are known for such P2Y receptors (**Figure i11)**. Unfortunately, selective-subtype P2-receptor antagonists are not yet available. Otherwise, they are some selective agonist for one or more than one P2Y receptor, for example: MRS2179 is a selective $P2Y_1$ agonist and with a high-affinity to $P2Y_1$ in various known species (Houston, et al 2006). $P2Y_2$ and $P2Y_4$ receptors, both are activated by UTP, and have no definitive antagonists available, even so there are

some molecules often used as slightly selective antagonists of these receptors, such as Reactive Blue 2 or Suramin (trypanoside suramin (8-(3-benz-amino-4-methylbenzamido)-naphthalene-1,3,5-trisulfonic acid),(Jacobson, Boeynaems 2010). Suramin, is used as a broad-spectrum non-selective antagonist of P2Y receptors, and it is known that interacts with G-proteins (Freissmuth, Boehm et al. 1996). This drug not only antagonizes P2Y receptors, but also antagonizes some P2X receptors such as $P2X_{1,\ 2\ and\ 3}$. Despite its poor competitiveness and antagonizing skills, and not being able to discriminate between P2X and P2Y receptors, it is the most used drug (Dunn, Blakeley 1998).

However, some specific antagonists are described for the $P2Y_{12}$ receptor, such as AR-C67085, which has a $pEC_{50}$ of 5.05, (Communi, et al 1999) or NF157, which has a $pK_i$ value of 7.35, for $P2Y_{11}$. The last one derives from the non-selective P2 antagonist Suramin (Ullmann, et al 2005).

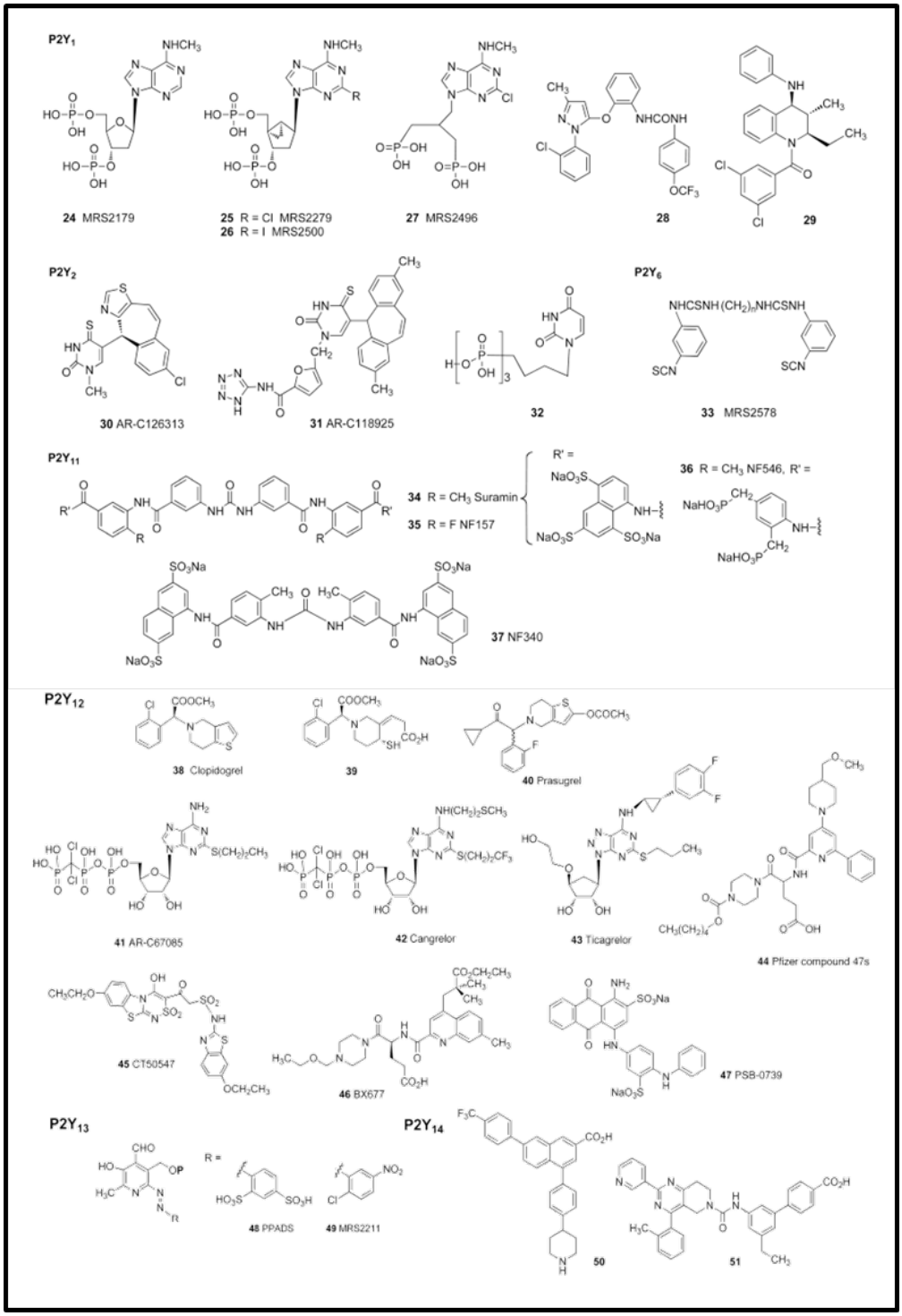


**Figure i11: Nucleotides and non-nucleotides that have been useful antagonists in the study of P2Y receptors.**(Jacobson, Boeynaems 2010)

## 2.2. P2Y receptors in the nervous system

It is known that purinergic system signaling plays a unique role in neuronal-glial interactions, besides, all the known glial cells display sensitivity to ATP and its analogues (Jarvis 2010). For the moment, we know that glial cells express mRNA for all known P2 receptors except for $P2X_5$ (Hanisch, Kettenmann 2007, Farber, Kettenmann 2006). In the nervous system, ATP is released from neurons, as well as from neuroglia. ATP excites microglial cells generating an inward rectifying current that contains both rapid and slowly desensitizing components. Farber et al, described in 2006 the presence of functional purinergic receptors using the patch-clamp technique in acute slices of adult mouse brain (Farber, Kettenmann 2006), as seen in **Figure i12**. P2Y and P2X receptors were also identified on ameboid microglial cells from early postnatal acute brain slices (Boucsein, Zacharias et al. 2003). ATP and possibly other nucleotides released after inflammatory and death cells or under normal physiological conditions, can act as communication molecules in the different cells of the nervous system.

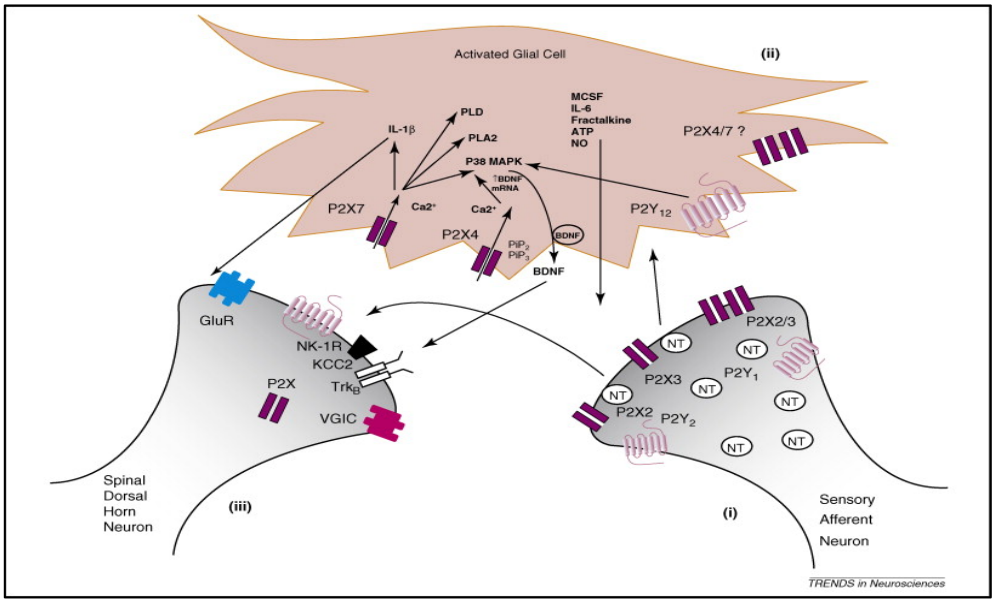


**Figure i12: Presence of purinergic signaling receptors and pathways in neuronal-glial circuits.** Nucleotides and it's derivatives act as an extracellular molecules at all levels of communications within neuronal-glia networks. (i) Presynaptic neurotransmitter release is activated by activation of P2X receptors. (ii) Activation of microglial cells at the level of the spinal cord by ATP stimulation of P2X7 receptors that led to multiple downstream signaling events including p38 MAPK phosphorylation. (iii) Extracellular accumulation of BDNF results in several excitatory post-synaptic events (Farber, Kettenmann 2006).

## 2.2.1. Signal pathways through P2Y receptors

Extracellular nucleotides can act both as paracrine or autocrine modulators and their appearance in extracellular fluid can result from cell lysis, exocytosis or efflux through membrane transport proteins. The principal role of these extracellular nucleotides is to mediate signalling pathways by acting on purinergic receptors.

Focusing on P2Y receptors, many pathways could be activated by this receptor activation. Activated pathway depends both, on the nucleotide and on the type of P2Y receptor. It is well known that different P2Y subtypes are coupled to specific G proteins as shown in **Figure i6 and i13**(Erb, Liao et al. 2006). All cloned P2Y receptors ($P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$) are coupled to G proteins **(Figure i13)**, and through the inositol phosphate ($IP_3$) pathway, they led to phospholipase C (PLC) activation, over-expression of inositol phosphates and mobilization of $Ca^{2+}$ from intracellular stores (Kügelgen, Wetter 2000). Multiple signal transduction pathways have been shown to be involved in calcium mobilization, such us activation of protein kinase C, phospholipase $A^2$ or $Ca^{2+}$ sensitive ion channels. The specific transduction pathway seems to depend not only on the P2Y subtype, but also on the cell type expressing each receptor (Ralevic, Burnstock 1998). The response time of P2Y receptors is longer than that of the P2X receptors because P2Y receptors activation involves second-messenger system (Communi, Janssens et al. 2000).

| Receptor | Agonist (human) | G protein | Main effector molecules |
|---|---|---|---|
| $P2Y_1$ | ADP | $G_{q/11}$ | PLC (+), $Ca^{2+}$ release |
| $P2Y_2$ | ATP, UTP | $G_{q/11}$ | PLC (+), $Ca^{2+}$ release |
| | ATP, UTP | $G_o$ | PLC (+), $Ca^{2+}$ release |
| | | | Rac (+) |
| | ATP, UTP | $G_{12}$ | RhoA (+) |
| $P2Y_4$ | UTP | $G_{q/11}$ | PLC (+), $Ca^{2+}$ release |
| | UTP | $G_o$ | PLC (+), $Ca^{2+}$ release |
| $P2Y_6$ | UDP | $G_{q/11}$ | PLC (+), $Ca^{2+}$ release |
| $P2Y_{11}$ | ATP | $G_{q/11}$ | PLC (+), $Ca^{2+}$ release |
| | ATP | $G_s$ | AC (+), increased cAMP |
| | UTP | $G_o$ | PLC-independent $Ca^{2+}$ release |
| $P2Y_{12}$ | ADP | $G_i$ | AC (−), decreased cAMP |
| | ADP | $G_{12/13}$? | RhoA (+) |
| $P2Y_{13}$ | ADP | $G_{i/o}$ | AC (−), decreased cAMP PLC (+), $Ca^{2+}$ release |
| $P2Y_{14}$ | UDP-glucose | $G_{i/o}$ | PLC (+), $Ca^{2+}$ release |

**Figure i13: P2Y receptor subtypes, agonists, G-protein coupling and main effector molecules.** (Erb, Liao et al. 2006)

From all the known P2Y receptors, only three of them are known to be activated through uridine nucleotides: $P2Y_{2,4}$ are activated by UTP and $P2Y_6$ is activated by UDP. Besides, these receptors induce MAPK-dependent or independent pathways, once activated, as shown in **Figure i14**. Stimulation of PLC results in the activation of tyrosine kinases, ending in the activation of c-jun or c-*myc,* via the Ras-MAPK cascade or in otherwise in an $IP_3$- mediated release of calcium from intracellular stores.

P2Y receptors are present in different cell types, as explained before in this manuscript. The response to GPCR activation varies do both, to the cell type specific expression of effector proteins and to the cross-talk occurring between various signalling pathways (Liebmann 2004). In the follow diagram (**Figure i14**) we can see that the $P2Y_2$ receptor activation, through UTP or ATP, induces $IP_3$ that in turn mediates $Ca^{2+}$ release from the endoplasmic reticulum. Finally, this $Ca^{2+}$ is the responsible of the activation of three MAPK: ERK1/2, p38 and JNK.

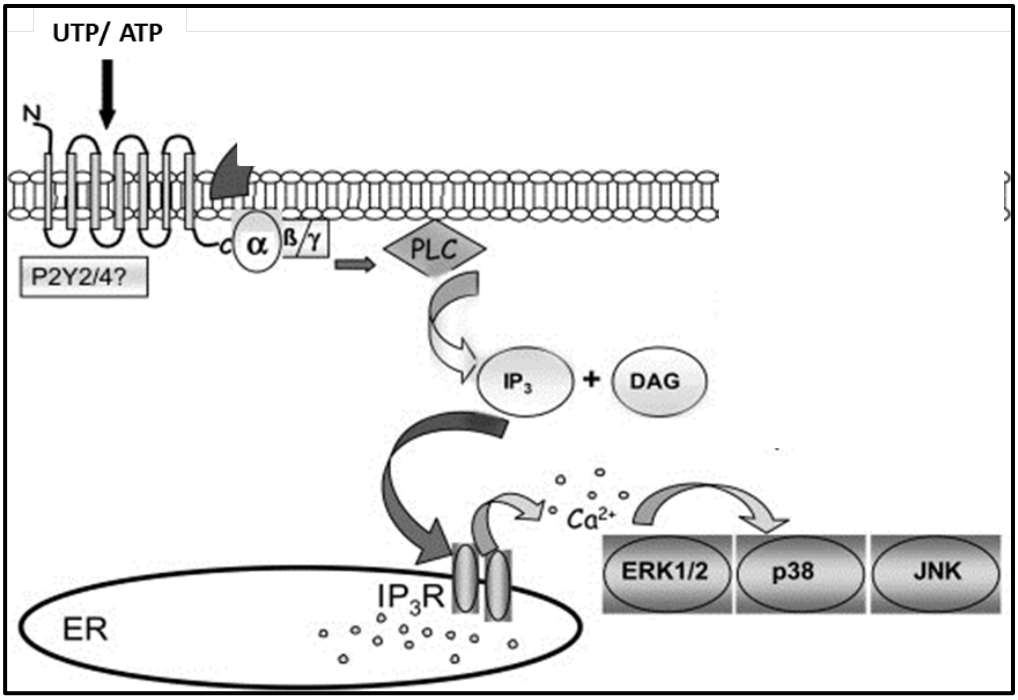


**Figure i14: Schematic model depicting UTP stimulation of MAPKs.** The $P2Y_2/_4$ purinergic receptor-mediated pathway by which UTP is induce PLC activation, and modulate ERK 1/2, p38 and JNK ½ activation, through the calcium release from the endoplasmic reticulum. Adapted from (Liebmann 2004)

#### 2.2.1.1. MAPK family

P2Y receptor stimulation has been shown to activate MAPK (kinase protein activated by mitogen) in a multiple range of cell types including microglia (Potucek, Crain et al. 2006, Shigemoto-Mogami, Koizumi et al. 2001). In multiple cell types, there are clearly evidence suggesting that $P2Y_{1,2,12,13}$ receptors are involved in ERK ½ activation (Potucek, Crain et al. 2006, Marteau, Communi et al. 2004, Muscella, Giovanna et al. 2003). $P2Y_2$ receptors, activated by UTP, have also been studied in monocytes and macrophage cells, in which it has been seen that $P2Y_2$ activation led to ERK ½ phosphorylation, but anyway there is little information of the exact mechanism of UTP action in microglia and nervous system.

All MAPKs, contains a Tyrosine-X-Threonine motifs (X is any amino acid) in the activation domain and are activated through kinase cascade. MAPKKKs (MAPK kinase kinase) activates MAPKK (MAPKs kinase) which finally, activates MAPK by the phosphorylation of threonine and tyrosine residues located at the activation domain, as we can see in the figure below (**Figure i15**). MAPK family can be subdivided into three groups: kinase regulated by extracellular signals (ERK ½), which displays a threonine-glutamine-tyrosine motif, p38 MAPK isoforms which have a threonine-alanine-tyrosine motif and finally JNK which present a threonine-proline-tyrosin motif (Kim, et al. 2004).

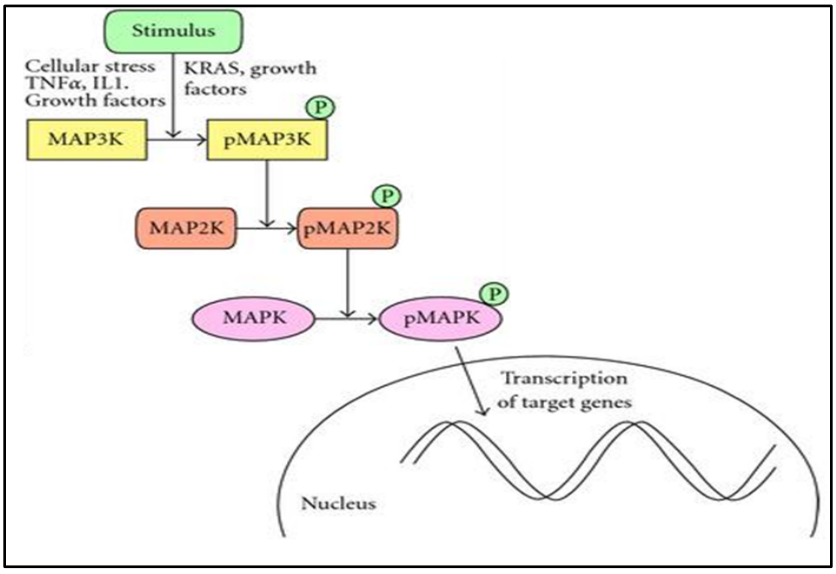


**Figure i15: General scheme of MAPK activation by phosphorylation.** MAPKKK is phosphorylated only in one site, MAPKKK activation leads to MAPKK activation, which can be phosphorylated in both, Ser or Thr residue. Finally, MAPKK activation leads to MAPK phosphorylation in both one Thr and Tyr residue (Kim, et al. 2004).

MAPKs protein family is a highly conserved module, and it has been shown for years to play a very important role in various cellular functions, including cell proliferation, survival, differentiation and migration, as shown in **Figure i16**. Extracellular stimuli such as growth factors, mitogens or nucleotides, induce sequential activation of MAPKs kinase kinase (MAPKKK) followed by MAPKs kinase activation (MAPKK) and finally MAPK activation. At least, four members of the MAPKs family have been identified nowadays: extracellular signal-regulated kinase ½ (ERK ½), p38 and c-Jun N-terminal kinase (JNK) and ERK 5 (Nishimoto, Nishida 2006, Nishida, Gotoh 1993, Sturgill, Wu 1991, Wang, Touener 2006). As with many other signaling pathways, complex regulatory mechanisms are used to direct the functional outcome mediated by MAPKs.

ERK ½ is found to be mainly responsive to stimulation, by a broad range of growth factors (Ramos, 2008); while p38 and JNK are most popularly called stress-activated MAPKs (SAPKs) due to their activation by physical, chemical and physiological stressors such us thermic shock or ultraviolet radiation between others. MAPKs form a complex signalling network, which can be induced by a large array of external stimuli, and can achieve highly specific cellular effects through multitudes of regulatory mechanisms (Rose, Force et al. 2010). Several studies support the involvement of ERK/MAPK signalling in a lot of aspects of neural development and function (Urushihara, Kinoshita 2005, Newbern, Xiaoyan et al. 2011).

The MAPK signaling generally refers to four cascades, each of them defined by the final level of the pathway. Extracellular signal regulated kinases 1 and 2 (ERK ½), ERK 5, c-Jun-N-terminal kinases (JNK) and p38 (**Figure i16**). In the nervous system, ERK ½ is the primary MAPK cascade activated by trophic stimuli and has been shown to modulate proliferation, migration and growth (Newbern, Xiaoyan et al. 2011). Schwann cell development is dependent on extracellular factors such us nucleotides and growth factors that act through tyrosine kinase receptors, which are capable of activating PI3K, PLC, ERK ½, JNK or p38 (Newbern, Xiaoyan et al. 2011). ERK ½ have been shown to regulate the survival of early and also mature Schwann cells in vitro (Li, Tennekoon et al. 2001, Parkinson, Lanner et al. 2002). On the other hand, some studies suggest that ERK ½ signaling regulates in some way Schwann cell myelination, among the exacts mechanisms have not yet been elucidated. (Maurel, Salzer 2000, Li, Tennekoon et al. 2001).

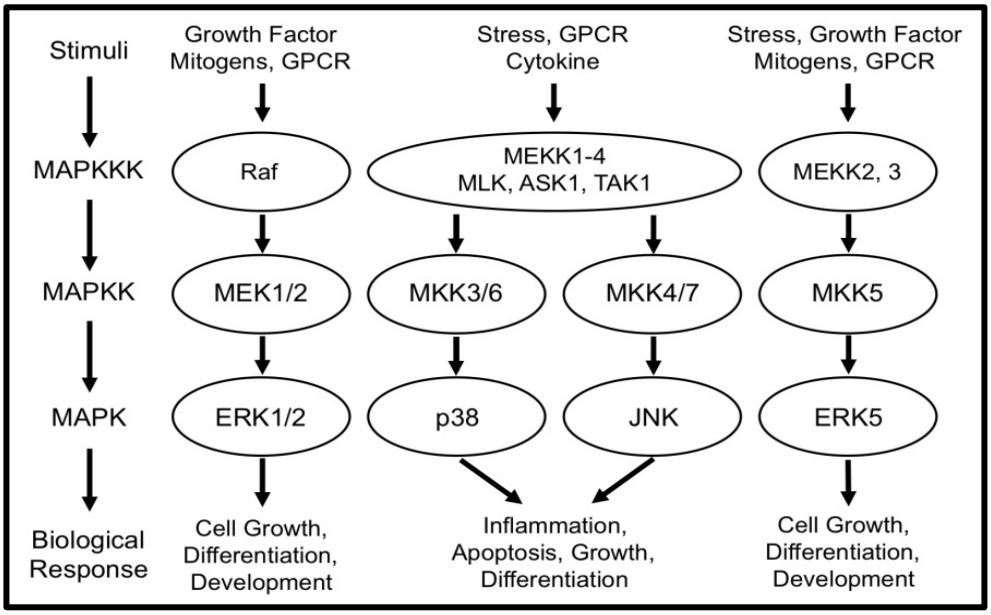


**Figure i16. Mitogen-activated protein kinase (MAPK) signaling.** Different stimulus such us growth factors, mitogens or stress, can result in MAPKKK activation by phosphorylation, which in turns results in MAPKK activation also by phospholrylation and finally MAPK activation, and it results in different biological responses such us cell growth, differentiation or development between other. (Rose, Force et al. 2010).

### 2.2.1.2. Role of nucleotides and MAPK in cell migration

Extracellular nucleotides have been characterized as important chemotactic factors for different cell types (Goepfert, Sundberg et al. 2001, Chaulet, Desgranges et al. 2001). The role of purines and pyrimidines in wound healing has been suggested before. Different studies have shown that both ATP and UTP promoted healing of wounds made in confluent monolayers of epithelial cells (Boucher, Rich et al. 2010, Abbracchio, Burnstock 1998), immune cells (Junger 2011), and renal cells (Klawitter, Hofmann et al. 2007) or leukocytes, between others (Linden 2011). On the other hand, propagation of cell injury signals also depends on activation of extracellular P2 receptors in rat epithelial cells. If cell injury is mechanically induced and stimulated with ATP, they initiate different signalling pathways, one involving intracellular communication via gap junctions, and another involving extracellular communication via activation of P2 receptors linked to $Ca^{2+}$ propagation (Sponsel, Breckon et al. 1995). Also, extracellular ATP promotes cell migration through both autocrine and paracrine pathways, there are some studies made in neutrophils which show that ATP is released in response to stress stimulation (Corriden, Insel 2012). In microglia, extracellular ATP promotes chemotactic cell migration response through P2 receptor activation. (Miller, Stella 2009). Some other studies have

elucidated that cross talk between P2Y receptors and EGFR plays a pivotal role in cell migration and wound closure in epithelial cells (Boucher, Rich et al. 2010).

Many pathways converge to activate the different MAPKs **(Figure i17)**, being this kind of proteins a crucial regulator of the pathways involved in cell proliferation and migration (Pearson, Robinson et al. 2001, Meintanis, Thomaidou et al. 2001). JNK generally was considered to play roles in inflammation, differentiation, apoptosis and insulin resistance (Barr, Bogoyevitch 2001). However, accumulating evidence also implicates JNK pathway in regulation of cell migration in several cell types. For example, JNK activation is highly associated with cell migration induced by EGF in many types of carcinoma cells (Hauck, C.R et al, 2001). It has also been studied that chemical inhibitors of JNK, such as SP600125, inhibit cell migration of epithelial cells, fibroblasts, cortical neurons or Schwann cells (Huang, Rajfur et al. 2003, Javelaud, Laboureau et al. 2003). So, all these studies collectively implicate JNK in the control of cell migration in a broad range of cell types and in several developmental processes. On the other hand, p38 is involved in inflammation, apoptosis and cell differentiation (Ono, Han 2000). Different cell types, such as smooth muscle cells, corneal epithelial cells or fibroblasts, treated with p38 chemical inhibitors (SB203580 and SB202190), show a significant inhibition in their migration rate (Ono, Han 2000, Hedges, Dechert et al. 1999, Sharma, He et al.).

Finally ERK, the most extensively studied subfamily of MAPKs has been implicated in the migration of numerous cell types including fibroblasts and some carcinoma cells (Huang, Rajfur et al. 2003, Huang, Jacobson et al. 2004), Huang, C. 2004, Reddy, K.B. 2003). It has been shown that the over-expression of ERK, through different growth factors such as VEGF, FGF, EGF and other stimuli, induce to an increment of the migration rate in different cellular types (Anand-Apte, Zetter et al. 1997, Klemke, Cai et al. 1997). On the contrary when ERK is inhibited through specific pharmacological drugs, such as PD98059 or U0126, no increase is observed. These results directly relate the ERK expression with cellular migration. (Lai, Chaudhary et al. 2001, Degryse, Orlando et al. 2001, Cuevas, Abell et al. 2003). For the moment, implication of MAPK in glial cell migration is poorly understood.

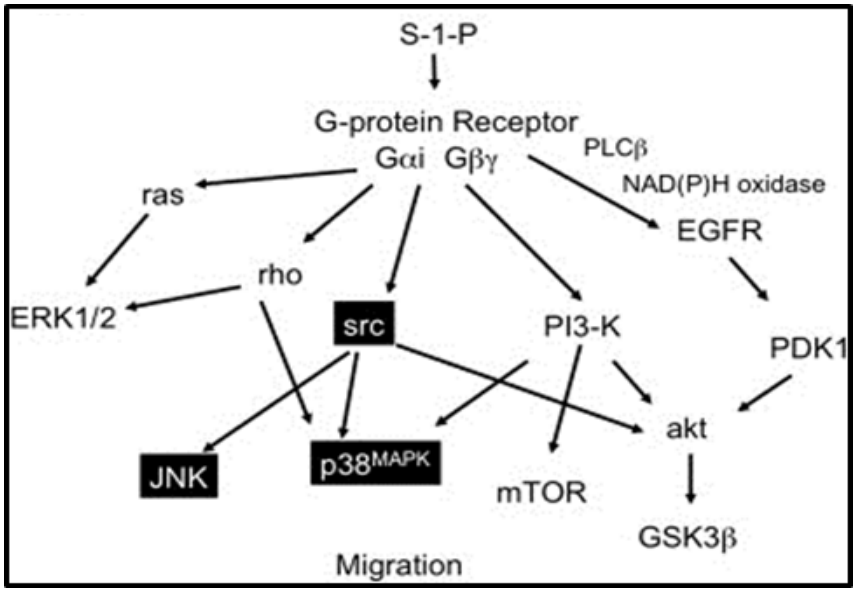


**Figure i17: Different pathway that induces MAPK activation and a migratory phenotype.**

# 3. MATRIX METALLOPROTEINASES

In the sixties, Jerome Gross and collaborators described for the first time an activity that was able to degrade collagen fibers in a tadpole (Gross 1964). Thereafter, it began to be described a large family of proteins capable of degrading components of the extracellular matrix, which are nowadays known with the name of Matrix Metalloproteinases (MMPs). MMPs are a family of enzymes responsible for the proteolytic processing of extracellular matrix structural proteins and regulate cell migration.

## 3.1. Structure

This family consists at least in 23 structurally related zinc-dependent endopeptidases (Kessenbrock, Plaks et al. 2010). As shown in **Figure i18**, this family shares specific functional and structural components, which includes a hydrophobic signal peptide for secretion (Pre), a propetide domain for enzyme latency (Pro), a catalytic domain with highly conserved zinc-binding sites (catalytic) and in most of the MMPs a hemopexin-like domain (PEX) located at the C-terminal region, which is linked to the catalytic domain via a flexible hinge region. This region is known to bind to endogenous tissue inhibitors of MMPs (TIMPs), and it is also involved in MMP activation (Bauvois 2012).

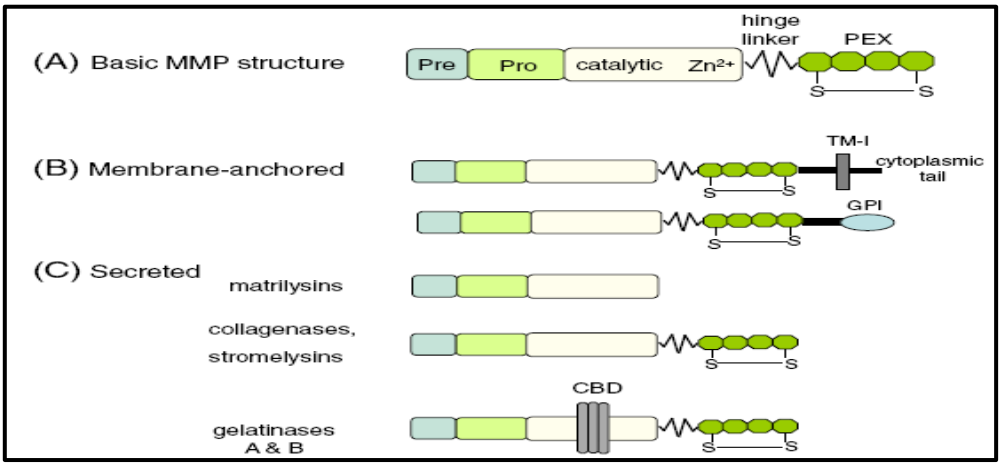


**Figure i18: Structures of the MMP family.** A) General domain structure of MMP family, they are formed by a signal peptide (Pre), a propetide domain (Pro) and a catalytic domain with a $Zn^{2+}$ binding region. B) MT_MMP include membrane anchored MMPs localized in the cell surface through the C-terminal region. C) Secreted MMPs have similar structures and contains repeats of fibronectin type III like domains. Adapted from (Bauvois 2012).

## 3.2. Types of Matrix metalloproteinases

The MMPs family includes membrane anchored MMPs (20b) or secreted MMPs (20c), as we can see in the figure above (**Figure i18**), The membrane anchored MMPs are known to be located at the cell surface by the C-terminal domain (TM-I) or in turn by a glycosylphosphatidylinositol anchor (GPI).

In contrast, secreted MMPs are always secreted as latent pro-enzymes, and this subfamily includes collagenases, stromelysins, matrilysins and two gelatinases (A and B) (Bourboulia, Stetler-Stevenson 2010). The pro domain in this MMPs family is removed by endopeptidases and this re-movement leads to the MMP final activation. TIMPs are known to inhibit most of the secreted MMPs (Brew, Nagase 2010). On the other hand, gelatinases differ from most of the other MMPs ought to their collagen-binding domain (CBD) in the catalytic region (**Figure i18, C**). This domain is known to be composed of three fibronectin type III repeats and is involved in the binding of collagen substrates, elastin, fatty acids and thromospondins (Bjorklund, Koivunen 2005).

The main known role of MMPs is the selectively degradation of several components of the extracellular matrix (ECM) and the posterior release of the growth factors and cytokines that reside in the ECM (Roy, Yang et al. 2009, Hatfield, Reikvam et al. 2010). MMPs are able to degrade almost all the components present in the extracellular matrix (ECM), such as collagen, elastin, fibronectin and laminin (Hartung 2000). On the other hand, MMPs are also known to activate various latent growth factors, cytokines and chemokines and cleaving cell surface proteins (cytokine receptors, cell adhesion molecules, the urokinase receptor, etc…). Due to all of that mentioned above and through their proteolytic activity, MMPs play crucial roles in migration, invasion, metastasis and regulation of signaling pathways that control cell growth, survival, migration, invasion, inflammation and angiogenesis (Kessenbrock, Plaks et al. 2010, Kelin, Bischoff 2011, Roy, Yang et al. 2009, Bauvois 2012).

The expression, secretion and activity of the different MMPs are finely controlled in normal cells. Concretely, MMPs expression is regulated at both transcriptional and post-transcriptional level. Several factors influence the transcription genes coding for these endopeptidases, which include cytokines, growth factors, hormones, oncogenes and tumor promoters between others (Brikedal-Hansen, Moore et al. 1993, Westmarck, Kahari 1999). Nowadays, it is

known that both cytokines and growth factors are able to regulate the expression of metalloproteinases through the MAPK pathway. Activation of AP-1 and ETS transcription factors by MAPK are responsible for the maximum expression of MMPs (Westmarck, Kahari 1999).

Described so far, there are 25 known MMPs (**Figure i19**), which are classified in function of their principal substrate. Their molecular structure is quite similar between all them.

| GENE | COMPLET NAME | LOCATION | SUBSTRATE DESCRIPTION |
|---|---|---|---|
| MMP-1 | Interstitial collagenase | secreted | Col I, II, III, VII, VIII, X, gelatin |
| MMP-2 | Gelatinase-A | secreted | Gelatin, Col I, II, III, IV, Vii, X |
| MMP-3 | Stromelysin 1 | secreted | Col II, IV, IX, X, XI, gelatin< |
| MMP-7 | Matrilysin, PUMP 1 | secreted | membrane associated through binding to cholesterol sulfate in cell membranes, substrates include: Casein, fibronectin, laminin, Col IV, gelatin |
| MMP-8 | Neutrophil collagenase | secreted | Col I, II, III, Vii, VIII, X, aggrecan, gelatin |
| MMP-9 | Gelatinase-B | secreted | Gelatin, Col IV, V |
| MMP-10 | Stromelysin 2 | secreted | Col IV, laminin, fibronectin, elastin |
| MMP-11 | Stromelysin 3 | secreted | Col IV, fibronectin, laminin, aggrecan |
| MMP-12 | Macrophage metalloelastase | secreted | Elasin, fibronectin, Col IV |
| MMP-13 | Collagenase 3 | secreted | Col I, II, III, IV, IX, X, XIV, gelatin |
| MMP-14 | MT1-MMP | membrane-associated | type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin |
| MMP-15 | MT2-MMP | membrane-associated | type-I transmembrane MMP; substrates include gelatin fibronectin, laminin |
| MMP-16 | MT3-MMP | membrane-associated | type-I transmembrane MMP; substrates include gelatin, fibronectin, laminin |
| MMP-17 | MT4-MMP | membrane-associated | attached; substrates include fibrinogen, fibrin |
| MMP-18 | Collagenase 4 | Unknown | Unknown |
| MMP-19 | Stromelysin-4 | Unknown | Unknown |
| MMP-20 | Enamelysin | secreted | Unknown |
| MMP-21 | X-MMP | secreted | Unknown |
| MMP-23A | CA-MMP | Unknown | Unknown |
| MMP-23B | - | membrane-associated | Unknown |
| MMP-24 | MT5-MMP | membrane-associated | Unknown |
| MMP-25 | MT6-MMP | membrane-associated | Unknown |
| MMP-26 | Matrilysin-2, endometase | Unknown | Unknown |
| MMP-27 | MMP-22, C-MMP | Unknown | Unknown |
| MMP-28 | Epilysin | Membrane anchored | Unknown |

**Figure i19. MMP classification.** Name, substrate description and cellular localization are shown. Adapted from (Brikedal-Hansen, Moore et al. 1993)

At a posttraductional level, the biological activity of MMPs is regulated by their state of activation. Many MMPs are secreted from cells in latent form as zymogens (pro-MMPs). For the activation of these MMPs there is first a rupture of the zinc-cysteine bond followed by the amino-terminal pro domain loss. For many MMPs, proteolytic activation begins in the extracellular space by serine proteases or other members of the MMPs family (Nagase 1997).

Active MMP may remain localized on the cell surface through binding with membrane molecules and this leads to a more focused ECM degradation. The regulation of MMP activity is strictly controlled at three different levels: gene transcription, pro-enzyme activation, and activity of tissue inhibitors of metalloproteinases (TIMPs). Various cytokines, such as interleukine-1, transforming growth factor-β or growth factors can directly induce or suppress MMP expression at transcriptional level (Hartung 2000). However, the regulatory effect is more likely to be dependent on the MMP signal transduction/synthesis cascade within a stimulated cell type, rather than on the cytokine acting as a ligand because the same cytokine can have either an inducible or suppressive effect on MMP expression in different cell types. Once synthesized MMPs are secreted as inactive zymogens (**Figure i20**). The activation of most of these propeptides involves sequential exogenous or endogenous cleavage steps, destabilizing the cysteine-zinc interaction, modifying the enzyme conformation, and allowing further exogenous or auto-catalytic processing to the final active form. The active form is subjected to inhibition by TIMPs, which are ubiquitously expressed, in the extracellular media.

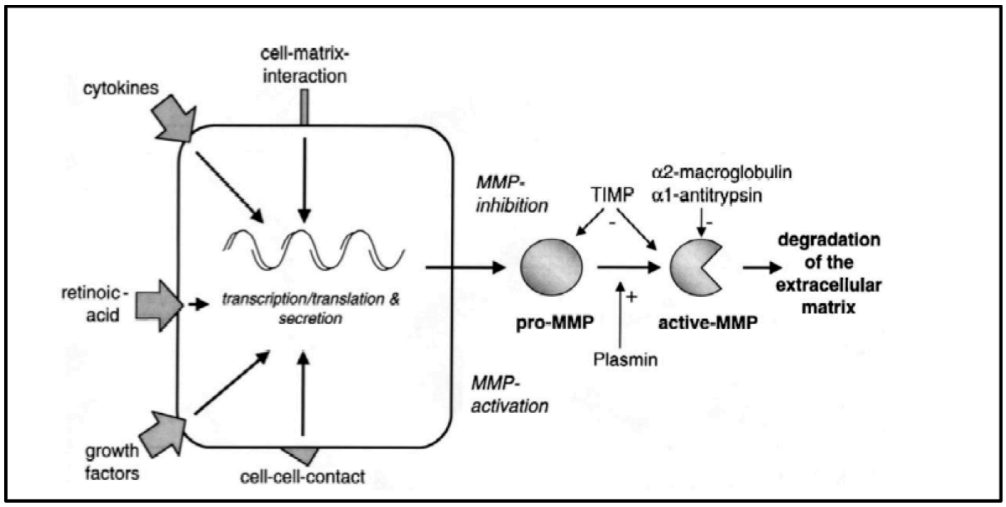


**Figure i20: Regulation of MMP synthesis and secretion.** These processes are dependent on various factors. Most MMPs are secreted as inactive zymogens that require extracellular activation. Adapted from (Hartung 2000).

ECM degradation is a very important step in many processes, such us wound healing, angiogenesis, invasion or metastasis between others and MMPs so as their inhibitors seem to be indispensable in many of them (Shapiro, Khodalev et al. 2010, Giannelli, Falk-Marzillier et al. 1997, Wilson, Hoeppner et al. 1997, Woessner 1994). Since MMPs seem to be important for ECM degradation, they must have a finely regulation, in order to avoid tissue destruction or cell invasion.

## 3.3. Gelatinase MMPs

Gelatinase MMP's are principally MMP-2 (or A) and MMP-9 (or B). AS we have seen before, and as we can see in the figure below (**Figure i21**), these two MMPs have similar structures. Both of them are secreted as inactive zymogens: proMMP-2 is known to have a molecular weight of 67 KDa. In contrast, proMMP-9 has a molecular weight of 92 KDa. Both of them suffer a cleavage of the pro-domain yielding to the active form of the metalloprotease MMP-2 with 60 KDa and MMP-9 with 82 KDa (Toth, Gervasi et al. 1997).

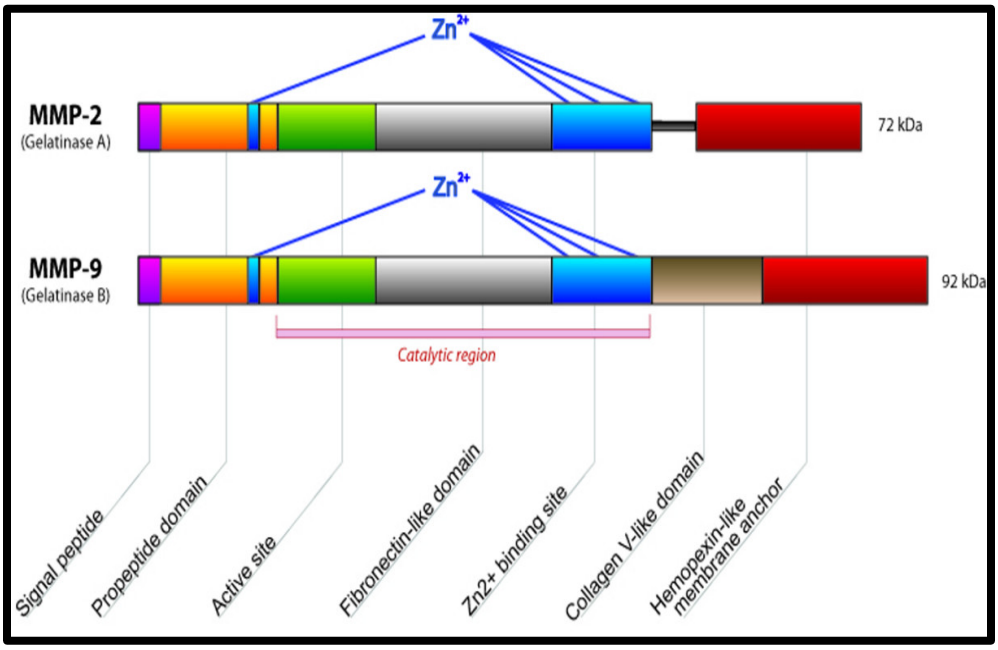


**Figure i21. Schematic structure of MMP-2 and MMP-9.** The catalytic site contains three essential zinc ion binding sites. At the zymogen stage, a cysteine residue within the pro-domain interacts with zinc to prevent substrate binding. The haemopexin domain mediates interaction with enzyme substrates. Specific to the gelatinases is the fibronectin-like domain, which further facilitates substrate binding. (Tveita, et al. 2008).

Several mechanisms are known to stimulate the activation process of these MMPs. The activation of proMMP-2 on the cell surface occurs through the formation of a molecular complex containing proMMP-2 (via the PEX domain), MT1-MMP (via the catalytic domain) and TIMP-2. This cell surface interaction leads to clustering of proMMP-2 near a free TIMP, which in turn activates MT1-MMP, and this finally starts the activation of proMMP-2. It has been studied that MMP-2 can also be activated by MMP-1, MMP-7, thrombin and activated protein C (Fridman, R.: Toth, M.: Chvyrkova, S.O.: Meroueh, S.: Mobashery, S. 2003). Also, in 1994, Sato et al. describes that the anchored matrix metalloproteinase MMP-14 was the promoter of MMP-2 activation (Sato, Takino et al. 1994).

In the other hand, gelatinases differ from most of the other members of the MMPs family, in that they have a collagen-binding domain (CBD) within the catalytic domain (**Figure i18C**). This CBD domain is composed of three-fibronectin type II repeats and its known to be involved in the binding of collagen substrates, elastin, fatty acids and thrombospondins (Bjorklund, Koivunen 2005). Activated gelatinases are able to degrade various components of the ECM (**Figure i22**) such us collagens or gelatins and non-matrix proteins such us some interleukines or growth factors (EGF), (Bjorklund, Koivunen 2005, Fridman, R.: Toth, M.: Chvyrkova, S.O.: Meroueh, S.: Mobashery, S. 2003).

Gelatinase substrates.

| Substrates | MMP-2/gelatinase A | MMP-9/gelatinase B |
|---|---|---|
| ECM substrates | Collagens I, IV, V, VII, X and XI | Collagens III, IV and V |
| | Gelatin | Gelatin |
| | Tenascin | Elastin |
| | Elastin | Vitronectin |
| | Fibronectin | Entactin |
| | Laminin-5 | |
| Other substrates | proTGF-β | proTGF-β |
| | proIL-1β | proTNF-α |
| | proTNF-α | IL-2Rα |
| | proHB-EGF | ICAM-1 |
| | FGFR-I | EGFR-1 |
| | IGFBP-3, -5, -6 | Kit ligand |
| | CXCL12/SDF-1 | CXCL1/GRO-α |
| | CCL7/MCP-3 | CXCL4/PF4 |
| | CX3CL1/fractalkine | CXCL8/IL-8 |
| | KISS-1 | CXCL9/MIG |
| | | CXCL11/ITAC |
| | | CXCL12/SDF-1 |
| | | α1 proteinase inhibitor |
| | | Plasminogen |
| | | KISS-1 |
| | | IFN-β |

**Figure i22: The different substrates of MMP-2 and MMP-9 in the ECM and other substrates.** (Tveita, et al. 2008).

### 3.3.1. Role of gelatinases on the nervous system

MMPs activity is important for the proper development of a broad range aspect of the neural microenvironment (Yong, Power et al. 2001). Some of the members of the MMPs family has been shown to play some functional roles on the nervous system. And, although matrix metalloproteinases are increasingly being implicated in several pathologies, it is not year clear what role they play in normal neurobiological processes.

Some recent *in vitro* studies suggest that at least two processes in the pathogenesis of inflammatory demyelination are mediated by MMPs: MMP-9 seems to promote migration in T lymphocytes which implies a new important role for MMPs in the process of T cell migration from the blood to the nervous system (Leppert, Waubant et al. 1995). On the other hand, MMPs appears to be involved also in the process of demyelination. Myelin basic protein (MBP) and P0 are both also substrates of proteolytic MMP activity (Chandler, Coates et al. 1995). MMP-9 is an intriguing MMP family member, found in the adult nerve only after injury (Chattopadhyay S, Shubayev 2009). It has been demonstrated that dominant negative MMP-9 gene knockout mice (MMP9 -/-) have remarkable protection from peripheral Wallerian degeneration, due probably to MMP-9 control of myelin protein degradation and macrophage migration into the injured sciatic nerves (Shubayev, Angert et al. 2006, Chattopadhyay, Myers et al. 2007).

In contrast, upregulation of MMPs has been attributed to the pathogenesis of experimental and clinical peripheral nerve damage (Leppert, Hughes et al. 1999), but their role in Schwann cell survival, migration and regulation of trophic signaling is not really understood. Upregulation of MMPs has been linked to the pathogenesis of peripheral nerve damage (Demestre et al., 2004; Leppert et el., 1999; Platt et al., 2003; Shubayev and Myers 2002). In rat sciatic nerve, after crush and during regeneration, a rapid and transient increase in MMP-9 has been localized at and immediately distal to the site of injury; whereas an increase in MMP-2 activity was delayed, and maintained during nerve regeneration proximally and distally to the injury site. This activity coincides with periods of axonal elongation, suggesting that it could act to facilitate axonal extension along the nerve matrix (Platt, Krekoski et al. 2003). Therefore, the modulation of MMPs activity may be a relevant target to enhance regeneration in demyelinating diseases of the nervous system (Lehmann, Köhne et al. 2009).

MMP-28 has been studied to act as a suppressor of myelination, and the inhibition of this metalloprotease may be beneficial in the promotion of myelin repair, as we can see in **Figure i23** (Werner, Dotzalf et al. 2008). Some studies show that MMP-28 expression is predominantly neural and its downregulation precedes myelination. MMPs activities are known to regulate molecules related to this process, such as Neuregulin, Bace-1 or ErbB receptors, represented in the figure below (Werner, Dotzalf et al. 2008).

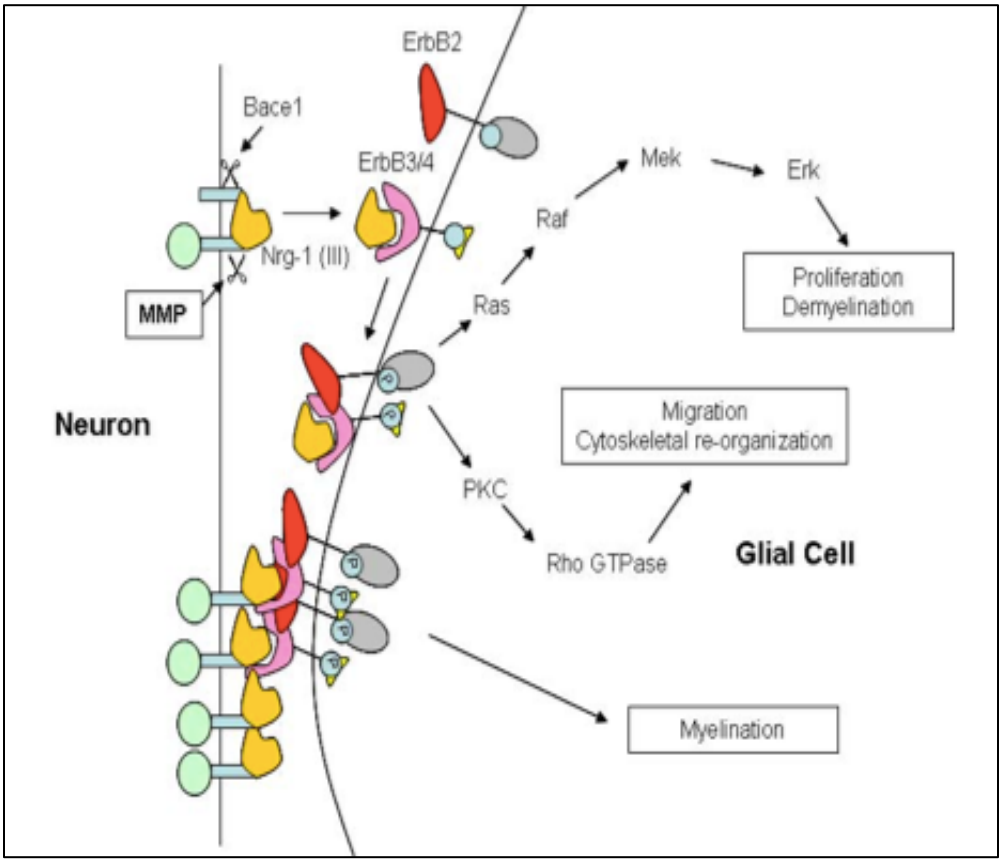


**Figure i23: Myelination signaling pathway.** Neuregulin signaling can led to myelinating, proliferative or migratory response depending on some external factors such as proteolysis of metalloproteases. Adapted from (Werner, Dotzalf et al. 2008).

Moreover, nerve biopsies from patients with Guillain-Barré syndrome show increased MMP-9 and MMP-7 mRNA expression. This upregulation on the messenger level was also associated with an increased in the gelationlytic activity (Kieseier, Clements et al. 1998). More recently, MMP-2 and MMP-9 augmented reactivity was reported in nerve biopsies from patients with chronic inflammatory demyelinating polyradiculopathy (Leppert, Hughes et al. 1999).

### 3.3.2. Role of gelatinases on cell migration

Cell migration is an extremely complex process that requires the temporal and spatial coordination of multiple mechanisms such as actin polymerization leading to forward membrane extensions, formation and releases of focal adhesion acting as traction points, and myosin motor activity leading to production of contractile force (Lauffenburger, Horwitz 1996). Although cell migration is crucial in normal physiological functions like embryonic development, inflammatory immune response, wound repair or angiogenesis, it is also known that this process plays critical functions in cancer and metastasis.

On the other hand, secretion of extracellular proteases plays an important role in immune functions, wound healing and cancer cell invasion. The degradation and remodeling of the extracellular matrix (ECM) are essential stages of migration, invasion in normal and tumor cells. Two types of proteolytic enzymes primarily mediate these processes: the plasminogen activator system (uPA) and the matrix metalloproteinases (MMPs) (Stetler-Stevenson W.G., Aznavoorian et al. 1993). Matrix metalloproteinases are often thought to facilitate migration and metastasis by breaking down barriers formed by extracellular matrix (ECM), but a clear demonstration of such role for the MMPs is limited to only a few members (Parks, Wilson et al. 2004, Sabeh, Li et al. 2009).

Many extracellular signals converge on the pathway of proteins belonging to the serine/threonine kinase family known as MAPK, which are known to play important roles in cell proliferation, differentiation or inflammation between others. As explained before in this manuscript, several evidence suggests that these kinases are also crucial in cell migration (Fu, P.: Jiang, X.: Arcasoy, M.O. 2009). Experiments with inhibitors strongly suggest the involvement of cell signaling pathways in MMP-9 mediated cell migration. For example, some studies using JNK inhibitor SP600125 blocked MMP-9 mediated dendritic cell migration (Hu, PIK et al. 2006), whereas the MAPK inhibitor PD8059 and the PI3K inhibitor LY-294002 inhibited MMP-9 induced epithelial cell migration (Dufour, Sampson et al. 2008).

Recent studies have described the signaling properties of MMP-2 and MMP-9, by using siRNA and enzyme inhibitors against MMPs targets. These gelatinases are clearly involved in cell survival, cell migration and tumor angiogenesis through different pathways, as shown in **Figure i24**. Also, some studies demonstrate the role of matrix metalloproteases in the epithelial cell migration (Chen, Parks 2009).

The expression of matrix-degrading proteolytic enzymes, such as PAs and MMPs, could be regulated by JNK and p38 signals in order to promote migration in cells (Koochekpour, Sartor et al. 2005, Hecht, Heider et al. 2007). Also, earthworm extract is known to promote Schwann cell migration through the activation of Pas and MMPs mediated by ERK ½ and p38 MAPK pathways (Chang, Shih et al. 2009).

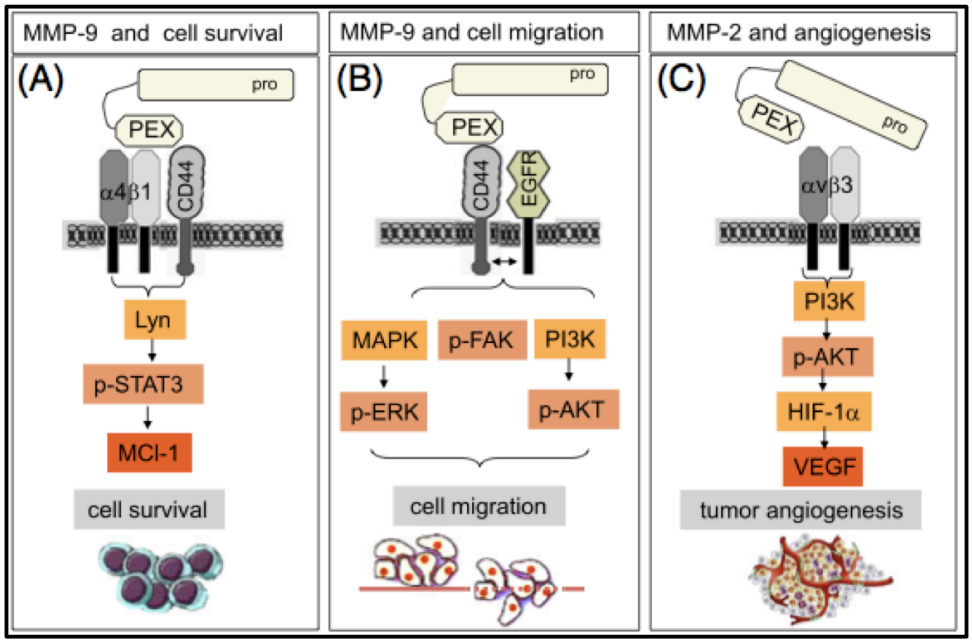


**Figure i24: Schematic representation of the signaling pathways induced by gelatinases and integral receptors.** A) MMP9 interacts via PEX domain with α4β1 and CD44 on B-CLL cells, leading to Lyn activation, STAT3 phosphorylation, which is essential for lymphocyte cell survival. B) PEX domain of MMP-9 interact with CD44 on tumor epithelial cells leading to activation of EGFR and posterior activation of MAPK, PI3K and FAK, which in turn induces epithelial cell migration. C) Interaction of proMMP-2 with integrin αvβ3 on lung cancer cells activate PI3K/Akt pathways leading to the activation of hypoxia-induced transcription factor-1α, which induces VEGF activation and all this drives to tumor angiogenesis and progression (Bauvois, 2012).

# IX. MATERIALS AND METHODS

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## 1. CELL CULTURES

### 1.1. RT4D6P2T cell line

Rat schwannoma cell line RT4-D6P2T (Schwannoma cells) was purchased from the European Collection of Cell Cultures (#93011415; ECACC, Salisbury, UK) and maintained in DMEM High Glucose medium supplemented with 2 mM L-Glutamine, 50 U/mL Penicillin, 50 mg/L Streptomycin, and 10% (v/v) DBS (Donor Bovine Serum). Culture cells were incubated in a 5% $CO_2$ humidified atmosphere at 37°C. This cell line express typical Schwann cells markers such us S100.

#### 1.1.1. Cell growth and preservation:

Schwannoma cells were maintained in **Growth Media** (DMEM High Glucose supplemented with 10% DBS, 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin) to expand, and the medium was replaced for **Deprivation media I** (DMEM High Glucose supplemented with 1% DBS, 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin), or **Deprivation media II** (DMEM High Glucose supplemented with 0% DBS, 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin), at least for two hours before the treatments.

Cell line was growth in **Growth media,** at 37ºC in a humidified atmosphere and 5% of $CO_2$. To subculture, cells were trypsinized with 0.25% Trypsin-EDTA (25200 Life Technologies) for 5 min at 37ºC. Trypsin reaction was blocked by the addition of **Growth Media**. Cells were centrifuged at 1.000 rpm for 5 minutes and seeded at the required density (**Table m1**).

To preserve the cell line, cells were frozen in liquid nitrogen. To do that, cells were trypsinized, counted and frozen at a density of 1 x $10^6$ cells/mL in **Frozen Media**(DMEM High Glucose supplemented with 20% DBS, 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin and 10% DMSO)**.** Cell suspension was transferred into criotubes and stored in a Cryo Freezing (Nalgene) container with isopropanol at -80ºC during 48h. Later cells were transferred to a liquid Nitrogen tank (-196ºC) for their storage.

## 1.2. Primary Schwann cell primary culture

### 1.2.1. Schwann cell isolation.

Schwann cell isolation was made as described before (Johnson, Taniuchi et al. 1988) with little variations. Schwann cells were isolated from the sciatic nerves of 3-8 days old Sprague-Dawley rat puppies. Puppies were obtained from the Animal Housing Service of the Universitat Autonoma de Barcelona, following the ethical rules on handling animals in experimental and other scientific purposes.

Schwann cells were obtained by incubating the shredded and cleaned sciatic nerves in **Digestion Media I** (PBS-$Ca^{2+}$, 0.01% Collagenase and 0.01% BSA), for 1h and 45min at 37ºC, mixing occasionally. Then, cell suspension was centrifuged at 1000 rpm for 10 min, supernatant was removed and cells were incubated with 1 mL of **Digestion Media II** (PBS + 0.25% Trypsina), for 15 min at 37ºC. Finally, cell suspension was centrifuged at 1000 rpm for 10 min, supernatant removed and cells resuspended in 1 mL of **Primary Cell Growth Media** (DMEM High Glucose, supplemented with 10% DBS, 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin, 20 µM Forskoline and 20 µg/mL Pituitary extract).

After chemical dissociation with **Digestion Media I and II**, we proceeded to perform mechanical dissociation, with three different diameters of Pasteur pipette (Big, medium and thin). Dissociated cells were resuspended in **Primary Growth Media** and plated at a density of 70.000 cells/mL on 6 $cm^2$ dishes for 3-4 hours, in order to eliminate contaminating fibroblasts, since fibroblast adheres faster on the plate. After that time, the media with non-adhered cells was collected and plated, using a 6 well plate dish with coverslips, previously coated with 0.01 % (v/v) Poly-L-lysine and 4 g/L Laminine, with the aim that the cells adhere to the plate.

After three days, medium was replaced completely for fresh **Primary Growth Media**. One week later, Schwann cells were slow trypsinized for 5-15 minutes, in order to eliminate any contaminating fibroblast monolayer. Purified Schwann cells were maintained in **Primary Growth Media**, at least for 1 week before making any experiment (**Figure m1**).

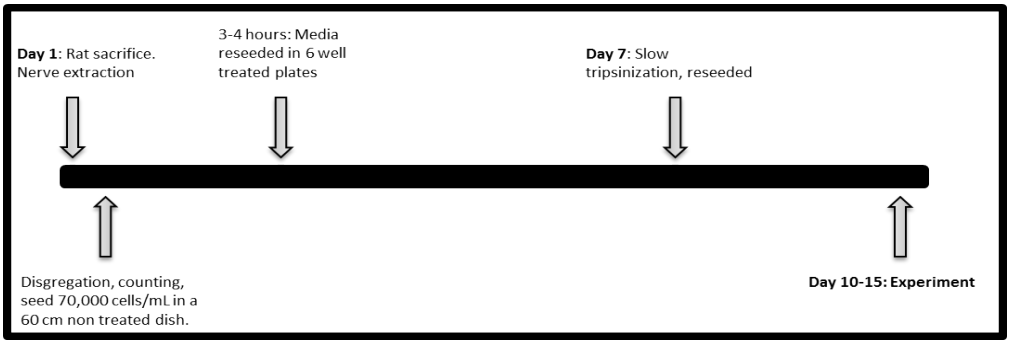


**Figure m1 Primary Schwann cell culture scheme.** Temporally scheme of the days of culture, is showed. At day one, nerve extraction is performed, and Schwann primary cells are led to growth until day 7, in which a slow trypsinization is performed and cells are reseeded. Between day 10 or 15 Schwann primary culture can be used for further experiments.

### 1.2.2. Primary Schwann cell media

Primary Schwann cells were maintained in **Primary Growth Media** (DMEM High Glucose, supplemented with 10% DBS 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin, 20 µM Forskoline and 20 µg/mL Pituitary extract) at least for one week after cell isolation was performed. After that, to perform the different experiments, media was replaced for **Primary Deprivation Media I** (DMEM High Glucose, supplemented with 1% DBS 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin, 20 µM Forskoline and 20 µg/mL Pituitary extract)**,** in metalloproteases experiments, and with **Primary Deprivation Media II** (DMEM High Glucose, supplemented with 0% DBS 2mM Glutamine, 0.25 g/L Penicillin/Streptomycin, 20 µM Forskoline and 20 µg/mL Pituitary extract) for migration experiments.

## 1.3. Cell density and treatment

### 1.3.1. Cell density:

Schwannoma cell line or Schwann primary cultures were seeded at different densities, plates and volumes in function of the technical procedure to perform (**Table m1**). In order to seed them, cells were trypsinized, counted using the Newbauer chamber and seeded on the corresponding plate.

| EXPERIMENTAL PROCEDURE | Cell density (cells/mL) | Type of Plate | Volume (µl) | Coverslips |
|---|---|---|---|---|
| **Wound Healing** | 250.000 | 100 cm | 5000 | No |
| **Boyden Chamber** | 150.000 | 24 well | Upper: 350<br>Lower: 700 | Yes |
| **Zymography** | 250.000 | 6 well | 1000 | No |
| **Gelatin activity** | 200.000 | 24 well | 500 | Yes |
| **Western Blot** | 50.000 | 6 well | 1000 | No |
| **Immunofluorescence** | 200.000 | 24 well | 500 | Yes |
| **Cell transfection** | 200.000 | 24 well | 500 | No |
| **RNA for PCR or qPCR** | 150.000 | 6 well | 1000 | No |

**Table m1. Cell density, plates and volume used in function of the type of experiment to perform.** There is also mentioned if coverslips were needed or not.

## 1.3.2. Pharmacological cell treatment

Both, Schwannoma cell line or Schwann primary cultures, were treated with different nucleotides and/or with different inhibitors, all of them listed below (**Table m2**). In selected experiments, inhibitors were added to the culture media for 30 minutes before nucleotide treatment.

| | REFERENCES | | CONCENTRATION |
|---|---|---|---|
| **NUCLEOTIDES** | **Commercial House** | **References** | |
| UTP | **Sigma** | **U6875** | **250 µM** |
| CMPF | **Ferrer** | **666230** | **1 g/L** |
| UMP | **Ferrer** | **3003378** | **250 µM** |
| UDP | **Ferrer** | **3000408** | **250 µM** |
| CMP | **Ferrer** | **3000098** | **1.7 mM** |
| **INHIBITORS** | | | |
| GM6001 | **Millipore** | **CC1000** | **10 µM** |
| Suramin | **Sigma** | **S2671** | **100 µM** |
| SB203580 | **Sigma** | **S8307** | **10 µM** |
| SP600125 | **Sigma** | **S5567** | **20 µM** |
| U1206 | **Sigma** | **U120** | **10 µM** |

**Table m2. Cell treatments and inhibitors.** It is shown the used concentration of each nucleotide or inhibitor, so as the reference and the commercial house for each.

# 2. METHODS IN MOLECULAR BIOLOGY METHODS

## 2.1. Polymerase Chain Reaction

### 2.1.1. RNA extraction

Total RNA from 150.000 Schwannoma cells was isolated using TRIzol® RNA isolation kit (Invitrogen Life Technologies, 15596018), which allows the separation of DNA, RNA and protein based on the formation of different phases (aqueous and organic).

After cell treatment, media was aspirated and Trizol® added to each well (1mL in 60mm plates). Cells were scrapped and 200 µL of chloroform was added to each mL of cell suspension. Samples were mixed on the vortex for 30 seconds, and phases were separated by centrifugation at 12.000 rpm for 15 min at 4ºC. The upper aqueous phase (500-600 µL) was transferred to a new eppendorf, and 500 µL of ice-cold Isopropanol added to each tube, in order to allow samples to precipitate overnight at -20ºC. Afterwards, samples were centrifuged again at 14.500 rpm for 15 min at 4ºC, the supernatant decanted and the pellet washed with 500 µL of ethanol 75%. Once more, samples were mixed in a vortex for 30 seconds, and centrifuged at 14.500 rpm for 10 min at 4ºC. At last, supernatant was decanted again, and pellet RNA dried at 37ºC for 15 min. RNA was suspended in 25 µL of RNAase free water ($H_2O_{DEPC}$) and solubilized at 55-60ºC for 15 min.

Finally, the concentration and purity of the RNA obtained, was quantified by spectrometry at $A_{260/280nm}$ [1 ($A_{260}$) unit of RNA = 40 µg/mL, in a spectrometry micro plate reader (Biotek Synergy HT)]. Optimal purity values were considered in $A_{260/280nm}$ ratio between 1.8-2.1.

### 2.1.2. Reverse transcription (RT)

Total RNA was reverse transcribed using the 5X iScript cDNA Synthesis Kit (Bio-Rad 170-8890), following the manufacture instructions. RNA (1µg) was mixed with 4 µL of 5X iScript Reaction Mix and 1 µL of the iScript reverse transcriptase. The obtained mix was submitted in a Termocycler (MyCycler, Biorad), to the following protocol to obtain cDNA: 5min at 25ºC, 30 min at 42ºC and 5 min at 85ºC.

| Gene | Accession number (GeneBank) | Direction | Primers | cDNA (bp) |
|---|---|---|---|---|
| MMP2 | NM_031054.2 | Forward<br>Reverse | 5'AGGGAATGAGTACTGGGTCT3'<br>5'CAGTTAAAGGCAGCGTCTAC3' | 474 |
| MMP9 | NM_031055 | Forward<br>Reverse | 5'CGGAGCACGGAGACGGGTAT3'<br>5'TGAAGGGGAAGACGCACAGC3' | 537 |
| MMP7 | NM_012864.2 | Forward<br>Reverse | 5'CGTGATCTCCCCTTGCGAAGCC3'<br>5'CGCACTTCAGTGGGAACAGGCG3' | 407 |
| MMP14 | NM_031056.1 | Forward<br>Reverse | 5'TGGCTTCTCCCACCCTCATAGCTTG3<br>5'GAGTGACGGGAGGCTGCCAAC3' | 237 |
| MMP28 | NM_001079888 | Forward<br>Reverse | 5'TGGTGACTGGCTTGAGCCTCCTG3'<br>5'CCATAGCTGGAAGGCGGCACG3' | 485 |
| $P2Y_2$ | NM_017255.1 | Forward<br>Reverse | 5'CTGCCAGGCACCCGTGCTCTACTT3'<br>5'CTGAGGTCAAGTGATCGGAAGGAG3 | 190 |
| SOX10 | NM_019193.2 | Forward<br>Reverse | 5'ACTCTTGTAGTGAGCCTGGA3'<br>5'TCCGGACTACAAGTACCAAC3' | 148 |
| c-jun | NM_021835.3 | Forward<br>Reverse | 5'GCGGCTGAAGTTGGGCGAGT3'5'GG<br>5'GTTAGCCTGGGCTGTGCG3' | 240 |
| KROX20 | U78102.1 | Forward<br>Reverse | 5' CACTGTTCTCCGAGTTCTG3'<br>5'ACAGAGGACACTTGCAACAC3' | 104 |
| NFKp65 | NM_001276711.1 | Forward<br>Reverse | 5'CTTCTCTCCCCGGAATACT3'<br>5'GGCCATATGTGGAGATCA3' | 563 |
| GAPDH | NM_012992 | Forward<br>Reverse | 5'TGGGAAGCTGGTCATCAAC3'<br>5'GCATCACCCCATTTGATGTT3' | 78 |
| NFKp105 | AA858801.1 | Forward<br>Reverse | 5'GAGCTCCGAGATAATGACAG3'<br>5'GCCTTCTATAGGTCCATCACT3' | 175 |

**Table m3: Primer sequences and molecular weight of the detected PCR products.**

## 2.1.3. - Polymerase Chain Reaction

The cDNA products (250ng) were amplified by PCR, with 0.4 U of Taq polymerase mix (Genecraft, Cologne, Germany), 20 µM of each specific primers, 2.5 mM of dNTPs, 2.5mM of $MgCl_2$ and the corresponding volume of Reaction Buffer 10X and sterile water. The mix was submitted in a Termocycler (MyCycler, Biorad), using a specific programme and primers for each of the analyzed gene (**Table 4** and **3**, respectively). Primers were selected using the BLAST database.

## 2.1.4. Quantitative Real Time PCR

The cDNA template was measured by quantitative Real Time PCR, which was assessed using SYBR Green incorporation (SYBR® Green Kit, Biorad) in the double DNA strain. cDNA (0.1 ng/µL) was mixed with the appropriate primers concentration (250 nM each, **Table m3)** and iQ SYBR Green Mix (1X). All samples were analysed in respect to the housekeeping GAPDH gene, as internal control. Different PCR conditions were used to study the different genes, as explained in **Table m4**. Gene expression was analysed by the ΔΔCt method.

| Gene | PCR Program | | | Cycles |
|---|---|---|---|---|
| | Desnaturalization | Hybridation | Extension | |
| MMP2 | 1' a 95ºC | 3' a 62ºC | 3' a 71ºC | 25 |
| MMP9 | 1' a 95ºC | 3' a 65ºC | 3' a 72ºC | 30 |
| MMP7 | 1' a 95ºC | 3' a 62ºC | 3' a 72ºC | 30 |
| MMP14 | 1' a 95ºC | 3' a 65ºC | 3' a 72ºC | 25 |
| MMP28 | 1' a 95ºC | 3' a 65ºC | 3' a 72ºC | 30 |
| P2Y2 | 1' a 95ºC | 3' a 65ºC | 3' a 72ºC | 25 |
| ID2 | 1' a 95ºC | 3' a 62ºC | 3' a 71ºC | 30 |
| c-jun | 1' a 95ºC | 3' a 62ºC | 3' a 71ºC | 30 |
| Pax3 | 1' a 95ºC | 3' a 62ºC | 3' a 71ºC | 30 |
| Sox2 | 1' a 95ºC | 3' a 62ºC | 3' a 71ºC | 30 |
| Tubuline | 1' a 95ºC | 3' a 65ºC | 3' a 71ºC | 25 |
| GAPDH | 1' a 95ºC | 3' a 56ºC | 3' a 71ºC | 25 |

**Table m4: PCR program used for each gene.** The programs are used both the same, for polymerase chain reaction and for quantitative real time PCR.

### 2.1.5. PCR products validation

PCR products, obtained in section 2.1.3 and 2.1.4, were visualized using DNA electrophoresis in Agarose Gels. DNA fragments migrate through a gel matrix electrophoresis, induced toward the anode due to negative charge of the sugar-phosphate backbone of the nucleic acid. Gels used to check the amplification PCR products contain 1-3% (w/v) agarose (Pronadisa, 8065) dissolved on TAE Buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8). Intercalating agent for DNA, Ethidium Bromide was used at a final concentration 0.005 % (w/v) before matrix solidification. PCR products were mixed with 1X Loading Buffer [0.021% (v/v) Bromophenol Blue, 0.021% (v/v) Xylene Cyanol, 0.02M EDTA at pH 8 and 50% (v/v) Glycerol]. Electrophoresis was carried out at 120 V for 45 min. Amplified DNA fragments were visualized using Gene Flash Synegene Bio Imaging system (Syngene).

## 2.2. shRNA MMP2 generation

Small hairpin RNA or short hairpin RNA (shRNA) is an RNA sequence that makes a tight hairpin turn, which can be used to silence target gene expression. This expression of shRNA in cells is typically accomplished by delivery of plasmids or through viral or bacterial vectors. The promoter choice is essential to have robust shRNA expression (Reynolds, Leake et al. 2004).

### 2.2.1. Design and selection of shRNA MMP-2 sequences

pLVTHM-shRNAi-MMP2-GFP vector, was constructed to express short interfering RNAs to silence MMP-2 expression. A sequence targeting for MMP-2 was selected based on the rules for shRNA susceptibility proposed by Tuschl's group (Reynolds, Leake et al. 2004) and using the shRNA prediction program from the Bioinformatics group of the Whitehead Institute for Biomedical Research. pLVTHM vector has a EF1-alfa promoter (blue rectangle in the **Figure m3**), which is a constitutive promoter of human origin that can be used to drive ectopic gene expression in various *in vitro* and *in vivo* contexts (Reynolds, Leake et al. 2004).

Two complementary DNA oligonucleotides (Roche, Berlin, Germany) were annealed to produce a double-stranded DNA fragment encoding a 19-nucleotide sense strand, 9-nucleotide loop, and 19-nucleotide antisense strand of the MMP2 target or of a random sequence, as shown in the figure bellow (**Figure m2**).


**Figure m2. shRNA for MMP2 generation.** Selected sense strand and antisense strand primers used to generate shRNA against MMP2 (NM_031054).

The sequence to generate the shRNA against MMP-2, is a MMP-2 target sequence that corresponds to bases 2173–2191 of the MMP-2 mRNA (GenBank accession number NM_031054). The sequences of the MMP2 shRNA are as follows:

**1) Sense Strand.**
5'CGCGTCCCCGGAGATACAATGAAGTAAATTAAGAGATTTACTTCATTGTATCTCCTTTTT GGAAAT-3'

**Antisense Strand**
5'CGATTTCCAAAAAGGAGATACAATGAAGTAAATCTCTTGAATTTACTTCAT TGTACTCCGGGGA-3'.

Also, a plasmid carrying a random sequence was used as a shRNA control (shRandom). Sequences for the shRNA-Random are the follows:

**2) Sense Strand.**
5'GATCCCCGCAGTGCAATATCGGAAACTTCAAGAGAGTTTCCGATATT-GCACTGCTTTTT3'

**Antisense Strand.**
5'AGCTAAAAAGCAGTGCAATATCGGAAACTCTCTTGAAGTTTCCGATATTGCACTGCGGG 3'

The duplex DNAs of shRNA-MMP2 and shRNA-Random were cloned into the *ClaI* and *MluI* sites of the pLVTHM vector, which are marked with a red rectangle in the **Figure m3**.

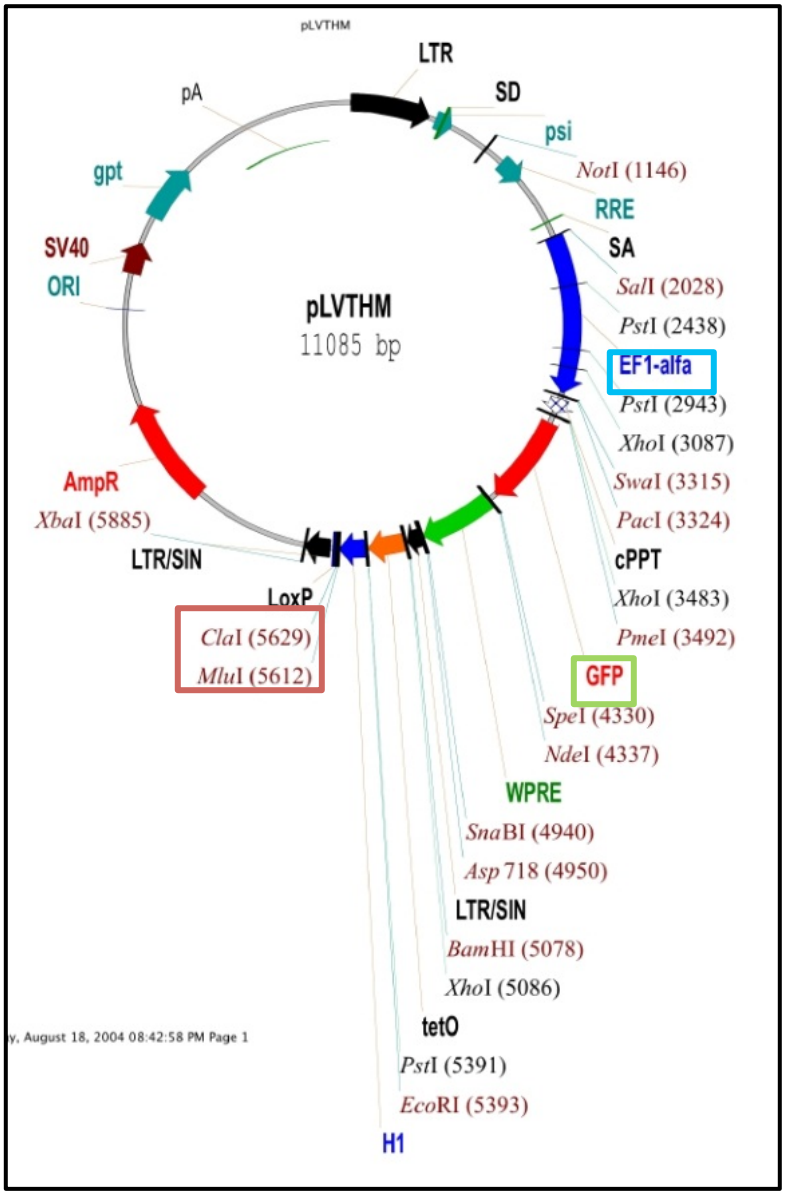


**Figure m3 pLVTHM vector used for shRNA of MMP2 generation**. ClaI and MluI sites (marked with a red rectangle) are located after the EF1-alfa promoter (marked with a blue rectangle) and the GFP region (marked with a green rectangle).

## 2.2.2. Cloning MMP-2 sequences into pLVTHM vector.

In order to generate the shRNA against MMP-2, the selected sequences were cloned into the pLVTHM vector. The backbones of the vector so as the different restriction sites, are shown in **Figure m3**.

### 2.2.2.1: Digestion of pLVTHM vector with ClaI and Mlu

The pLVTHM vector was first digested with ClaI (Fermentas, #ER0145) overnight. Then, pLVTHM vector was digested again overnight with MluI (Invitrogen, Cat. No. 15432-016) and later, digested vector was purify using the GeneClean Turbo kit (Fisher Cat.#1102-600) following the manufacturers' instruction. Finally, DNA concentration and purity was quantified by spectrometry using a nanodrop spectrophotometer (Syngene), reading DNA concentration at $A_{260/280\ nm}$. [1 ($A_{260}$) unit of DNA= 50 µg/mL]. Optimal purity values were considered in a ratio between 1.7 and 2.

### 2.2.2.2: Oligo annealing and ligation.

3 µg of each oligo for MMP-2 was mixed with annealing buffer (100 mM NaCl, 50 mM Hepes at pH 7.4). The mix was submitted to the following PCR program: 95ºC for 4min, 80ºC for 4min, 75ºC for 4min, 70ºC for 10min, 60ºC for 20min, 50ºC for 30min, 37ºC for 45min and finally 10ºC for 60 min.

Ligation of the pLVTHM vector with the annealed oligos was performed using the Rapid DNA Ligation Kit (Fermentas, #1422). 50 ng of each vector, together with 0.9 ng of the annealed oligos, were added to 5 U/µL of T4 ligase so as with 5U/µL of T4 ligase buffer. This mix was incubated at 22ºC for 15 min in order to perform the correspondent ligation between pLVTHM vector and MMP-2 annealed oligos.

### 2.2.2.3: Transformation of the ligated vector on DH5α competent cells.

Then obtained ligation was then transformed. For that, 5µL of the ligation were mixed with 50 µL of ice thaw DH5α competent cells. The mix was incubated for 30 min in ice, and a heat shock of 20 seconds was performed at 42ºC.

Finnally, 150 µL of the obtained transformation was seeded on LB Agar Petri dishes with Ampicilin (60 µg/mL). Dishes were let overnight at 37ºC to grow. Next

day, the obtained colonies were isolated and grown in 5 mL of LB Ampyclinie O/N at 37ºC.

2.2.2.4: Glicerates, Miniprep preparation and verification

Glycerates were made of each colony, using 20% of glycerol. The glycerates were kept at -80ºC. Right after, minipreps were performed for each colony using Wizard Plus Miniprep FNA Purification System (Promega, A7500), following the manufacturer's instruction. The obtained DNA was eluted with 100 µL sterile water, and the final DNA concentration was quantified by spectrometry at $A_{260/280}$ using a nanodrop (Syngene). Finally, the above-obtained minipreps were checkup using two independent restriction analyses:

- Restriction analysis with **ClaI and MluI** was performed. For that, 1.5 µg of DNA was mixed with 1.5 µL of ClaI (Fermentas, #ER0145) and 1.5 µL of MluI (Invitrogen, Cat. No. 15432-016), with the correspondent volume of Buffer 10X (Fermentas, 15241002). The mix was incubated 3 h at 37ºC and digestion was visualized in 2.7 % agarose gels, as explained in Section 2.1.5 of M&M. The predicted bands were: 11 Kb (backbone of the vector) and 67Kb when the insert is correct; or 11 Kb (backbone of the vector) and 17 pb if there is no insert.

- Restriction analysis with **ClaI and EcoRI** was performed. For that, 1.5 µg of DNA was mixed with 1.5 µL of ClaI (Fermentas, #ER0145) and 1.5 µL of EcoRI (Fermentas, 15202013), with the correspondent volume of Buffer 10X (Fermentas, 15241002). The mix was incubated 3 h at 37ºC anf Finally digestion was visualized in 2.7 % agarose gels. The predicted bands were: 11 Kb (backbone of the vector) and 301pb when the insert is correct and 11 Kb (backbone of the vector) and 236pb if there is no insert.

The obtained results for the different colonies were the follows for the first restriction analysis (**Figure m4**). With this restriction analysis (with ClaI and MluI) it seems that all the obtained colonies have correctly acquired the insert.

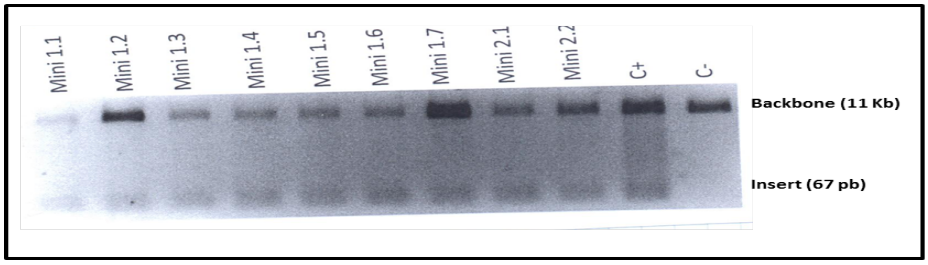


**Figure m4: Restriction analysis of the obtained colonies with ClaI and MluI.** Agarose gel was performed to check the obtained minipreps. Correct minipreps shows a band at 67 pb which corresponds to the insert and a band at 11 kb which corresponds to the vector backbone.

A second restriction analysis with ClaI and EcoRI was performed to each of the obtained miniprep. In the figure bellow (**Figure m5**), the obtained results of the restriction analysis are shown. All the tested colonies contain the insert, but colonies 1.2 and 1.7 show a most intense band at 301 pb, which clearly indicates the presence of the insert in the backbone. So we selected clone 1.2 and 1.7 to test the silence efficiency in Schwann cells, as explained in section 3.2 of this manuscript.

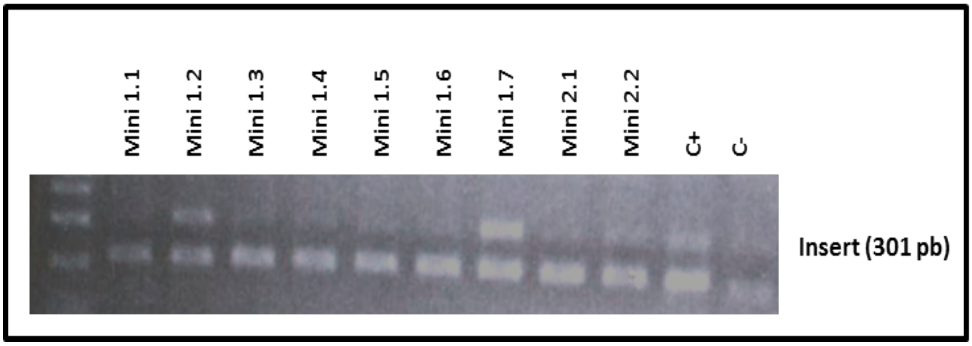


**Figure m5. Restriction analysis of the obtained colonies with ClaI and EcoRI.** A band at 301 pb indicates that the insert is correct inserted into the vector.

## 2.3. shRNA P2Y2 obtention

shRNA against $P2Y_2$ was kindly given to us by Maria Teresa Miras Portugal from the Universidad Complutense de Madrid.

This shRNA $P2Y_2$ was generated against the human sequence of $P2Y_2$ receptor, and taking in account that our Schwannoma cell line is from rat, the first step was to check the homology between human and rat sequences.

So, first a BLAST alignment between the Human $P2Y_2$ sequence (BC012104.1) and the Rat $P2Y_2$ sequence (NM_017255.1) was performed. BLAST alignment reveals an 85 % of identity between the human and the rat $P2Y_2$ sequence. Taking a look to the specific sequence used to design this shRNA (**Figure m6**), we can see that between the human sequence (in yellow in the figure) and the rat sequence (in blue in the figure) there is only a gap of difference. This gap is located at position 582 in the Human sequence (marked with a red rectangle in the **Figure m6**). shRNA against $P2Y_2$, was constructed into the pSuper.gfp/neo vector. This vector has a H1 promoter, which is a favorable promoter with properties for the expression of short nucleic acid sequences such as shRNA. In the figure below (**Figure m7**) restriction sites of pSuper.gfp/ neo vector are shown. Finally, the chosen sequence for the $P2Y_2$ receptor mRNA was the follows: 5'- AACGAGGACTTCAAGTACGTGCT – 3', which is located at the position 549 of the Human Clone BC012104 of $P2Y_2$ (yellow box in the **Figure m6**).

```
Query  477  CAGGGCAATGGCAGCAGGCCTGGACTCCTGGAATAGTACCATCAATGGCACCTGGGAGGG  536
            |||||| ||||||||| ||||| | ||||||||   ||||||||||||||||||| ||
Sbjct  461  CAGGGCGATGGCAGCAGACCTGGGCCCCTGGAATGACACCATCAATGGCACCTGGGATGG  520

Query  537  GGACGAACTGGGTTACAAATGTCGCTTCAACGAGGACTTCAAGTATGTGCTGCTGCCCGT  596
            ||| || ||||| ||||  || ||||||||||||||||||||||||||||||||||| ||
Sbjct  521  GGATGAGCTGGGCTACAGGTGCCGCTTCAACGAGGACTTCAAGTACGTGCTGCTGCCTGT  580

Query  597  GTCCTATGGCGTGGTGTGCGTGCTCGGGCTGTGCCTGAACGTCGTGGCCCTCTACATCTT  656
            |||||| ||||||||||||||||| |||||||| ||||||| |||||| ||||||||||
Sbjct  581  GTCCTACGGCGTGGTGTGCGTGCTTGGGCTGTGTCTGAACGCCGTGGCGCTCTACATCTT  640

Query  657  CCTGTGCCGCCTCAAGACCTGGAACGCCTCCACCACCTACATGTTTCACCTGGCAGTTTC  716
            | ||||||||||||||||||||||| || |||||||| || ||||| |||||||| || ||
Sbjct  641  CTTGTGCCGCCTCAAGACCTGGAATGCGTCCACCACATATATGTTCCACCTGGCTGTGTC  700

Query  717  TGACTCTCTCTACGCAGCCTCCCTGCCGCTGCTGGTTTATTACTACGCCCAGGGTGACCA  776
            |||  | || || || ||||||||||||||||||||| |||||||||||||  || |||||
Sbjct  701  TGATGCACTGTATGCGGCCTCCCTGCCGCTGCTGGTCTATTACTACGCCCGCGGCGACCA  760

Query  777  CTGGCCATTTAGCACAGTGCTCTGCAAGCTGGTGCGTTTCCTTTTCTACACTAACCTCTA  836
            |||||| || ||||| ||||||||||||||||||||| ||||| |||||||| ||||| ||
Sbjct  761  CTGGCCCTTCAGCACGGTGCTCTGCAAGCTGGTGCGCTTCCTCTTCTACACCAACCTTTA  820

Query  837  CTGCAGCATCCTCTTCCTCACCTGCATCAGCGTGCACCGGTGCCTGGGGGTCCTGCGCCC  896
            |||||||||||||||||||||||||||||||||||||||||||| ||||| ||| | || ||
Sbjct  821  CTGCAGCATCCTCTTCCTCACCTGCATCAGCGTGCACCGGTGTCTGGGCGTCTTACGACC  880
```

**Figure m6: BLAST aligment between Human P2Y$_2$ sequence (Query) and Rat P2Y$_2$ sequence (Sbjct).** Comparing the human P2Y$_2$ sequence with the rat P2Y$_2$ sequence, in the selected region to design the shRNA between the human sequence (in yellow in the figure) and the rat sequence (in blue in the figure) there differs only one nucleotide in position 582 of the sequence (marked with a red rectangle in the figure.

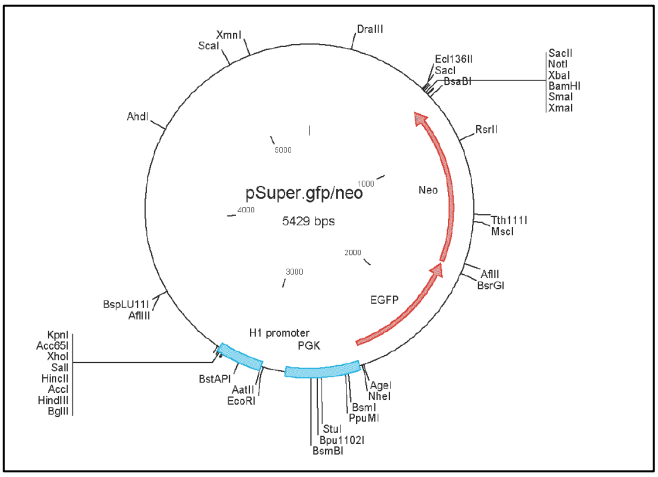


**Figure m7: pSuper vector used for shRNA of P2Y2 generation.** Backbone of the vector is shown, so as the restriction sites of this vector.

# 3. METHODS IN BIOCHEMISTRY AND MOLECULAR BIOLOGY

## 3.1. Cell migration studies

### 3.1.1. Wound Healing

Wound Healing assay is a simple, inexpensive, and one of the earliest developed methods used to study directional cell migration *in vitro*. It is a very useful tool to reproduced cell migration during Wound Healing *in vitro* (Liang, Park et al. 2007, Rodriguez, Wu et al. 2005). The basic steps involve creating a "wound or scratch" in a cell monolayer, as shown in **Figure m8**, and capturing images at the beginning and at regular intervals during cell migration until the wound is closed, and finally compare the obtained images to quantify the migration rate of the cells (Rodriguez, Wu et al. 2005, Liang, Park et al. 2007).

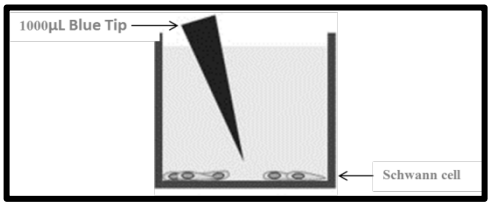


**Figure m8 Wound Healing assay**. Cells are seed until a confluence of 80% is reached and a scratch is performed using a blue pippete tip.

Schwann cells were seeded at a density of 250.000 cells/mL (**Table m1**), and once a monolayer was obtained (80% of confluence), a scratch was performed using a blue pipette tip (1000 µL). Thereafter plates were carefully washed twice with PBS, in order to eliminate any detached cell. Fresh **Deprivation Media I** was then added and pictures were taken at different times (every 2-4 hours), until the wound was closed. Finally, % of occupancy of the scratch performed in Schwann cells was measured using TSScratch v1.0 program (Matlab, Zurich). The rate of migration (velocity) was expressed as occupancy (%)/h

## 3.1.2. Boyden Chamber

The Boyden chamber system is a useful tool, used for the study of cell migration and invasion. As shown in **Figure m9**, it is based in a cylindrical cell culture insert with a known pore size, placed inside the well of a cell culture plate. (Valster, Tran et al. 2005). Cells are seeded in the bottom part of the insert, and then chemoattractant treatment is located at the bottom part of the plate.

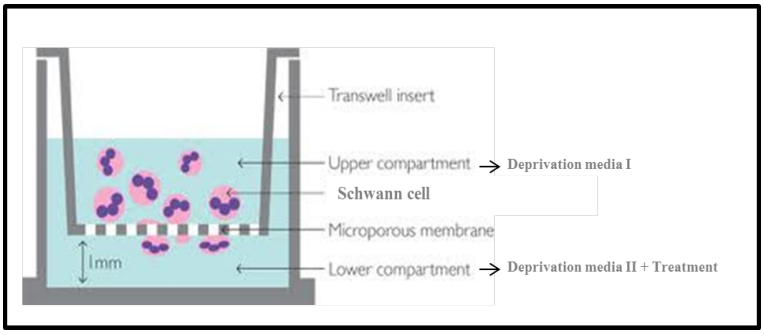


**Figure m9 . Scheme of Boyden Chamber System. Cells** are seeded in the top of the insert with Deprivation Media I, and it the bottom part of the insert, chemoatractant was added with Deprivation Media II.

Migration of Schwann cells was determined using an 8.0 µm pore BD BioCoat™ Cell Culture Inserts (BD Biosciences). Schwann cells, at a density of $1.5x10^5$ cells/insert were seeded into the upper chamber in **Deprivation Media I**. **Deprivation media II** with 250 µM UTP, was placed in the lower chamber as chemoattractant. Cells were incubated at 37ºC, for the corresponding times and thereafter, the upper surface of each membrane was cleaned with a cotton swab in order to remove any non-adhered cell, and invading cells on the lower surface of the membrane were fixed in 70% (v/v) ice cold ethanol for 20 min at 4ºC. Fixed cells were stained with 0.2% (w/v) Crystal Violet. Finally, the numbers of cells were manually counted in at least four random fields under light microscopy. Results are represented as fold increase in cell migration in treated Schwannoma cells in comparison to control Schwannoma cells (non-treated).

## 3.2. Cell transfection

Cell transfection is a useful technique used to introduce foreign genetic material into cultured mammalian cells using non-viral methods (Catesa, Harrisa et al. 2004). Three different vectors were used to transfect Schwann cells: first pLVTHM-shMMP2 clone 2.1 to silence MMP-2 (section 2.2); second pSUper shP2Y2 to silence $P2Y_2$ receptors (section 2.3); and third pLVTHM Random vector (section 2.2) as a transfection control vector.

For cell transfection, Schwann cells were seeded at 200.000 cells/mL in 24 well plates, as indicated in **Table m1**. Cells were incubated for 48 hours at 37ºC. Transfection protocol was performed as recommended by the manufactures instructions: Lipofectamine 2000 Reagent (Invitrogen) was mixed with 4 µg of the DNA to transfect; the mix was led 20 minutes at Room Temperature in order to form the DNA- liposomes complexes. The mix was then added to cell media, and cells were incubated for 6 h at 37ºC. Thereafter, medium was replaced for fresh **Growth Media**. Cells were maintained in this media for 24 h, and finally they were treated for the different experiments.

Transfection efficiency was measured by taking pictures of the cells after 24h of transfection. The number of cells, with inserted GFP (transfected cells), in front of the total number of cells was counted. Valid transfection values were considered if at least a 60% efficiency of transfection was obtained.

## 3.3. Gelatin Zymography

Matrix Metalloproteinase activity was assessed by zymography as described previously (Ma, Chen et al. 2001). Zymography is an electrophoretic technique, based on SDS-PAGE that includes a substrate co-polymerized with the polyacrylamide gel, for the detection of enzyme activity. SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) separates proteins according to their molecular weight, based on their differential rates of migration through a sieving matrix (a gel) under the influence of an applied electrical field.

### 3.3.1. Sample obtention and gel electrophoresis

Schwann cells were cultured at $2.5\times10^6$ cells in 10 $cm^2$ plates and incubated for 48 hours. Then, cells were serum deprived (**Deprivation media II**) for 6 hours, and finally medium was replaced at new for **Deprivation media II**, with the correspondent treatment and for the correspondent times. Conditioned media was collected and centrifuged for 5 min at 1000 rpm, in order to eliminate any floating dead cell. The cleared conditioned media was concentrated 10 folds in Centricon Ultra Filters 10 KDa (Millipore). Protein concentration in the media was measured using Bradford technique (section 3.5.2) and 20 µg of these total proteins, were resolved in zymography gels (SDS-PAGE).

For zymography, gels were prepared always freshly at 10% (v/v) of Polyacrylamide (**Table m6**) and 0.1% of Gelatin (Sigma G9391), Casein (Sigma C7078) or Collagen (Sigma C8919), in 1 mm crystals. Electrophoresis was carried out in vertical format electrophoresis cell system (BioRad) with electrophoresis buffer (**Table m6**) at 80V for 30 minutes, followed by 100V for 6 hours, all in ice. 20 µg of total protein in the media were resuspended in loading buffer (250 mM Tris–HCl pH 6.8, 8% SDS, 40% glycerol, 0.004% bromophenol blue buffer). And ressuspended proteins were electrophoresed in SDS-PAGE gels containing 0.1 % .

### 3.3.2. Digestion and staining

After electrophoresis, gels were washed for three times during 30 min in Washing Buffer (100 mM Tris-HCl (pH 7.5) and 2.5% Triton X-100), in order to remove the excess of SDS. Finally, gels were incubated for 16 h at 37ºC and constant agitation, in digestion buffer (100 mM Tris-Cl (pH 7.5), 5 mM $CaCl_2$ and 200 mM NaCl), to allow substrate proteolysis. Bands corresponding to MMP activity were visualized by negative staining using 0.25% Coomassie Brilliant Blue R250 (Bio-Rad, CA, USA) for 6 hours and destained for 30 min in a solution of 10% Acetic Acid and 25% Isopropanol, which enhances the bands obtained. A photo of the digested gels was taken using G-Box Syngene transilluminator software, and densitometry was performed using ImageJ software (National Institutes of Health, USA).

## 3.4. Protease activity in vitro

In situ zymography is a laboratory technique, which enables the localization of matrix-degrading metalloproteinase (MMP) activity in cell culture or histological sections (George, Johnson 2010).

Schwann cells were seeded over coverslips pretreated with Poly - Lysine at a density of 200.000 cells/mL, and incubated for 48 hours in a humidified $CO_2$ chamber. Medium was replaced for **Deprivation Media I,** and the correspondent treatment for 12 hours. Later, medium was replaced and cells were washed three times with Reaction Buffer, consisting of 0.5 M Tris-HCl,1.5 M NaCl, 50 mM $CaCl_2$, 2mM Sodium Azide (pH 7.6), all in ice. Then, cells were treated with 100µg/mL DQ-Gelatin (D12054, Life Technologies) at 37ºC for 2 hours and 30 minutes in the dark. It has to be in the dark because DQ-Gelatin is a fluorogenic substrate used to detect protease activity in vitro with high sensitivity. Afterwards, cells were washed twice with ice cold PBS, coverslips were mounted with the anti-fading medium Fluoromount (F4680, Sigma) and the fluorescence reaction product was visualized using a fluorescence microscope (Leika DM IRB, Wetzlar, Germany). At least, four photos of four random fields were taken of each coverslip, the fluorescent green spots of each photo were manually counted and representative pictures of each field are shown.

## 3.5. Western Blot

Western Blot (or Immunoblot), was described for the first time by Towbin in 1979 (Towbin, Staehelin et al. 1979), and actually it is a routine laboratory technique used for protein analysis. Specificity of union between antibody and antigen allows it to detect a unique protein between lots of them.

### 3.5.1. Cell lysis

Cultured cells were keeping in ice for 10 minutes before washing twice with ice cold PBS. Cells were lisated by scrapping with cold RIPA buffer, which contains 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% (v/v) NP-40 with proteases and phosphatases cocktail inhibitors (Sigma), 1 mM both. Cell lysates were sonicated three times for 10 seconds in ice, in order to break all cellular membranes and structures. Finally, cell lysates were kept at -20C until their use.

### 3.5.2. Protein Quantification

Total protein concentration of cell lysates or conditioned media was determined using the Bradford reagent (Bradford et al, 1976). This method is based on quantification of absorbance between 465 and 595 nm, given when Comassie Blue G-250 (161-0406, BioRad) binds to the proteins. Bovine serum albumin (BSA) was used as standard, at well-known increasing concentrations (5, 10, 15, 25 and 35 µg/mL). In a 96 well microplate, straight pattern, cell lysates or conditioned media were seeded in triplicate. Protein problem samples were seeded on a 1/100 final dilution. 50 µL of Bradford reagent was added to each well, and samples were homogenized. Absorbance was read at 595 nm using a micro well plate reader Synergy HT (Biotek®). The concentrations of samples were calculated from the equation of the standard curve.

### 3.5.3. Gel electrophoresis and transference

Both whole cell lysates (30 µg) or protein media (20 µg), were prepared and analyzed by western blotting (Towbin, Staehelin et al. 1979). Briefly, 30 µg of cell lysates or 20 µg of conditioned media, were prepared in sample Buffer (**Table M5**), and thereafter samples were boiled for 5 min at 95ºC before loading, in order to denature the proteins. Samples (either cell lysates or protein media), so as protein standard weight were resolved in SDS–Acrylamide gels with a density between 10 or 12% (v/v) of acrylamide in 1.5 mm crystals. After electrophoresis, proteins were transferred with constant amperage (400mA) for 2 hours with the Mini Trans-Blot Electrophoretic transfer Cell (BioRad) to PDVF activated membranes (**Table M5**) in ice. Once transference was finished, membranes were blocked for 1 hour in constant agitation with blocking buffer (**Table M5**).

### 3.5.4. Immunodetection

Membranes were incubated O/N at 4ºC with the primary antibody at the correspondent dilution, diluted in TBS-Tween with 5% (v/v) of BSA and 0.01 % (v/v) sodium azide (**Table m6**). Membranes were washed three times for 10 minutes with **washing buffer** (**Table m5**). Finally they were incubated with the secondary antibody (**Table m6**) conjugated to peroxidase (HRP), diluted 1:5.000 in **blocking buffer** for 1 hour at room temperature, in continuous agitation. Membranes were

washed three times with **washing buffer** and, finally, membranes were revealed with Luminata classic ECL quimioluminiscent reactive (Millipore, WBLUC500).

| Materials and Reactive for Electrophoresis | | | |
|---|---|---|---|
| **Reactive name** | **Reactives** | **Concentration** | **pH** |
| **Electrophoresis Buffer** | Tris<br>Glicine<br>SDS | 250 mM<br>2 M<br>1% (v/v) | 8.3 |
| **Sample Buffer 5X** | SDS<br>Tris<br>DTT<br>Glicerol | 0.4. % (v/v)<br>0.5 M<br>20 mM<br>25 % (v/v) | 6.8 |
| **Stacking Gel (3%)** | Acrylamide bis solution<br>Tris<br>SDS<br>APS<br>TEMED | 30 % (v/v)<br>0.5M<br>10% (v/v)<br>10% (v/v)<br>10% (v/v) | 6.8 |
| **Separating Gel (10%)** | Acrylamide bis solution<br>Tris<br>SDS<br>APS<br>TEMED | 30 % (v/v)<br>0.5 M<br>10% (v/v)<br>10%<br>10% | 8.8 |
| **Molecular weight standard** | Precision Plus Standard Dual Color. Weights from 10 KDa to 250 KDa. | | |
| | **Materials and Reactive for Transference** | | |
| **Transfer Buffer** | Tris<br>Glicine<br>MetOH | 250 mM<br>2 M<br>10 % (v/v) | 8.3 |
| **Membrane** | PVDF (Polyvinylidene difluoride Hybond-P, Millipore) | | |
| | **Materials and Reactive for membrane blocking** | | |
| **Washing Buffer (TBS-Tween)** | Tris<br>NaCl<br>KCl<br>Tween | 5 mM<br>150 mM<br>100 mM<br>0.2 % (v/v) | 7.4 |
| **Blocking Buffer** | Skimed Milk dissolved in washing buffer | 5% (w/v) | |

**Table m5:Reactive, buffers and reagents used for Western-Blot.** Reactive are subdivided into materials and reactive used for electrophoresis, used for transference or used for blocking

| Primary Antibodies | | | | | |
|---|---|---|---|---|---|
| **Protein** | **Origin** | **Dilution** | | **Comercial house** | **Ref** |
| | | **WB** | **IF** | | |
| **Phospho ERK½ (Thr202/Tyr204)** | Rabbit polyclonal | 1:1000 | 1:500 | Cell Signalling | #9101 |
| **Phospho- p38 (Tyr182/Thr180)** | Rabbit polyclonal | 1:1000 | | Abcam | Ab4822 |
| **Phospho- JNK (Thr183/Tyr185)** | Rabbit polyclonal | 1:1000 | | Cell Signalling | #9251 |
| **ERK ½** | Rabbit polyclonal | 1:1000 | | Cell Signalling | #9102 |
| **p38** | Rabbit polyclonal | 1:1000 | | Abcam | Ab27986 |
| **JNK** | Rabbit polyclonal | 1:1000 | | Cell Signalling | #9252 |
| **S100** | Rabbit polyclonal | | 1:100 | Sigma | S2644 |
| **GAPDH** | Mouse polyclonal | 1:20000 | | Ambion | AM4300 |
| **MMP-2** | Rabbit polyclonal | 1:1000 | 1:200 | Abcam | Ab37150 |
| **$P2Y_2$** | Rabbit polyclonal | 1:1000 | | Abcam | Ab10270 |
| **Secondary Antibodies conjugated to HRP** | | | | | |
| **Anti Rabbit (IgG) HPR** | Goat polyclonal | 1:5000 | | Calbiochem | 401315 |
| **Anti Mouse (IgG) HPR** | Goat polyclonal | 1:5000 | | Calbiochem | 401207 |
| **Secondary Antibodies conjugated to fluorocromes** | | | | | |
| **Alexa Fluor Goat 594 Anti Mouse IgG (H+L)** | Goat polyclonal | | 1:1000 | Invitrogen | A11005 |
| **Alexa Fluor Goat 488 Anti Mouse IgG (H+L)** | Goat polyclonal | | 1:1000 | Invitrogen | A11001 |
| **Alexa Fluor Goat 594 Anti Rabbit IgG (H+L)** | Goat polyclonal | | 1:1000 | Invitrogen | A11012 |
| **Alexa Fluor Goat 488 Anti Rabbit IgG (H+L)** | Goat polyclonal | | 1:1000 | Invitrogen | A11008 |

**Table m6: Primary and secondary antibodies used for Western Blot and Immunofluorescence.** It is shown the origin specie, the used dilution and application so us the commercial house and reference of each used antibody. WB: Western Blot; IF: Immunofluorescence; HRP: Peroxidase.

## 3.6. Immunochemistry

### 3.6.1. Cell fixation and permeabilization:

Cells were washed twice with PBS 1X (phosphate buffer saline, pH= 7.5) and fixed with **Fixed Solution** (**Table m7**) for 15 min at Room Temperature. Thereafter, fixed solution was replaced for **Permabilization Solution** (**Table m7**) for 5 min at -20ºC in order to permeabilize cell membranes. From here, cells can be stored for a long period of time in PBS wit sodium azyde at 4ºC.

### 3.6.2. Immunodetection:

Fixed cells were washed twice for 5 minutes with **Washing Solution** (**Table M7**), and blocked with **Blocking Solution**. Blocking was performed at Room Temperature for 30 minutes in constant agitation. Once blocked, cells were incubated with the correspondent primary antibody diluted in blocking solution at the correspondent concentration (**Table m6**), for 120-180 min at Room Temperature in a humidified dark chamber. Finally, cells were washed three times with **Washing Solution**, for 5 minutes at Room Temperature and with constant agitation.

Thereafter, and ought to secondary antibodies are light sensitive; the following steps were performed in the dark. The cells were incubated with the correspondent secondary antibody and at the same time with Hoechst in order to stain cell nucleus, during 1h at Room Temperature in a humidified dark chamber. Finally, cover slips were washed 3 times with **Washing Solution**, and cover slips were mounted on slides using Gel Mount anti-fading medium Fluoromount (Sigma, F4680).

Mounted slides were kept at 4ºC until they were analyzed with fluorescence microscopy (Leica). At least four random fields were taken of each slide.

| Solution name | Reactives | Incubation Time (min) | Temperature (ºC) | Agitation (Yes/No) |
|---|---|---|---|---|
| **Fixed Solution** | 4% Paraformaldehyde+ 4% Sucrose + PBS 1X | 15 | 25 | Yes |
| **Permeabilization solution** | Ice cold methanol | 5 | -20 | No |
| **Blocking Solution** | 2% BSA + 2% Goat Serum + PBS 1X | 30 | 25 | Yes |
| **Washing Solution** | PBS 1X | 5 | 25 | Yes |

**Table m7: Immunofluorescence solutions**. Solution name is shown so us the components of each solution. Also the time and the temperature needed for each solution.

## 3.7. Statistical Analysis

Results are expressed as the mean ± SD (standard deviation media) of at least three independent experiments in all the obtained results.

Different groups were analyzed with variance analysis using ONE WAY ANOVA test, followed by a *Newman Keuls* post-test, using the statistic package of GraphPad Prism 5 program (San Diego, CA, USA). Significant values were considered when $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

# X. RESULTS

## SECTION 1: Effects of nucleotides on Schwann cell migration

# X. RESULTS

## 1. NUCLEO CMP FORTE INDUCES SCHWANNOMA CELL MIGRATION

Cell motility and migration are fundamental biological properties that contribute to the normal development and differentiation of the cell. Cellular migration is a complex process that requires a strong coordination of both cytoskeleton changes and extracellular pro-migratory stimulus.

Of the entire known pro-migratory stimulus, nucleotides have been described to play an important role in cell migration. Evidence in recent years indicate the importance of extracellular nucleotides and adenosine, in the regulation of homeostatic mechanisms and migration of different cell types such as, fibroblasts, endothelial cells, keratinocytes or neurons, between others, (Corriden, Insel 2012, Junger 2011, Linden 2011).

### 1.1. Schwannoma cell wound repair is induced by Nucleo CMP forte

Nucleo CMP forte (CMPF) is a drug composed mainly by different nucleotides. Due to the important role that nucleotides seems to have in the migration of different cell types, we were interested in study the effects of this drug in Schwannoma cell migration. To do that, functional studies of cell migration were performed using *in vitro* Wound Healing assay. Schwannoma cell monolayer was scratched and stimulated with 1 g/L of CMPF, and the wound closure was monitored until the scratch was completely closed.

Treatment of cells with CMPF resulted in a significant increase in the number of cells that migrated into the wounded area (**Figure r1)**. After 12 hours of wound scratch, the cells treated with CMPF had closed a 50% of the wounded area, while non-treated cells (Control, in the **Figure r1)** had only closed a 20% of the scratch. Moreover, after 20 hours of the scratch, CMPF treated cells had completely invaded the wounded area (100% occupancy), while non-treated cells had only closed a 50% of the area, in the same period

of time (20 hours). Not only so, non-treated cells never end to close the scratched area.

This result indicates that some of the components present in the Nucleo CMP drug trigger a change in Schwann cell motility, and an increasing in their migratory potency.

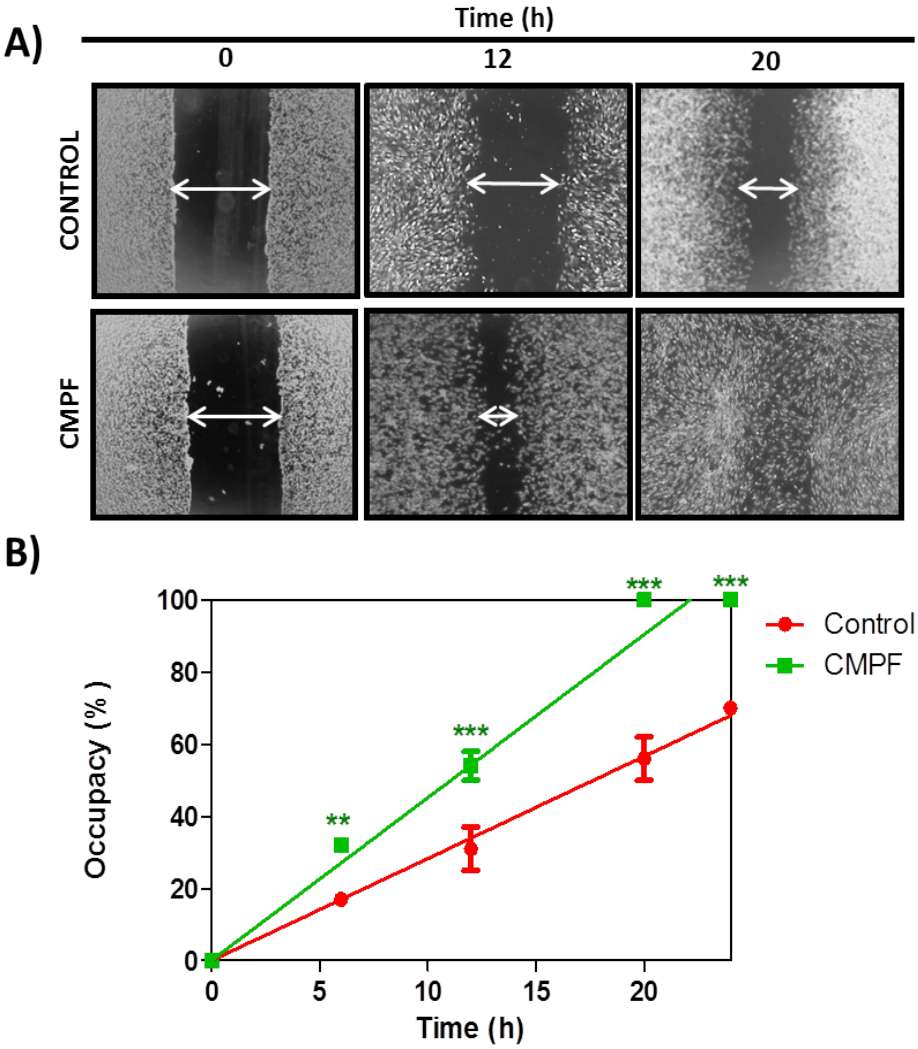


**Figure r1: Nucleo CMP forte induces Schwannoma cell wound repair. A)** Wound-Healing assay for Schwannoma cells. Monolayer cells were scraped and treated with Nucleo CMP forte (1g/L). Pictures were taken at 4X objective magnifications at the same area until wound was completely closed (0, 12 and 20 hours). **B)** Wound closure quantification. At least three independent experiments were performed and significant images are shown, values are represented as the Mean ± SD. Statistical significance: **p<0.01, ***p<0.001.

## 1.2. Study of Schwannoma cell wound repair with the different components of Nucleo CMP forte.

In order to elucidate which of the nucleotides present in the CMPF drug was the responsible of the observed Schwannoma cell wound repair (**Figure r1**), a new Wound Healing assay was performed. Schwann cells were stimulated with the different nucleotides of the drug, separately: uridine triphosphate nucleotides (UTP, 250µM), uridine diphosphate nucleotides (UDP, 250µM), uridine monophosphate nucleotides (UMP, 250µM) or cytidine monophoshpate nucleotides (CMP, 1.7 mM).

Comparing the percentage of occupancy between UTP, UDP, UMP and CMP in **Figure r2**, we could see that UTP showed a significant increase in the rate of migration in comparison to UDP, UMP and CMP. After 12 hours of the performed scratch, almost the 60% of it was closed in UTP treated cells. In contrast, Schwannoma cells treated with UDP or UMP had only closed a 40% of the scratch at the same time (12 hours). Finally, cells treated with CMP had only invaded a 30% of the wounded area after that time (12 hours). When we compared the wound invasion after 20 hours, we could observe that UTP treated cells had completely closed the affected area (100% occupancy), while the cells stimulated with UMP or UDP had only invaded an 80% of the scratch. At the end, CMP induced cells only closed the 50 % of it. So in resume, although UDP (yellow line in **Figure r2B**) and UMP (green line in **Figure r2B**) showed significant increases in the migration rate of Schwannoma cells, it seems clear that UTP (blue line in **Figure r2B**) showed the most significant increase in the Schwannoma cell wound repair.

So, our results indicate that all the nucleotides present in the drug improves Schwann cell wound repair. Of all the nucleotides tested, UTP is the most effective, followed by UDP, UMP and CMP respectively. Increasing rate in migration is as follows: **UTP>UDP>UMP>CMP**.

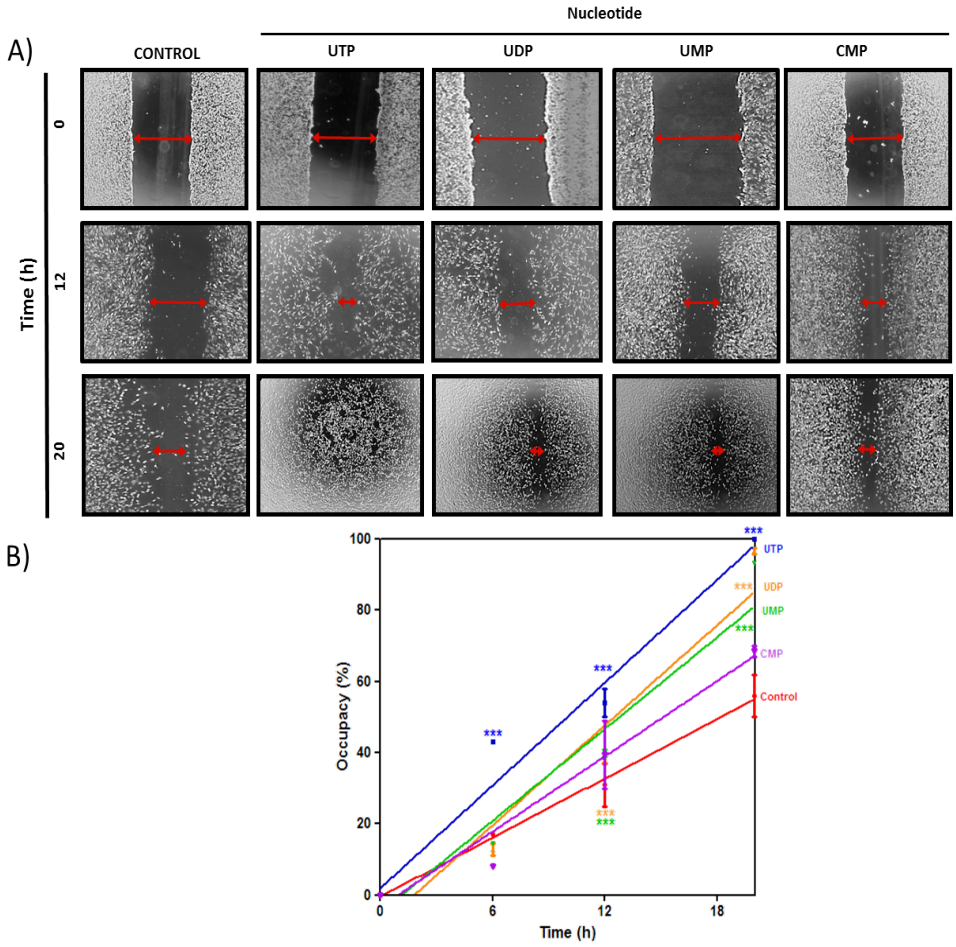


**Figure r2. Both uridine and citidine nucleotides induce Schwann cell wound repair. A)** Wound-Healing assay for Schwannoma cells. Monolayer cells were scraped and treated with UTP (250 µM), UDP (250 µM), UMP (250 µM) or CMP (1.7 mM). Pictures were taken at 4X objective magnifications on the same area, until wound was completely closed (0, 12 and 20 hours). **B)** Wound closure quantification of the % of occupancy from treated to non-treated cells. At least three independent experiments were performed and significant images are shown, values are represented as the Mean ± SD. Statistical significance * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

## 1.3. Schwannoma cell migration is induced by Nucleo CMP forte

Once elucidated that Nucleo CMP forte induces Schwannoma cell wound repair, we next wanted to study if this wound repair was due to an increase in Schwannoma cell migration. For that purpose we used the Boyden Chamber System. The Boyden Chamber, is a useful *in vitro* tool used to study cell migration on a permeable support, with a known pore filter and a chemotactic component (Lee, Chin et al. 2006), as described in **M&M Section 3.1.2**.

Schwannoma cells were seeded at the top of the insert and Nucleo CMP forte was added in the bottom chamber. Cells were led to migrate for 2, 4, 8 and 12h, and different pictures were taken of each insert. Migrated Schwannoma cells were manually counted and quantified. Our results indicated that CMPF induced a significant increase in Schwannoma cell migration (**Figure r3**). This significant improving in the migration rate of these cells started after 2 hours of cell treatment (*$p<0.05$), and reached the maximum between 8 and 12 hours of cell stimulation (**$p<0.01$).

The obtained results allow us to confirm that CMPF induce Schwannoma cell wound repair and migration.

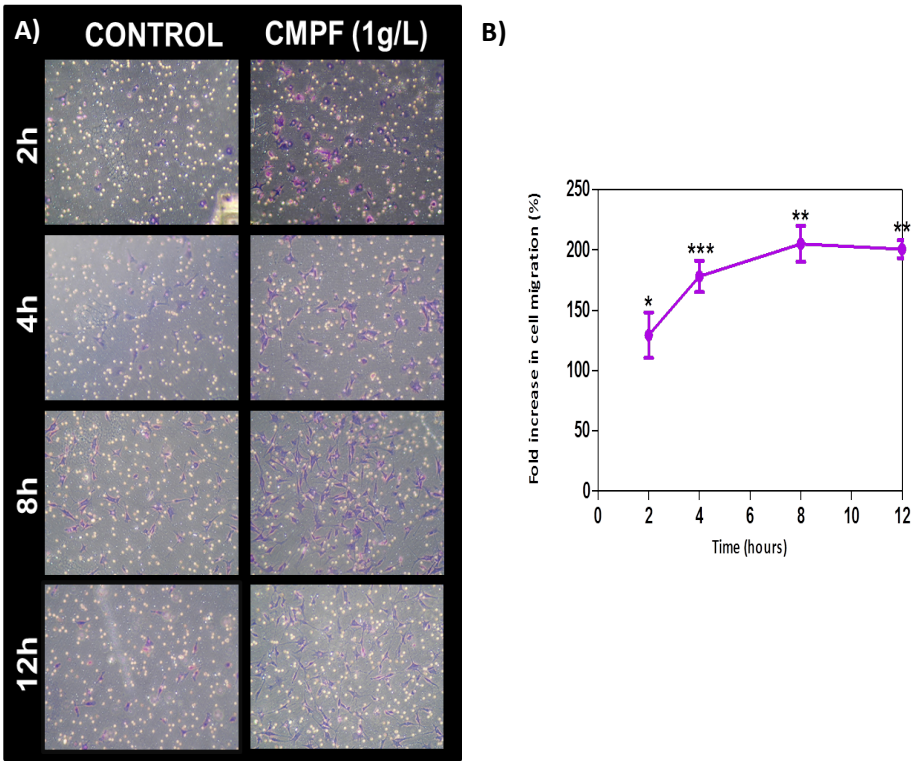


**Figure r3. Migration effects of Nucleo CMP forte on Schwannoma cells. A)** Cell migration was examined using the Boyden Chamber system after cell treatment with Nucleo CMP forte at different times. Pictures were taken at different times of cell treatment with CMPF (2, 4, 8 and 12h). **B)** Quantification values of the fold increase in Schwannoma cell migration. At least three independent experiments were performed and significant images are shown, values are represented as the Mean ± SD. Statistical significance * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

# 2. URIDINE TRIPHOSPHATE INDUCES SCHWANNOMA CELL MIGRATION

As we had seen that UTP is the main effector in inducing wound repair in Schwannoma cells, following experiments were performed only with UTP.

## 2.1. Schwann cell wound repair is induced by UTP

UTP enhanced wound repair in Schwannoma cells (**Figure r2**), so our next goal was to study if this wound repair was also enhanced in a Schwann primary culture, after UTP treatment. Both, Schwannoma cells and Schwann primary cultures, were scratched and treated with UTP (250µM). UTP treatment also induced cell wound repair in Schwann primary cultured cells, as it happens in Schwannoma cells (**Figure r4**). Both, cell types after UTP stimulation, had completely occupied the wounded area after 12 and 33 hours of scratch respectively. In contrast, at the same time non-treated cells had only closed a half of the performed wound (50% of occupancy).

These results clearly suggest a significant role for UTP in Schwann cell wound repair, both in the cell line and in the primary culture.

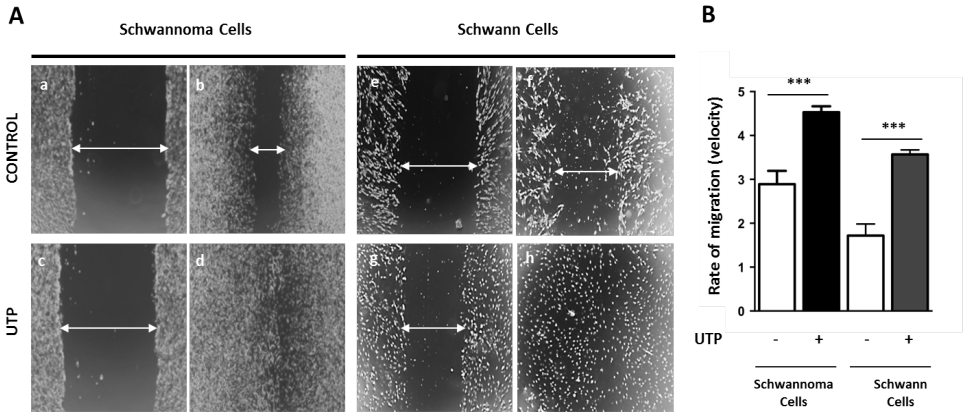


**Figure r4: UTP enhances Schwann cell wound repair.** A) Wound healing assay for Schwannoma cells and for Schwann primary culture: monolayer cells for both Schwann cultures were scraped and treated or not with 250 µM UTP. Micrographs are representative of at least three independent experiments (objective magnification 4X). B) Quantitative analysis of the rate of migration. Results are the mean of at least three independent experiments; values are represented as the Mean ± SD. Statistical significance: ***p<0.001.

## 2.2. Schwann cell migration is induced by UTP

Next we wanted to confirm if the enhancement in wound healing obtained after UTP treatment, was also due to an increase in cell migration, so as observed previously with CMPF (**Figure r3**). For that, a Boyden Chamber experiment was performed in Schwannoma cells stimulated with UTP.

This nucleotide impulse Schwannoma cells to migrate through the insert membrane (**Figure r5**). Moreover, the observed migration was time dependent and reached the highest rate after 8 hours of UTP treatment. Cell migration was slightly reduced after 12 hours of treatment, but in spite of this UTP treated cells suffered a significant increase in migration when compared with control cells (second column on **Figure r5A**).

With this result, we can conclude that UTP is the major responsible of the Schwannoma cell migration in a time dependent manner.

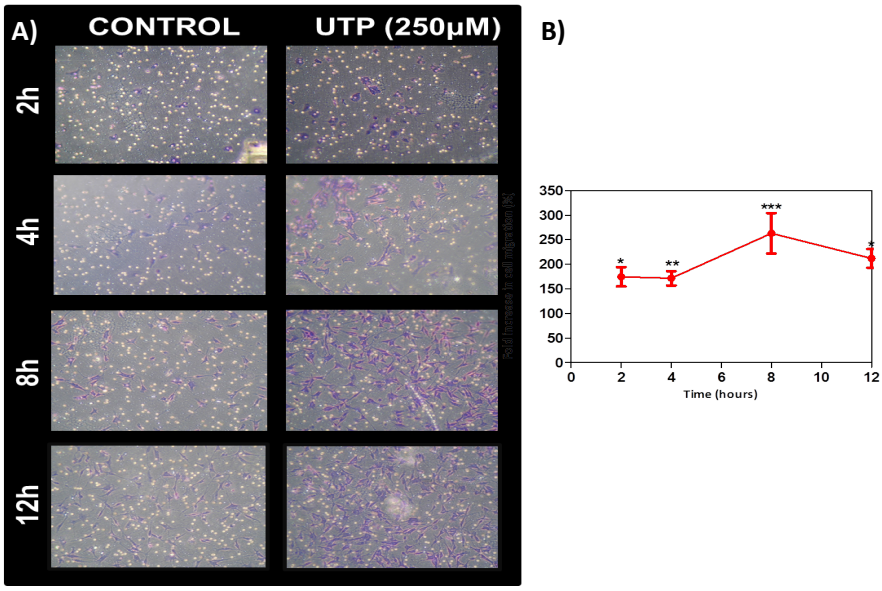


**Figure r5: UTP induces Schwannoma cell migration in a time dependent manner. A)** Schwann cell migration was examined using the Boyden Chamber system after treatment with UTP (250 µM) at different times. Pictures were taken at different times of experiment (2, 4, 8, 12h). **B)** At least, three independent experiments were made, and significant images are shown. *** $p< 0.001$ and ** $p<0.01$ and * $p<0.05$.

Later we studied if this increase in the cell migration, observed after UTP stimulation, was dose dependent. For that, we performed a Boyden Chamber assay using different UTP concentrations (0, 100, 250, 500 and 1000 µM). Schwann cells were led to migrate for 8 hours (time of the maximum migration rate, observed in **Figure r5**).

Progressively increases in UTP concentrations carried out progressively and significant increases in Schwannoma cell migration (**Figure r6**, ***p< 0.001, ** p<0.01 and * p<0.05). The maximum rate of cell migration was obtained after 8 hours and 500 µM of UTP treatment. The dose of 1000 µM did not show a higher migration rate, due probably to the toxic effects of UTP.

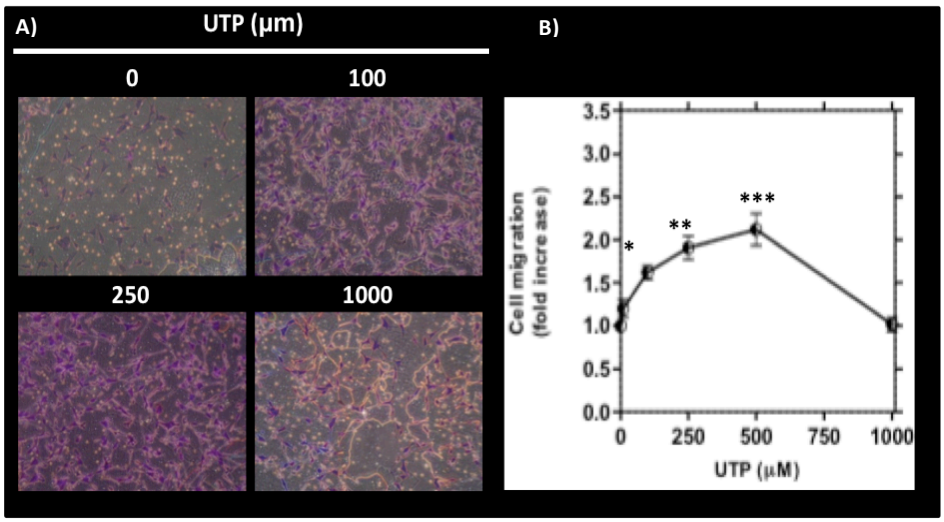


**Figure r6: Migration effects of UTP on Schwannoma cells are dose dependent A)** Schwannoma cell migration was examined using the Boyden Chamber system after treatment with UTP at different concentrations (0, 100, 250, 500 and 1000 µM). Pictures were taken after 8 hours of cell treatment. **B)** At least three independent experiments were performed and significant images are shown, the results are the mean ± SD. *** p< 0.001, ** p<0.01 and * p<0.05.

These results allow us to conclude that UTP is the major nucleotide of the CMPF drug responsible of the Schwann cell wound repair and migration, and that their effects are time and dose dependent.

## SECTION 2: UTP induced Schwann cell migration is mediated by the P2Y-MAPK pathway

# 3. P2Y RECEPTORS ARE INVOLVED IN SCHWANNOMA CELL WOUND REPAIR

It is well known that purinergic receptors are activated by nucleotides (Abbracchio, Burnstock 1998, Boeynaems, Communi et al. 2012). On the other hand, some authors described previously the involvement of purinergic receptors activation in cell migration and wound repair in epithelial cell lines (Boucher, Rich et al. 2010, Klepeis, Weinger et al. 2004). Finally, previous results of our research group had described the presence of functional $P2Y_{2,4 \text{ and } 6}$ receptors in Schwannoma cells (Martiañez, Carrascal et al. 2012)

## 3.1. Purinergic receptor activation is crucial for UTP induced wound repair in Schwann cells.

To verify if the migratory effects induced by UTP on Schwann cells were actually due to the activation of P2Y receptors, Schwannoma cells were scratched and exposed to CMPF or UTP in the presence of the non-selective purinergic receptor antagonist (Suramin, 100 µM). Pictures of the scratch were taken until the wound was completely closed and % occupancy was measured.

Treatment with Suramin almost completely blocked the wound repair induced by UTP or CMPF (**Figure r7)**. Schwann cells treated with CMPF or UTP had completely closed the scratch after 20 hours of stimulation (similar results as those shown previously **Figure r1 and r2**). In contrast, pretreatment of Schwannoma cells with Suramin, significantly reversed this migratory effects. The migration rate in cells pretreated with Suramin was the same that those observed in control cells; the % of occupancy was around the 50% after 20 hours of cell scratch (**Figure r7 B and C**).

These results describe a new role for the activated purinergic receptors in the Schwann cell wound repair induced by UTP.

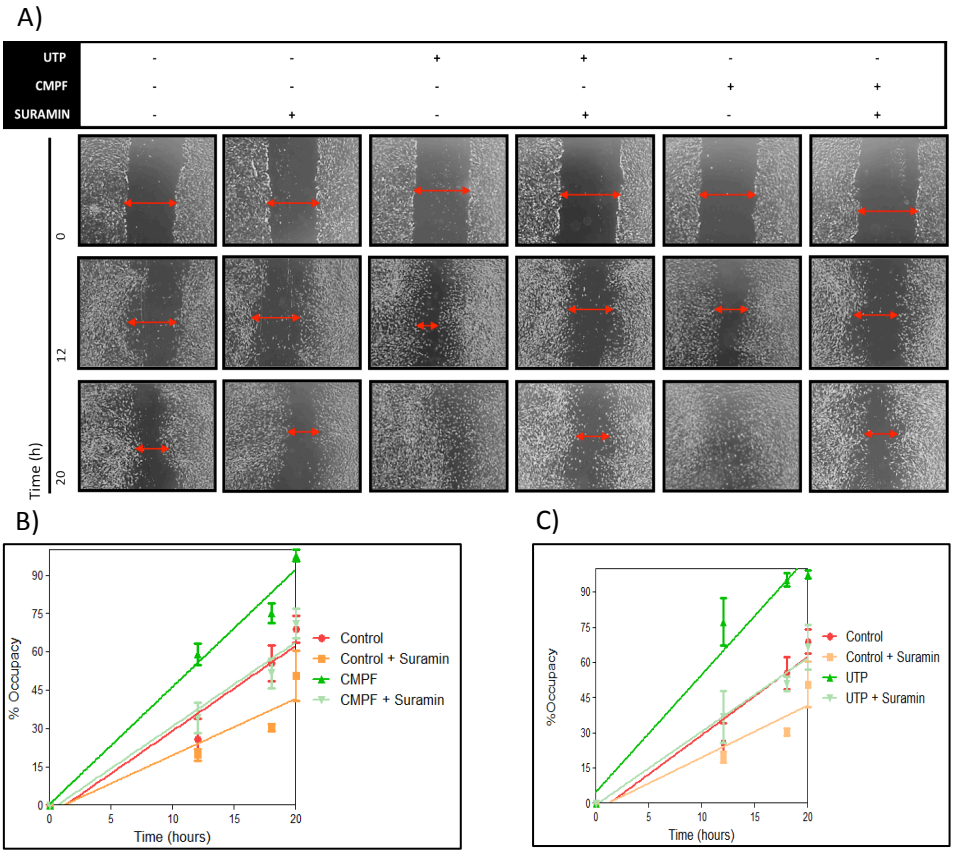


**Figure r7 Schwann cell wound repair is purinergic receptor dependent. A)** Schwannoma cells were scratched, pretreated with 100 µM Suramin (P2Y inhibitor), and thereafter treated with 250 µM UTP or 1g/L of CMPF. Different images were taken always at the same area until the wound was completely closed with an inverted microscope. Images shown are representative to 0, 12 or 20h after cell scratch. **B, C)** Quantitative analysis of % occupancy obtained for at least, three independent experiments is shown. Each bar represents the mean ± S.D (n=3).

## 3.2. P2Y2 receptor is the responsible of the Schwannoma cell wound repair induced by UTP.

Suramin is a non-selective inhibitor of Purinergic Receptors, and it is known to inhibit both P2X and P2Y receptors (Dunn, Blakeley 1998). In contrast, UTP seems to be the most implicated nucleotide in Schwann cell migration and wound repair. We next wanted to know, whether the purinergic receptor involved in the Schwann cell migration observed after UTP stimulation, was specifically the $P2Y_2$ receptor, which is UTP sensitive. For that, we used a small hairpin RNA against $P2Y_2$ ($shP2Y_2$ vector) that was kindly given to us by Dra. Maria Teresa Miras Portugal (Universidad Complutense de Madrid), see Materials and Methods section 2.3.

First, Schwannoma cells were transfected with the $shP2Y_2$ vector or in contrast with the shRandom vector (as a control), in order to check the silencing efficiency of the $shP2Y_2$ vector. After cell transfection, both RNA and cell lysates were collected. A Western Blot so as a Real-Time PCR was performed in order to quantifies the $P2Y_2$ protein and mRNA levels, respectively. Schwannoma cells transfected with the $shP2Y_2$ showed a decrease in the protein levels of the $P2Y_2$ receptor of approximately a 50%, in comparison to non-transfected cells or cells transfected with the shRandom (**Figure r8A**). mRNA levels of Schwannoma cells transfected with the $shP2Y_2$ showed a significant decrease in the $P2Y_2$ expression, in comparison to non-transfected cells or to cells transfected with the shRandom (orange bar in **Figure r8B**).

So, our results confirm that the $shP2Y_2$ is effective in silencing $P2Y_2$ receptor, both at mRNA and protein levels in the rat RT4-D6P2T Schwannoma cell line.

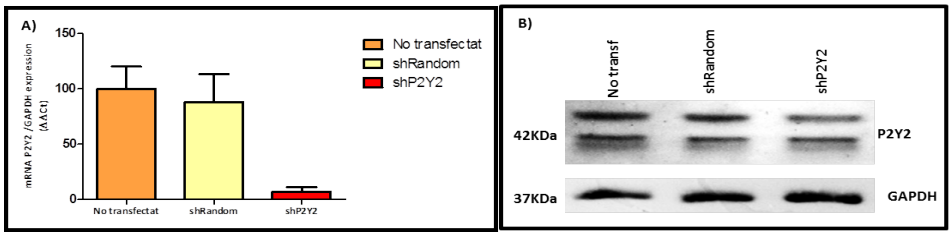


**Figure r8: shP2Y$_2$ validation. A)** RT4-D6P2T Schwannoma cells transfected with shRNA against nontargeting control (shRandom) or rat P2Y$_2$ gene (shP2Y$_2$). After 48h of cell transfection, P2Y$_2$ protein levels were determined by western blot. GAPDH was used as an internal loading control. **B)** RT4-D6P2T cells were transfected with shRandom or shP2Y$_2$ and after 48 h of cell transfection, P2Y$_2$ mRNA levels were determined by quantitative RT-PCR. GAPDH was used as an internal control.

To test whether the P2Y$_2$ receptors were involved directly in the wound healing improvement observed after UTP stimulation in Schwannoma cells, a wound-healing assay was performed in cells transfected with the shP2Y$_2$ vector. Our results indicate that Schwannoma cells transfected with the shRandom had more or less the same rate of migration than non-transfected cells, even after UTP stimulation (**Figure r9,** bar 1, 2, 3 and 4). On the other hand, cells transfected with the shP2Y$_2$ vector and treated with UTP (Barr 6 in **Figure r9B**), suffered a significant and drastic decrease in the cell wound healing capacity, in comparison to UTP treated cells ($^{###}p<0.001$, Barr 2 and 6 in **Figure r9B**).

This result clearly suggests that the ability of wound repair observed in Schwann cells in response to UTP is mediated directly by P2Y$_2$ receptors.

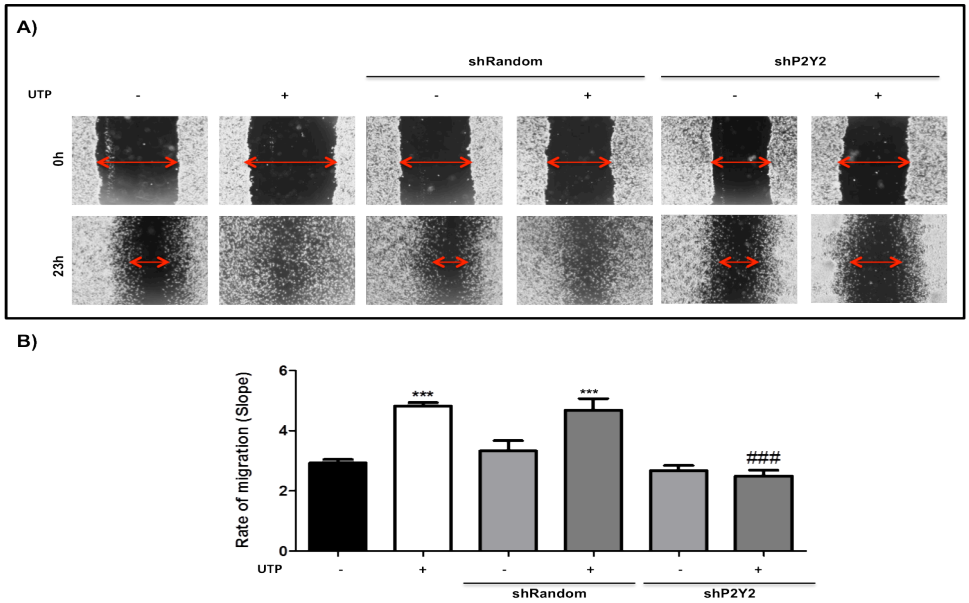


**Figure r9. UTP induced Schwannoma cell wound repair is dependent on $P2Y_2$ receptor. A)** Schwannoma cells were transfected with $shP2Y_2$ or shRandom vectors, then scratched using a sterile 1000 µL tip and finally treated with 250 µM UTP. Different images were taken at the same area until the wound was completely closed with an inverted microscope. Images shown are representative of 0 and 23 h after cell scratch. **B)** Rate of migration (slope) obtained for at least, three independent experiments is shown, each bar represents the mean ± S.D. Statistical significance: *** denote significant differences from control values with ***$p< 0.001$ respectively; ### denote significant differences from UTP values with $p< 0.001$.

# 4. IMPLICATION OF THE MAPKS PATHWAY IN UTP INDUCED SCHWANN CELL WOUND REPAIR

## 4.1. UTP induces MAPK biphasic activation in Schwannoma cells.

It is well known that Purinergic receptor have a mitogenic role on a variety of cell types like, endothelial cells, smooth muscle cells or neurons between others (Huang, Jacobson et al. 2004, Meintanis, Thomaidou et al. 2001). These receptors are sensitive to uridine nucleotides and can activate MAPK pathway through PKC activation (Graham, et al 1996). For that, we next wondered to known whether UTP stimulation induces any MAPKs signalling activation. For that, Schwannoma cells were exposed to 250 µM UTP at different times (5 min, 45 min, 2 h, 6 h, 12 h, 24 h and 48 h) and the activation of three MAPKs (ERK ½, JNK and p38) by phosphorylation, was analyzed using Western Blot.

UTP induced to a strong biphasic activation wave of the three-studied MAPKs, as measured by the ratio between the intensities of the phosphorylated versus the total MAPK protein bands (**Figure r10**). A first peak of activation was observed after 5 minutes of UTP stimulation, for the three analyzed MAPKs (**Early Activation**). This peak was maintained up to 45 minutes of UTP treatment. Thereafter, phosphorylation of the three MAPK decreased near the baseline levels. A second wave of MAPKs phosphorylation was observed again between 6 and 12 hours of UTP stimulation (**Late Activation**). This peak was sustained for up to 24 hours after UTP stimulation. Finally, MAPKs phosphorylation decreased again to baseline levels, after 48 hours of treatment. Of the three studied MAPK, ERK ½ was the one that showed the highly phosphorylation increasing rate (2.7 fold increase over the control samples), followed by JNK (2. 5 fold of the control) and p38 (2 fold of the control samples) in both activation peaks (Early and Late). The observed increases in MAPKs phosphorylation were statistically significant for all three-studied MAPKs (*$p<0.05$ and ***$p<0.001$).

The obtained results suggest that UTP activates P2Y receptors which initiates a downstream signalling cascades resulting in a biphasic phosphorylation of MAPKs, in a similar way, in the three analysed MAPKs (ERK ½, JNK and p38).

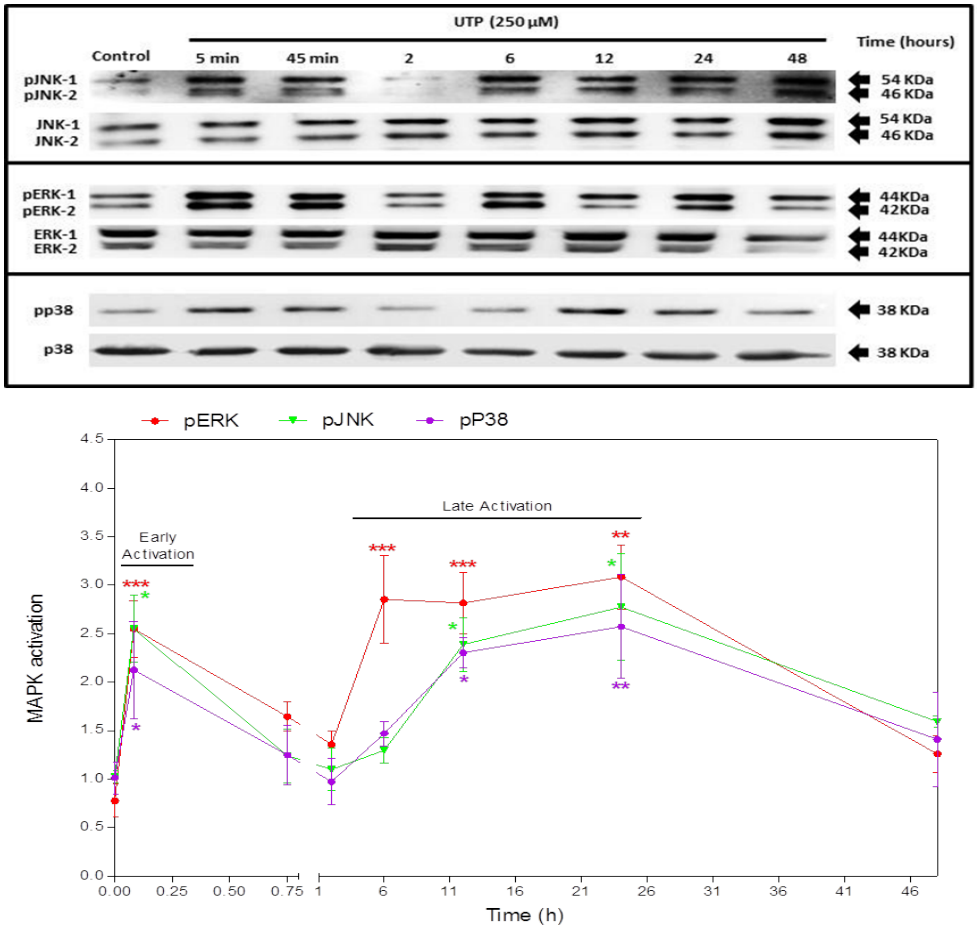


**Figure r10. Extracellular UTP induces MAPK biphasic activation in Schwannoma cells**. Cells stimulated with 250 µM UTP for the indicating times and cell lysates were collected. **A)** Representative western blotting experiments show biphasic activation of ERK ½, p38 and JNK. Equal amounts of protein (30 µg) were resolved in SDS-PAGE. Western blots were performed using antibodies against phosphorylated MAPK (ERK ½, JNK and P38). The same membranes were deshibrided and probed again using antibodies against total MAPK (ERK1/2, JNK, P38). **B)** Densitometry analysis of western blotting, the ratio of phosphorylated MAPK in front of total MAPK was measured for each time, and fold increase in MAPK phosphorylation was expressed taking the control value as 1. At least, three independent experiments were made for each MAPK. Representative blots are shown on the top of the figure, and group data averages are shown on the bottom of it. *, ** and *** denote significant differences from control values with $p<0.05$, $p<0.01$ and $p< 0.001$, respectively.

## 4.2. Schwannoma cell wound healing induced by UTP is MAPK activation dependent.

It has been previously described that MAPK need to be active in epithelial cell wound repair (Yang, Cranson et al. 2004, Klepeis, Weinger et al. 2004, Weinger, Klepeis et al. 2005). Also, MAPK and especially ERK ½ has been shown to regulate cell survival, migration and proliferation in different cell types (Huang, Jacobson et al. 2004, Nishida, Gotoh 1993). For that, we next aimed to known if the MAPK activation, observed in response to UTP stimulation, could be the responsible of the Schwannoma cell wound repair obtained after UTP treatment. To address this point, we analyzed Schwannoma cell wound repair in the presence of specific inhibitors for the different MAPKs: 10 µM of U1206 an inhibitor of MEK1/2 (upstream ERK ½ kinase), 10 µM of SB203580, an inhibitor of P38, or 20 µM of SP600125, an inhibitor of JNK, in the presence of UTP.

Comparing the migration rate obtained after UTP stimulation, with the migration rate obtained after the treatment with UTP plus any of the antagonists, we found that cells treated with each of the antagonists resulted in a dramatic decrease in the cell wound healing induced by UTP. Schwannoma cells treated with UTP in the absence of any antagonist, showed a significant increase in the cell wound repair in comparison with control cells (*** $p<0.001$, Line 1 and 2 of **figure r11**). In contrast, the treatment with any of the three-studied MAPK inhibitors plus UTP resulted in a drastic and significant decrease of the Schwannoma cell wound repair ($^{###}p<0.001$, Line 2, 3, 4 and 5 of **figure r11**).

So, these results suggest that the MAPKs activation through $P2Y_2$ receptor is needed for the wound healing potency, observed in Schwannoma cells after UTP stimulation.

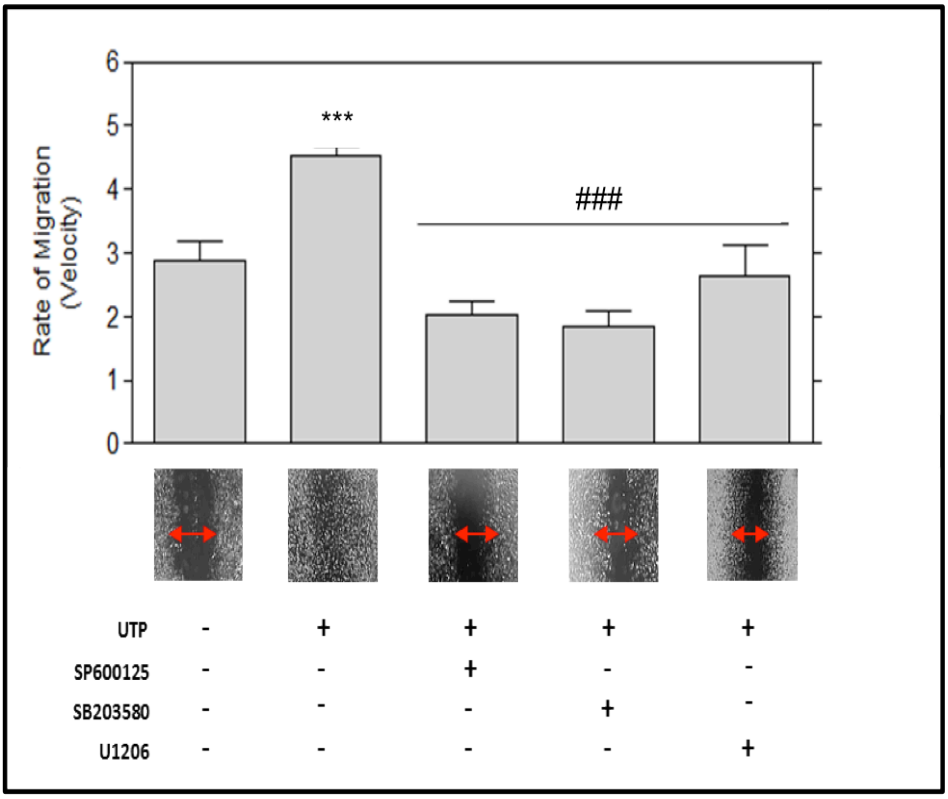


**Figure r11: MAPKs plays a critical role in Schwannoma cell wound repair induced by UTP**. Schwannoma cells were scratched and pretreated with different MAPK inhibitors (SP600125, a p38 inhibitor; SB203580, a JNK inhibitor and U1206, a Mek inhibitor). After 30 minutes of pretreatment, cells were treated with 250 µM UTP. Different images were taken always at the same area until the wound was completely closed, with an inverted microscope. The rate of migration (slope) obtained for at least, three independent experiments is shown in the top of the image, each bar represents the mean ± S.D (n=3). *** Denote significant differences from control values with $p < 0.001$ and ### denote significant differences from UTP values with $p < 0.001$.

## 4.3. Cross-talk between the different MAPKs pathways

Many references describe a possible cross-talk between different members of the MAPKs family (Shen, Godlewski et al. 2003), Boucher, Rich et al. 2010, Klepeis, Weinger et al. 2004, Weinger, Klepeis et al. 2005). In order to determine if there were any cross-talks between the three studied MAPKs (ERK ½, P38 and JNK), phosphorylation levels were assessed in the presence of specific inhibitors for each of the three MAPKs.

Schwannoma cells were seeded and pretreated for 30 minutes with different MAPKs inhibitors (10 µM of U1206, an inhibitor of MEK1/2; 10 µM of SB203580, an inhibitor of P38; or 20 µM of SP600125, an inhibitor of JNK; or with 100 µM Suramin, P2Y receptor inhibitor). Thereafter, cells were treated with UTP for 12h (Late MAPK activation peak **Figure r10**). Cell lysates were collected and 30 µg of total protein lysates were resolved in SDS-Page electrophoresis and probed against pERK, pP38 and pJNK antibodies. The same membranes were deshibrided and probed again with anti-ERK, anti-JNK and anti-P38, which recognizes both active and non-active forms of MAPKs (ERK ½ , JNK, P38).

Blocking purinergic receptors with Suramin caused a significant decrease in the phosphorylation levels of all three analyzed MAPKs ($^{\#\#}p<0.01$ and $^{\#\#\#}p>0.001$, third lane of **Figure r12A**), indicating that purinergic receptors activation is upstream of MAPK phosphorylation. These results were consistent with previous studies showing that the MAPKs pathways were downstream of purinergic receptor activation (Graham, et al 1996, Boucher, Rich et al. 2010).

Furthermore, our results also indicate that treatment with U0126 (MEK ½ inhibitor), not only inhibited ERK ½ but also pP38 and pJNK phosphorylation in a significant way, in comparison to the UTP obtained values ($^{\#\#}p<0.01$ and $^{\#}p>0.05$, lane six in **Figure r12A**. Besides, SB203580 (p38 inhibitor) inhibited not only P38, but also pJNK and pERK1/2 ($^{\#\#\#\cdot}p<0.001$, fourth lane in **Figure r12A**). Finally, with the pJNK inhibitor (SP600125), we observed a significant inhibition not only of pJNK, but also of pP38 ($^{\#\#\#\cdot}p<0.001$ and $^{\#\#}p<0.01$, fifth lane in **Figure r12A**).

These results confirm that activation of the purinergic receptors through UTP leads to MAPKs activation (ERK ½, JNK and p38), and suggest a possible cross talk between the MAPKs. It seems that, in some way, ERK regulates P38 and JNK activation. Also both p38 and JNK, need from each other phosphorylation for their own activation.

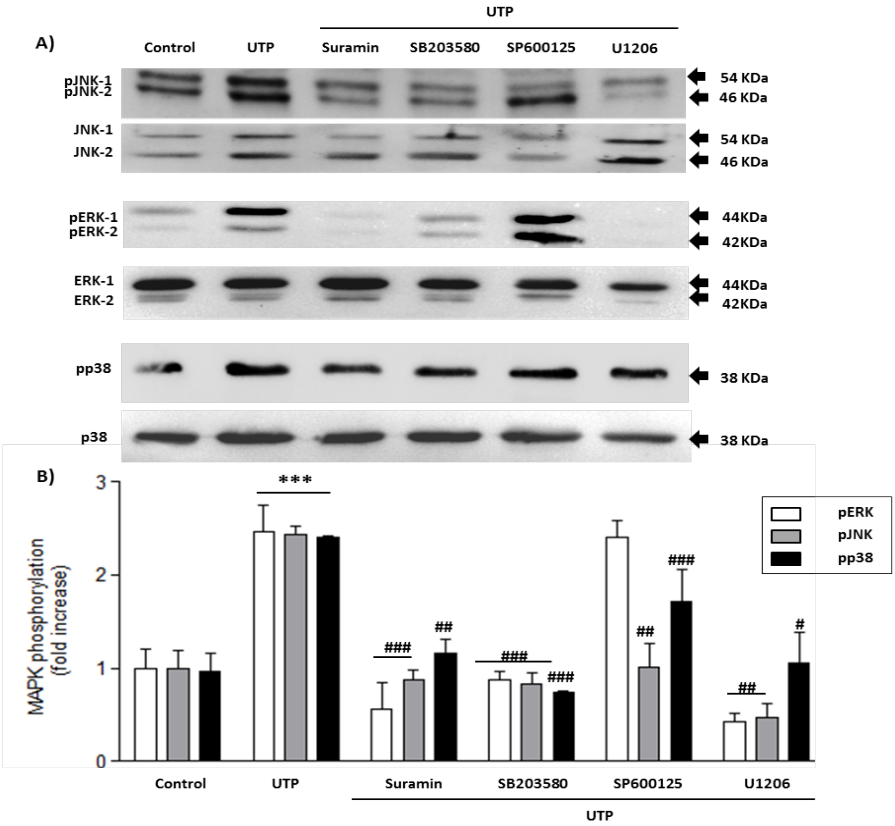


**Figure r12: Cross talk between MAPKs pathways in Schwannoma cells.** Before treating Schwann cells with UTP, we pretreated them with 10 µM of U0126, 10 µM of SB202190, 20 µM of SP600125 or 100 µM of Suramin during 30min. After 12 h of UTP treatment, cell lysates were collected, and equal amounts of protein (30 µg) were resolved in SDS-PAGE and blotted with anti-phosphoERK, anti-phosphoJNK and anti-phosphoP38. The same membranes were deshibrided and also probed with anti-ERK, anti-JNK and anti-P38, which recognizes both active and non-active forms of MAP kinases (ERK1/2, JNK, P38). At least, three independent experiments were made, and representative blots are shown on the top and group data averages are shown on the bottom of the figure. *** denote significant differences from control values with ***$p< 0.001$, and $^{\#\#, \#\#\#}$ denote significant differences from the UTP values with $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$.

## SECTION 3: Activation of MMP by UTP induces Schwann cell migration. Hypothetical pathway

# 5. MMPS EXPRESSION AND ACTIVATION THROUGH UTP IN SCHWANN CELLS

Matrix metalloproteinases (MMPs) are among the proteins that have the potential to promote cell migration and, in their active form, they are involved in wound healing and repair (Hsu et al., 2006). So, once described that Nucleo CMP forte and specially UTP induce Schwannoma cell migration and Wound Repair, and due to the important role that MMP's plays in matrix degradation and cell migration, we next aimed to study the expression and activation of MMPs in Schwann cells.

## 5.1. Study of MMPs mRNA expression

We first explored whether Schwann cells, expressed or not the mRNA of distinct MMPs (MMP-2, MMP-9, MMP-7, MMP-14 and MMP-28), in both Schwannoma cell line and Schwann primary culture. Specific primers for each metalloproteinases were designed using the GeneBank software. mRNA expression of the distinct MMPs was analyzed by PCR. The presence of the same MMP's was observed after PCR amplification in both Schwannoma cells and Schwann primary culture (**Figure r13**). They showed the presence of MMP-2, MMP-7, MMP-14 and MMP-28. In return, MMP-9 mRNA expression was not observed neither in the Schwannoma cell line nor in the Schwann primary culture.

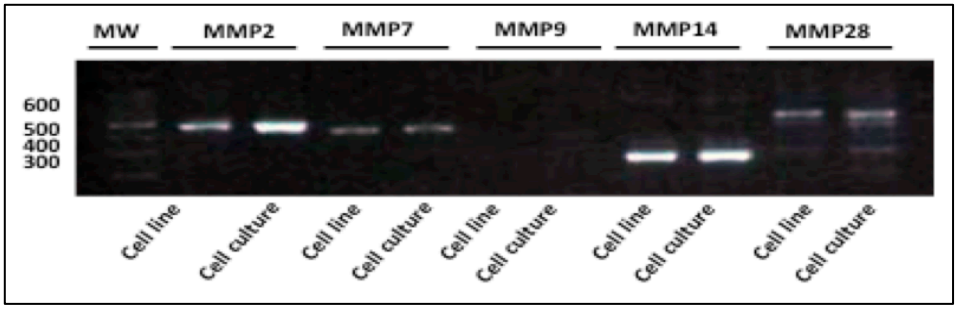


**Figure r13: mRNA presence of different MMP's in Schwannoma cell line and in Schwann primary culture.** mRNA levels of MMP-2, MMP-7, MMP-9, MMP-14 and MMP-28 were assessed by common PCR using specific primers for each of the analyzed MMP's.

## 5.1.1. UTP increases MMP-2 mRNA levels in Schwannoma cells.

Our next goal was to identify if UTP alters the MMP-2 mRNA levels of Schwannoma cells. For that, cells were seeded, treated with UTP (250 µM) at different times (30 min, 1h, 3h, 6h, 12h and 24h) and total RNA was collected, retrotranscribed and a Real Time PCR was performed using specific primers for MMP-2 (as explained in M&M Section 2.1.4). UTP induced a significant increase in the mRNA expression of MMP-2 (*p<0.05 and ***p<0.001), which started 1h after UTP treatment, and reached the maximum peak after 12 h of cell stimulation. 24 hours after UTP stimulation, MMP-2 mRNA levels decreased to the baseline control levels (**Figure r14**).

This result describes, for the first time, a role for UTP in MMP-2 mRNA expression in Schwannoma cells. Also, the maximum level of MMP-2 mRNA expression is reached after 12 hours of cell treatment, which coincides with the late MAPK peak (**Figure R10**).

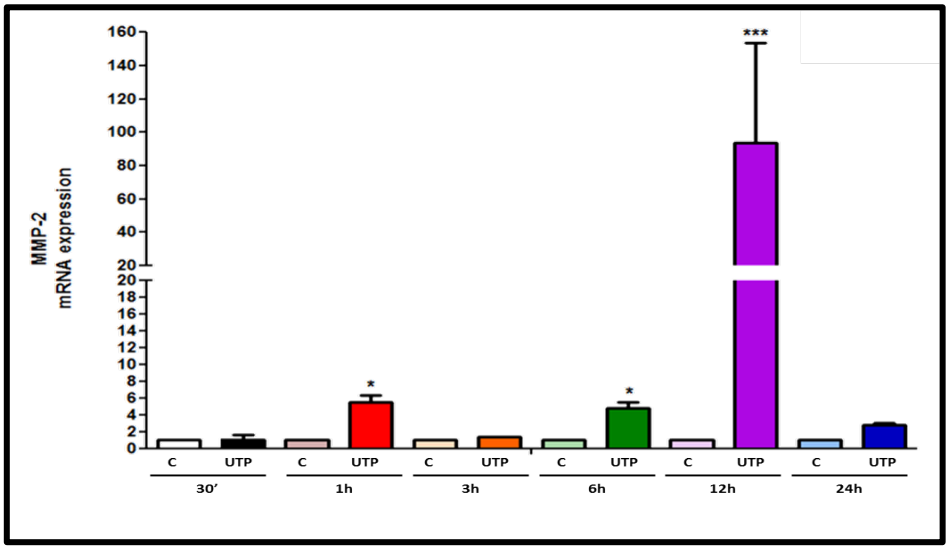


**Figure r14. UTP induces over expression of MMP-2 mRNA levels in Schwann cells at different times.** RT4D6P2T cells were treated with 250 µM of UTP for different times. Total RNA, was analyzed by real time PCR, using specific primers for MMP-2 and using GAPDH as housekeeping gene. We observed a significant increase of mRNA levels of MMP-2 after 1, 6 and 12h of UTP treatment. At least, three independent experiments were made, and results are shown as the Mean ± SD. *** p< 0.001 and * p<0.05.

## 5.2. Study of MMPs activity

Once elucidated that mRNA, of different MMP's, was present in Schwannoma cells (MMP-2, MMP-7, MMP-14 and MMP-28, **Figure r13**), we wanted to analyze the activity of these MMPs in the cell line, using the substrate zymography. This is a simple, sensitive, quantifiable and functional assay, used to analyze MMPs, that allows us to identify the activation of different MMPs based on the degraded substrate (Patricia A.M, Van Beurden et al. 2005). Three different types of zymography were used; Gelatin zymography, which is extremely sensitive and used for the detection of gelatinases such as MMP-2 and 9; Casein zymography that is principally suitable for the detection of MMP-1, MMP-7, MMP-12 and MMP-13, although MMP-9 can also be detected when it is present at high concentrations, but this zymography is less sensitive than the first. And, finally Collagen Zymography, is mainly used for analyze MMP-1 and 13, although MMP-2 and 9 can be detected at high concentrations. This last zymography is the less sensitive of them (Patricia A.M, Van Beurden et al. 2005, Kleiner, Stetler-Stevenson W.G. 1994).

Gelatin zymography allowed us to detect both isoforms of MMP-2, the latent one (Pro-MMP2, 67 KDa) and the active one (MMP2, 60 KDa), while we could not detect the presence of any of the other gelatin MMP's, such as MMP-9 (never present in the mRNA analysis, **Figure r13**). Casein zymography did not show the presence of any casein-degraded metalloproteinases, such as MMP-1, 12 or 7 (detected in the mRNA analysis), maybe because MMP-7 was present at very low levels or was not active in Schwannoma cells. Finally, in the collagen zymography, we detected MMP-2, in both the active and the latent form, but no other MMP's with collagen affinity such as MMP-9, 1 or 13 were present, as shown in **Figure r15**.

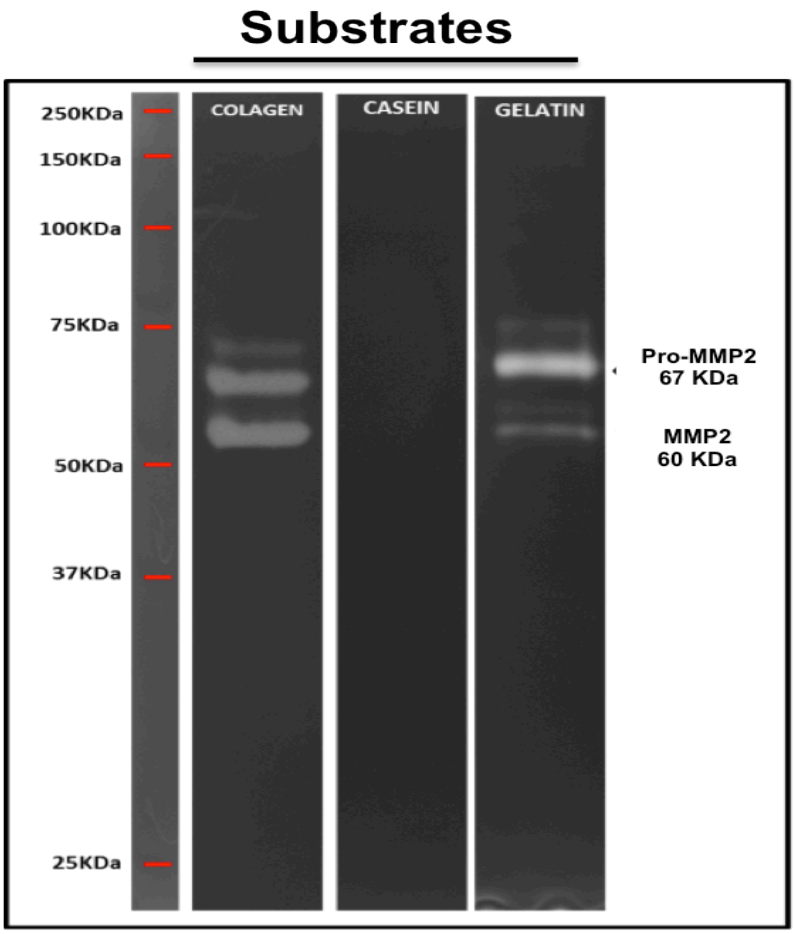


**Figure r15. Analysis of different substrate zymoraphy to identify MMPs.** Conditioned media of Schwann cells was submitted to Zymography electrophoresis using different substrates (Colagen, Casein or Gelatin).

### 5.2.1. Nucleo CMP forte induced MMP-2 activation in Schwannoma cells.

We next explored whether Nucleo CMP forte can induce and/or activate MMP-2 and MMP-9 (both gelatinases) in Schwannoma cells. For that, Schwannoma cells were treated with 1 g/L of Nucleo CMP forte at different times (6, 12, 24 and 48h). Cell-conditioned media was submitted to a gelatin zymography.

Expression of latent MMP-2 (molecular weight of 67 KDa) in Schwannoma cells was detected after 12 hours of cell treatment, and it was present at similar levels, in both untreated and CMPF treated cells. In contrast, active form of MMP-2 (which appears at 60 KDa) was only observed in Schwannoma cells treated with CMPF after 12 hours of stimulation. Also, the active form of MMP-2 was clearly enhanced in Schwannoma cells stimulated with CMPF, after 24 and 48 hours, indicating that some of the nucleotides present in the drug prompted the proteolytic MMP-2 activation in Schwannoma cells (**Figure r16**).

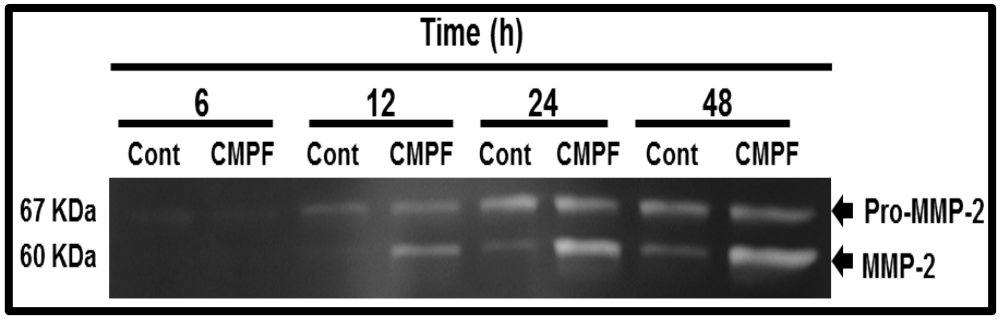


**Figure r16. Gelatin zymograhy of Schwann cells treated with Nucleo CMP forte.** MMP-2 is observed in both Pro and active form after cell treatment with Nucleo CMP forte for 12, 24 or 48 h.

## 5.2.2. UTP induced MMP-2 activation in Schwannoma cells

Once saw that Nucleo CMP forte induced MMP-2 activation, we wanted to know if UTP was also the responsible of this MMP-2 activation since this nucleotide was the major inductor of the Schwannoma cell wound repair and migration.

Schwannoma cells were treated with 250 µM UTP at different times (6, 12 and 24h). After cell treatment, conditioned media was collected and resolved in Gelatin Zymopgraphy gels. Expression of latent MMP-2 (67 KDa) in Schwannoma cells was detected after 12 and 24 hours of cell treatment, and it was presented at similar expression levels in both untreated and UTP stimulated cells (**Figure r17**). In contrast, active MMP-2 (60 KDa), was only detected in Schwannoma cells stimulated with UTP for 12 or 24 hours.

This result indicates that UTP stimulation induces a proteolytic MMP-2 activation in Schwannoma cells after 12 hours of cell treatment and at least until 24 hours of cell treatment.

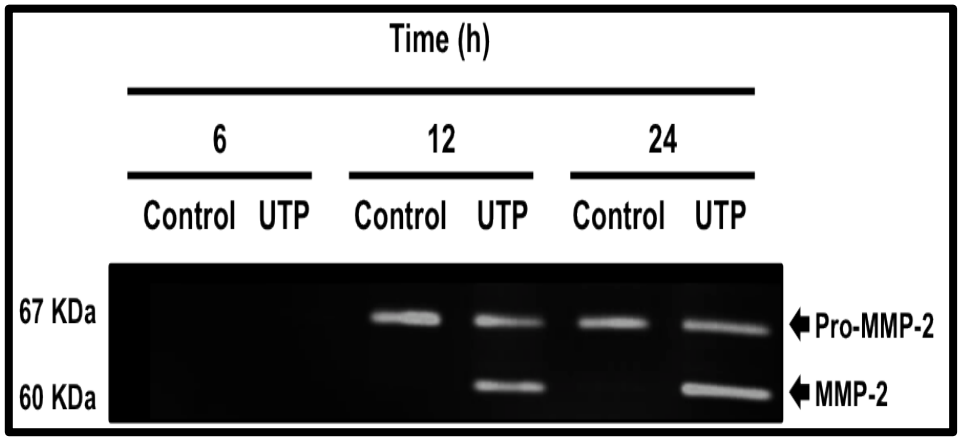


**Figure r17. Gelatin zymograhy of Schwann cells treated with UTP.** MMP-2 activation is observed after 12 and 24 h of UTP treatment. The band of 60 KDa corresponds to the active form of MMP-2 and the band of 67 KDa to the latent form of MMP-2.

To confirm the MMP-2 activation observed after UTP stimulation, we performed an *in situ* zymography (EnzChek kit, Molecular Probes). This technique is high sensitive and it is used to measure and localize gelatinase activity in cell cultures. In the *in situ* zymography, an increase in the fluorescence spots is proportional to the proteolytic activity of the cell (Mook, Van Oberveek et al. 2003, George, Johnson 2010, Wesley, Bove et al. 2006).

In order to perform the *in situ* zymography, Schwannoma cells were seeded and treated with UTP for 12 h, time in which MMP-2 is active after stimulation (**Figure r18**). The presence of gelatinase activity was highly enhanced after UTP treatment, observed by the increasing of fluorescence spots in Schwannoma treated cells (green spots in **Figure r18,** a and c), although the number of cells was similar in both conditions, as we can see in the bright field pictures (**Figure r18**, b and d).

So, taking together all the obtained results, we can confirm that UTP enhances MMP-2 activation and activity in Schwannoma cell line, after 12 hours.

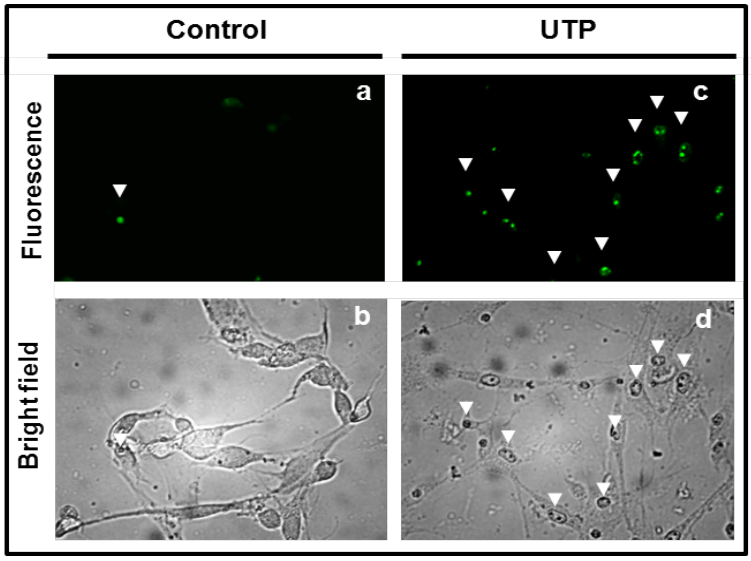


**Figure r18. In situ zymography of Schwann cells.** Gelatinase activity, in control (a, b) and treated (c, d) cells. Schwann cells were plated in chamber slides, serum deprived and treated for 12 h with or without UTP. Gelatinase activity was assessed by incubation with DQ-Gelatin and visualized by fluorescence microscopy (green). Pictures of the bright field were taken. Arrows indicate the localization of gelatinase activity in the cell.

## 5.3. Wound Healing enhances MMP-2 activation in response to UTP in Schwann cells.

It has been hardly described that MMP's are involved in the remodelling of tissues, cell migration and wound healing among other actions (Steffensen, Hakkinen et al. 2001, Pilcher, Wang et al. 1999, Page-McCaw, Ewald et al. 2007). We first elucidated that UTP induces MMP-2 activation in Schwann cells, then we wondered to known whether the injury, created by a scratch, was able to induce MMP-2 activation. Schwannoma cells or Schwann primary cultures were seeded, scratched and treated with UTP (250 µM). Conditioned media was collected 12 hours later, concentrated and 20 µg of protein resolved in a gelatin zymography gel.

MMP-2 activation (60KDa) was observed both after wound healing and/or UTP treatment (**Figure r19**, second, third and fourth lane). Surprisingly, when a wound scratch was performed in the UTP stimulated Schwannoma cells, MMP-2 activation was drastically increased, up to 80 times in front of control cells (***$p<0.001$, **Figure r19** first and fourth lane).

These results suggest that wound healing together with UTP strongly induces MMP-2 activation in Schwannoma cells.

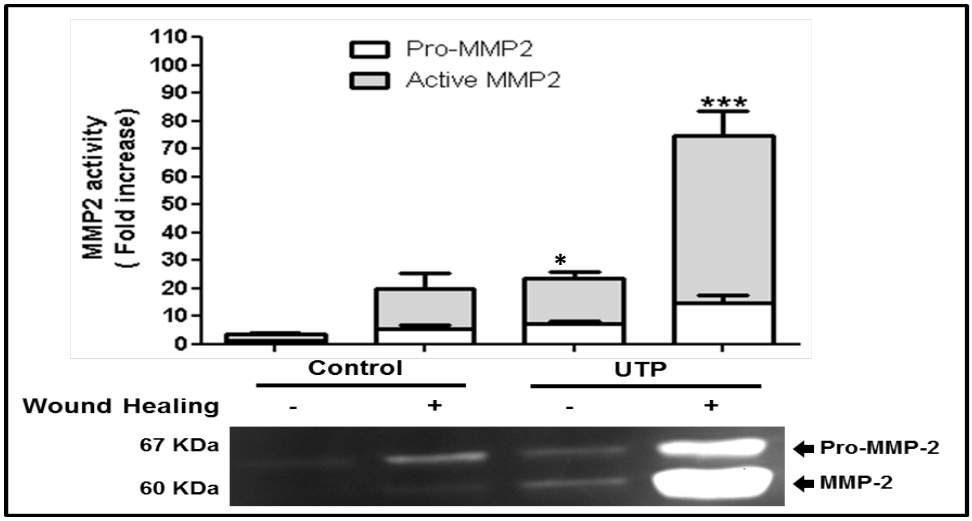


**Figure r19. Wound Healing enhances UTP effects on MMP-2 activation in Schwannoma cell line.** Cells were serum deprived, treated with UTP (250 µM) and an in vitro wound scratch was made. Conditioned media was collected after 12h. Equal amounts of protein (20µg) were analyzed by gelatin zymography. UTP induces MMP-2 activation (third lane), and this activation is hardly increased when we treated Schwannoma cells with UTP and performed a wound scratch into the culture dish (fourth lane). At least, three independent experiments were made, and a significant image is shown, statistical significance *** $p< 0.001$ and *$p<0.05$.

Next, we studied the MMP-2 activation after wound healing and UTP treatment in a Schwann primary culture. For that, Schwann primary cells were seeded, a scratch was made and cells were treated with UTP (250µM) for 33 hours (time in which the wound was completely closed, **Figure r3**). Thereafter, conditioned media was collected, concentrated and 20 µg of total media proteins were submitted to a gelatin electrophoresis.

After 33 hours the latent form of MMP-2 (Pro-MMP2, 67 KDa) was present in all the tested conditions (**Figure r20**). Interestingly, UTP treatment enhanced MMP-2 activation in Schwann primary culture in a significant way (**$p<0.01$, third lane of the image). At the end, this activation was significantly increased in cells scratched and stimulated with UTP (up to 4 fold over control cells, **$p<0.01$, fourth lane of the image).

So our results strongly suggest that, in Schwannoma cells and in Schwann primary culture, UTP induces MMP-2 activation and also that this is strongly activated after cell injury, showing a possible role of MMP-2 in cell migration.

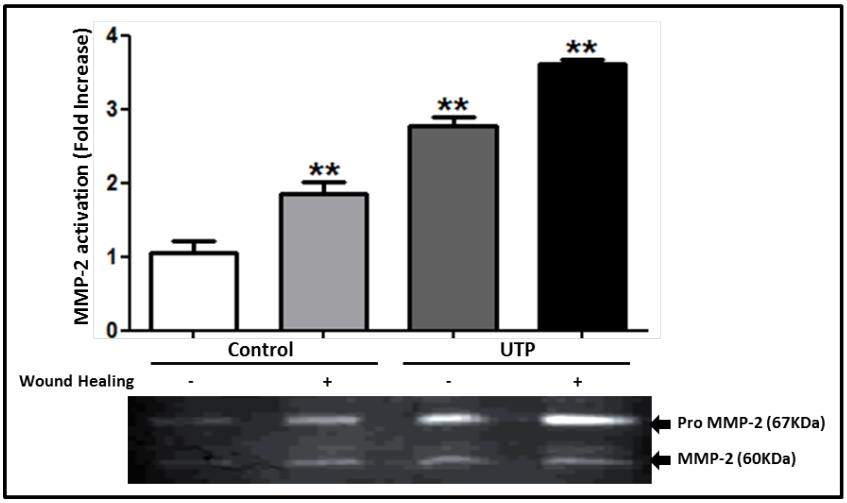


**Figure r20. Wound Healing enhances UTP effects of MMP-2 activation in Schwann primary culture.** Primary Schwann culture was seeded in 10 cm plates and growth until they reached an 80% of confluence. After, cells were serum deprived, scratched and treated with UTP (250 µM). Conditioned media was collected at 33h. Equal amounts of protein were analyzed by gelatin zymography. UTP induces MMP-2 activation, and this activation is hardly increased when we performed a wound scratch into the culture dish. At least, three independent experiments were made, and a significant image is shown. ** $p < 0.01$.

## 5.4. UTP induces changes in MMP-2 localization in Schwann cells.

To study the MMP-2 localization after UTP stimulation, an immunofluorescence against MMP-2 was performed, using a specific antibody (as explained in M&M, section 3.9). Both, Schwannoma cells or Schwann primary cultured cells were seeded over coverslips and treated with UTP (250 µM) during 12h for the cell line and during 33h for the primary culture.

As we could observe non-treated cells, in both the cell line and the primary culture, showed mainly a preinuclear MMP-2 localization (**Figure r21 A and E**). Conversely, both cell types treated with UTP (250 µM), showed not only an increasing in the MMP-2 protein intensity, but also a more cytoplasmatic localization of the MMP-2 **(Figure r21 B and F)**.

These results seem to indicate that UTP induces a significant increase in the MMP-2 expression and a differential MMP-2 protein localization, which is more located in the cytoplasm and philopodias of Schwann cells, after treatment.

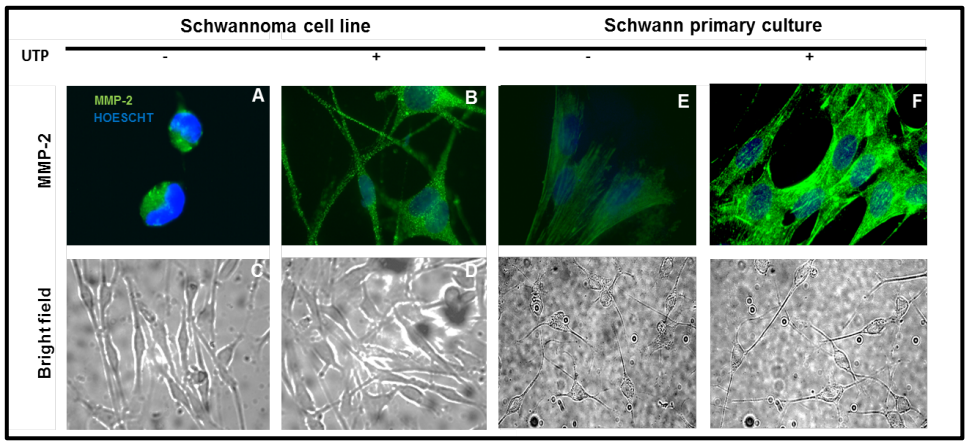


**Figure r21. UTP increases MMP-2 levels and induces a cytoplasmic localization in Schwann cells.** Immunofluorescence against MMP-2 was performed in Schwannoma cells and Schwann primary culture treated with UTP (B, F) or non-treated (A, E). Photos were taken at 60X. Bright fields were taken to confirm cell morphology.

# 6. SCHWANNOMA CELL WOUND HEALING INDUCED BY UTP IS DEPENDENT ON MMP-2 ACTIVATION

It has been hardly described that MMP's plays important roles in cell migration and wound healing in different cell types such us epithelial or cancer cells (Bjorklund, Koivunen 2005, Giannelli, Falk-Marzillier et al. 1997, Chen, Parks 2009). For that reason, we next wondered to known if MMP's are also important in the Schwann cell wound healing observed after UTP treatment. Then, we used a pharmacological MMP inhibitor, GM6001 that inhibits a broad spectrum of MMP's (MMP-1, MMP-3, MMP-2, MMP-8 and MMP-9), and the wound healing potency of Schwannoma cells was analyzed.

Cells were seeded, scratched, pretreated with GM6001 (10µM) and finally stimulated with UTP (250 µM). Pictures were taken at the same area until wound scratch was completely closed. The obtained results, shown in **Figure r22,** indicated that Schwannoma cells treated with UTP suffered a significant increase in Schwann cell wound repair, as seen before (**Figure r2,** and Second bar in **Figure r22B**). In contrast, pretreatment of Schwannoma cells with GM6001 reduced the cell wound repair, induced by UTP, at more or less the same rate of migration than those observed in control cells (First and third bar in **Figure r22B**).

These results suggest that MMP's plays an important role in the UTP induced wound repair in Schwann cells.

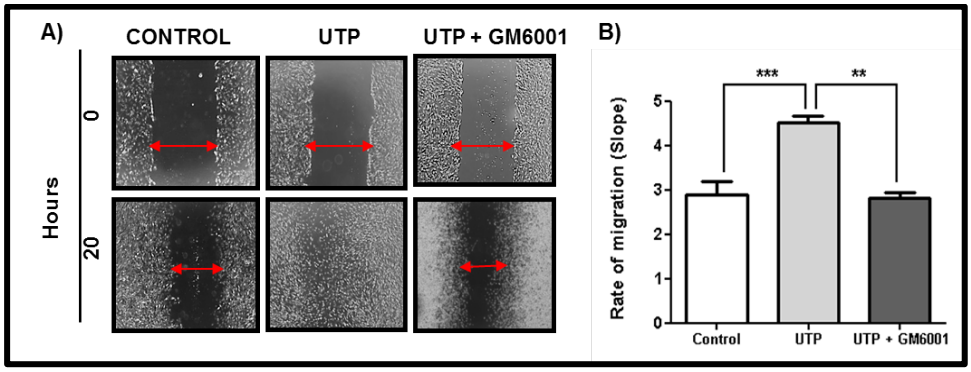


**Figure r22. MMP's play a critical role in Schwann cell wound repair induced by UTP**. A) Schwann cells were pretreated 30 min with 10 µM of GM6001, thereafter treated with 250 µM UTP, and a scratch wound assay was performed. Different images were taken always at the same area until the wound was completely closed. Images shown represent 0 and 20h of treatment. B) The rate of migration (slope) obtained for at least, three independent experiments is shown. Each bar represents the mean ± S.D (n=3). **, *** denote significant differences from control values with p<0.01 and p< 0.001 respectively.

As the GM6001 inhibitor is non-selective for MMP-2, and since we had previously elucidated that MMP-2 is activated by UTP in Schwann cells (**figure r17**), we designed a specific shRNA against MMP-2 (M&M Section 2.2), in order to confirm if it is involved directly in the Schwannoma cell wound repair.

Designed shMMP-2 was checked, to elucidate their effectiveness in silencing MMP-2. Schwannoma cells were transfected with shMMP-2 or shRandom vectors and, after 24 hours, cell media, cell lysates and RNA were collected. mRNA levels of MMP-2 were analysed by Real Time PCR, and the results showed that levels of MMP-2 were significantly decreased in cells with the shMMP2 (**Figure r23A** red bar). In contrast, mRNA levels of MMP-2 in the shRandom cells were similar to those observed in non-transfected cells (Bar yellow and orange respectively, **Figure r23A**). In a similar manner, the levels of MMP-2 protein in cells transfected with the shMMP2, were reduced at more than a 50%, in comparison to non-transfected cells or shRandom cells (**Figure r23B**).

Finally, we wanted to know if this shMMP-2 was useful decreasing the MMP-2 activation observed after UTP treatment. shRandom cells showed a similar MMP-2 activation than those observed in non-transfected cells after UTP stimulation (**Lane 2 and 4 of image r23 C**). Schwannoma cells transfected with the shMMP2 and treated with UTP, showed a significant decrease in the MMP-2 levels, both in the Pro MMP-2 (67KDa) and the activated MMP-2 (60KDa) forms, in comparison to control or to shRandom transfected cells.

All these results indicate that the designed shMMP-2 is effective in reducing the activation of MMP-2 induced by UTP, so as in silencing mRNA and protein levels of MMP-2.

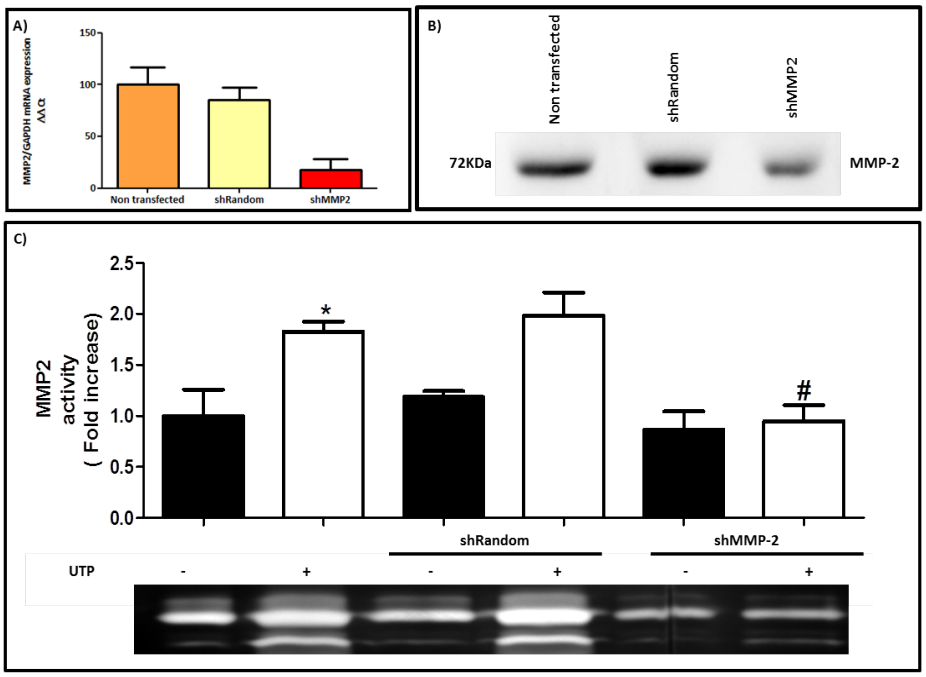


**Figure r23. shMMP2 silencing efficiency. A)** Real Time PCR was performed using specific primers against MMP-2, and mRNA levels were checked after cell transfection with both vectors. **B)** Western Blot against MMP-2 was performed after 24 hours of cell transfection with shRandom or shMMP2, and protein levels of MMP-2 were checked. **C)** Schwannoma cell line was transfected with both vectors (shRandom or shMMP2) and MMP-2 activity was assessed by gelatin zymography. * Denote significant differences from control values with $p<0.05$ and $^{\#}$ denote significant differences from UTP values with, $^{\#}p< 0.05$.

After, we wanted to study the possible role of MMP-2 on the Schwannoma cell wound repair induced by UTP. Cells were seeded and transfected with either the shMMP2 or the shRandom for 24 hours. Then, a wound scratch was performed and cells were treated with UTP (250 µM).

Schwannoma cells transfected with the shRandom vector, showed similar rates of migration than those obtained with non-transfected cells (**Figure r24**). However, shMMP2 transfected cells treated with UTP suffered a drastic reduction of the migration rate, in comparison with non-transfected or shRandom cells (**Lane 1, 4 and 6 of Figure r24B**).

Our results strongly suggest that MMP-2 is necessary for the increasing of Schwannoma cell wound repair, observed after UTP stimulation.

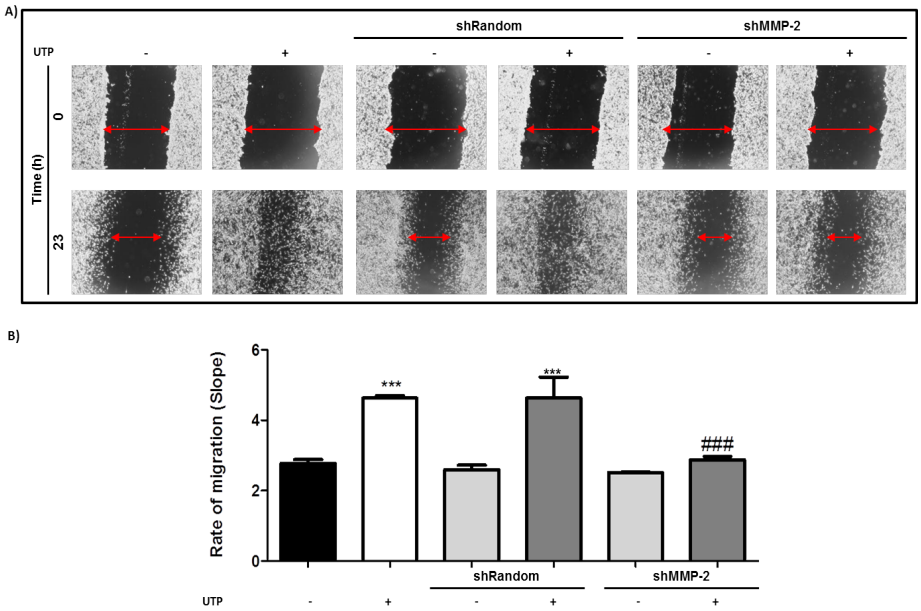


**Figure r24. MMP-2 plays a critical role in Schwannoma cell migration induced by UTP**. A) Schwannoma cells were transfected with shMMP2 or shRandom vectors. After transfection cells were scratched and treated with 250 µM UTP. Different images were taken always at the same area until the wound was completely closed with an inverted microscope. Images represent 0h and 23h of cell treatment. B) The rate of migration (slope) obtained for at least, three independent experiments is shown, each bar represents the mean ± S.D (n=3) and *** denote significant differences from control values with $p<0.001$, and ### denote significant differences from UTP values with $p< 0.05$.

# 7. MMP-2 ACTIVATION BY UTP DEPENDS ON THE PURINERGIC RECEPTORS AND MAPKS

Many research articles relation MAPKs with the activation of MMP's (Chattopadhyay S, Shubayev 2009, D'Alessio, Ferrari et al. 2008). To investigate whether the signalling pathway through P2Y receptors and MAPKs are involved in the MMP-2 activation, observed in Schwann cells after UTP stimulation, cells were pretreated with different MAPKs inhibitors (SB203580 a P38 inhibitor, SP600125 a JNK inhibitor and U1206 a MEK1/2 inhibitor), so as with Suramin (P2Y receptor inhibitor). After 30 min of pretreatment, cells were stimulated with UTP (250µM) for 12h (Late MAPK activation and maximum MMP-2 activation induced by UTP). The cell-conditioned medias were collected and 20 µg of total media proteins were resolved in a zymography gel.

As we can see in the figure below (**Figure r25**), all the tested conditions showed similar intensity bands of Pro-MMP2 (67 KDa). As well, a significant decrease in the MMP-2 activation, after UTP stimulation, was observed once cells were preincubated with any of the used inhibitors ($^{###}p<0.001$ First, fourth, fifth and sixth lane in **Figure r25**). Finally, GM6001 was used as a positive control of MMP inhibition, and Schwannoma cells pretreated with this, showed a significant decrease in the MMP-2 activation levels ($^{###}p<0.001$, last bar in **Figure r25**).

These findings strongly suggest that P2Y-MAPK pathway stimulation, induced by UTP in Schwann cells, is required for MMP-2 activation.

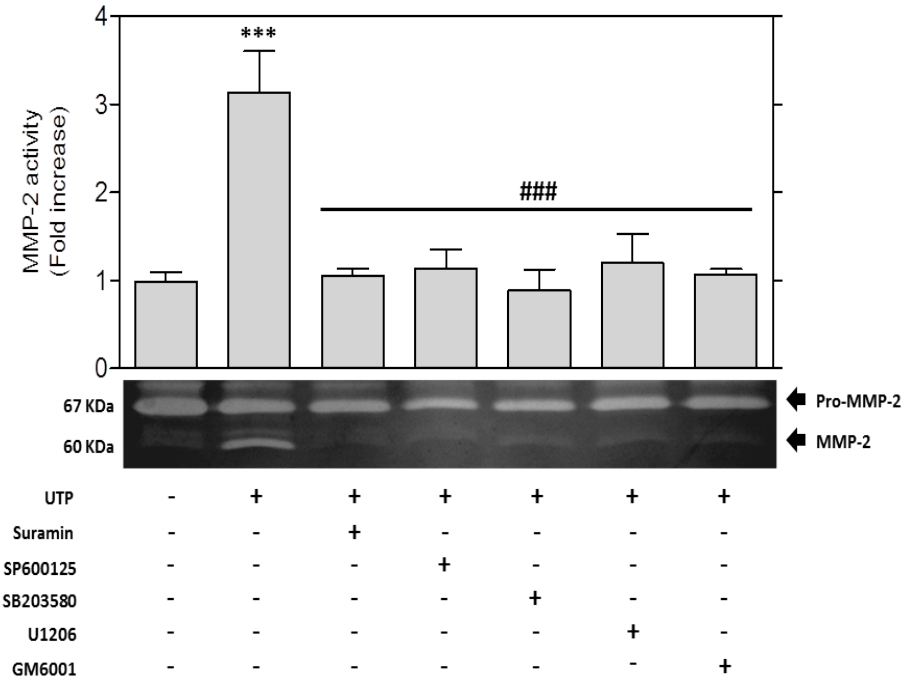


**Figure r25. Purinergic receptor activation and MAPK phosphorylation is necessary for MMP-2 activation by UTP.** Schwann cells were pretreated with MAPK inhibitors (SP600125, SB203580, U1206), Purinergic Receptor inhibitors (Suramin) and MMP's inhibitor (GM6001), and then treated with UTP for 12h. Zymography assay was performed with 20 µg of protein. At least, three independent experiments were made, and representative zymographies and group data averages are shown on the figure. *** denote significant differences from control values with *** $p<0.001$, and ### denote significant differences from UTP values with ###$p<0.001$.

We previously observed that Suramin inhibits the MMP-2 activation in Schwannoma cells. This pharmacological inhibitor is non-selective for P2Y receptors, then we wanted to analyze if the $P2Y_2$ receptor, which we had seen before that was directly involved in Schwannoma cell migration (**Figure r9**), was also involved in the MMP-2 activation through UTP. Cells were transfected with the $shP2Y_2$ or the shRandom and treated with UTP. After 12 hours, cell conditioned media was collected and 20 µg of total media proteins were submitted to zymography electrophoresis.

As we can see in the figure below (**Figure r26**), Schwannoma cells transfected with the shRandom and stimulated with UTP, showed a significant increase in the MMP-2 activation in comparison to non- stimulated cells (First and second raw of **Figure r26)**. Instead, Schwannoma cells transfected with the shP2Y$_2$ and treated with UTP, suffered a drastic reduction of the MMP-2 activation, in both the Pro and the active forms (60 and 67KDa band respectively, **Figure r26**).

These results clearly suggest that P2Y$_2$ receptor, is directly involved in MMP-2 activation after UTP stimulation in Schwannoma cells. Once the P2Y$_2$ receptor is silenced, not MMP-2 activation is obtained, even with UTP stimulation. So, MMP-2 activation is probably a downstream event that happens after P2Y$_2$ activation.

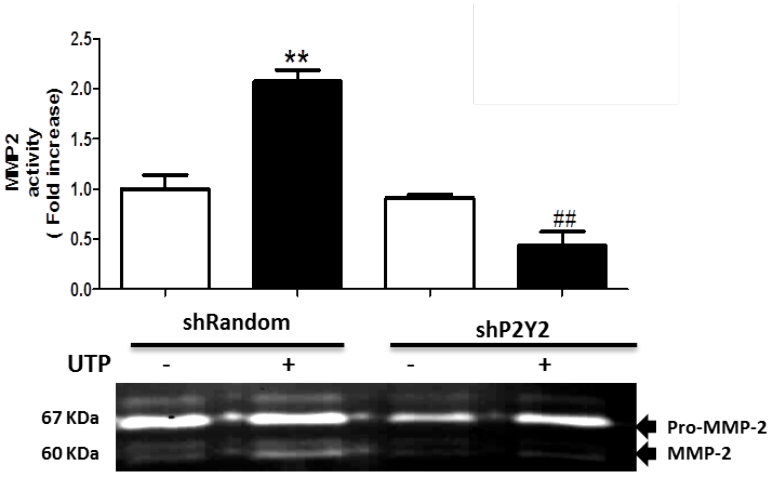


**Figure r26. P2Y2 receptor activation is necessary for MMP-2 activation by UTP.** Schwann cells were transfected with shP2Y2 or shRandom, and then treated with UTP for 12h. Zymography assay was performed with 20 µg of protein. At least, three independent experiments were made, and representative zymographies and group data averages are shown on the figure. ** denote significant differences from control values with ** $p<0.01$, and ## denote significant differences from UTP values with ##$p<0.01$.

# 8. UTP MAINTENANCE IS REQUIRED FOR BOTH THE LATE MAPK AND THE MMP-2 ACTIVATION

Taking into account that inhibition of any studied MAPKs induce an inhibition of the MMP-2 activation induced by UTP (**Figure r25**), our next question was if UTP ought to be constantly present in the medium in order to observe the late MAPK peak and the MMP-2 activation. To study that, Schwannoma cells were treated with 250 µM UTP for 45 minutes, thereby inducing the early MAPK activation. After that time, media was completely replaced for fresh media without UTP. Both, cell extract and conditioned medias were collected after 12 hours and analyzed by western blott for the three MAPKs (ERK ½, JNK or p38), so as by gelatin zymography.

Results showed that UTP is necessary to induce both Late MAPK phosphorylation (**Figure r27,** a, b and c) and MMP-2 activation (**Figure r27**, d). Once UTP was replaced from the cells, after 45 minutes of treatment, no Late Activation of any of the three-studied MAPKs was observed. Besides, we were not able to observe MMP-2 activation once UTP was replaced from the cells (lane 4 in **Figure r27**). MMP-2 levels in replaced media cells were similar to those obtained in control cells (compare lane 1 with lane 3 and 4 in **Figure r27**).

These results clearly indicate that the presence of UTP is constantly required to induce both MAPK late phosphorylation and MMP-2 activation.

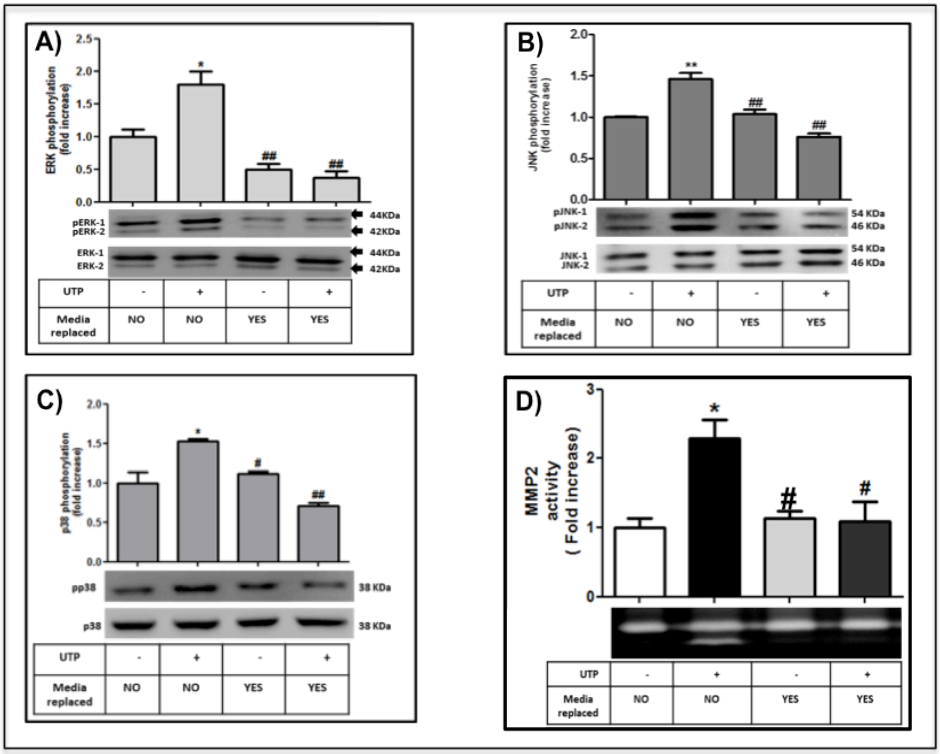


**Figure r27. Maintained UTP is necessary for the late MAPKs phosphorylation and the MMP-2 activation.**_Schwann cells were treated with UTP for 45 minutes and thereafter media was replaced for fresh media without UTP. Cell lysates and conditioned media were collected after 12h. **A**, **B** and **C**) cell lysates were collected, and equal amounts of protein (20 µg) were resolved in SDS-PAGE and blotted with anti-phosphoERK (A), anti-phosphoJNK (B) and anti-phosphoP38 (C). The same membranes were deshibrided and also probed with anti-ERK, anti-JNK and anti-P38, which recognizes both active and non-active forms of MAP kinases (ERK1/2, JNK, P38). **D)** Zymography was performed with 20 µg of protein. At least, three independent experiments were made, and representative images are shown on the bottom and group data averages are shown on the top of the figure. *, ** denote significant differences from control values with *$p<0.05$ and ** $p<0.01$, and $^{\#, \#\#}$ denote significant differences from UTP values with $^{\#}p<0.05$, $^{\#\#}p<0.01$.

# 9. LONG TIME MAPK ACTIVATION BY UTP IS MMP-2 DEPENDENT.

MAPKs and MMP-2 seem to play an important role in the Schwannoma cell wound repair induced by UTP (**Figure r11 and r24** respectively). Also, UTP actives both MAPK and MMP-2 (**Figure r10 and r16** respectively) therefore we wanted to study whether MMP-2 had any role on MAPKs activation. We performed two different approximations: in one hand, we studied if GM6001 (MMPs inhibitor) was able to block MAPKs activation induced by UTP; and in the other, we studied if the specific silencing of MMP-2 blocked the MAPKs activation induced by UTP. In both approximations, we analyzed the early and the late activation peaks of MAPKs.

First, Schwannoma cells were pretreated with GM6001 (10µM) for 30 minutes and thereafter with UTP (250 µM) for 5 minutes (Early activation peak of MAPK). Cell extracts were collected and the phosphorylation of three MAPKs (pERK, pP38 and pJNK) was analyzed by western blotting. The same membranes were also probed against anti-ERK, anti-JNK and anti-P38, which recognizes both active and non-active forms of MAP kinases (ERK1/2, JNK, and P38). Schwannoma cells treated with UTP for 5 minutes showed a significant increase in ERK ½, JNK and p38 phosphorylation of approximately 2 fold over the control cells **(***$p<0.05$ and ****$p<0.01$ in **second lane in figure r28 A, B or C**), as expected. In addition, Schwannoma cells pretreated with GM6001 (MMP inhibitor) and treated with UTP for 5 minutes, showed similar MAPKs activation rates than those obtained only with UTP treatment (approximately 2 fold over the control, **fourth lane in figure r28 A,B or C**).

These results suggest that MMPs activation is not involved in the MAPKs early activation due to UTP.

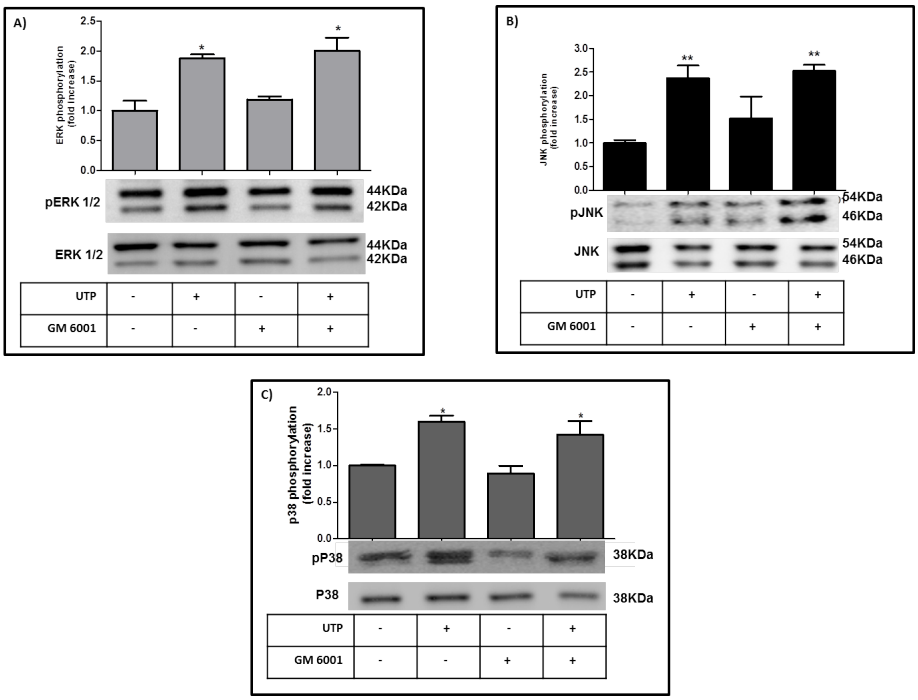


**Figure r28. MMP's do not inhibit Early MAPK activation due to UTP.** Schwann cells were treated with GM6001 for 30 minutes and thereafter with UTP for 5 min, cell extract were collected. A, B and C) cell lysates were collected, and equal amounts of protein (30 µg) were resolved in SDS-PAGE and blotted with anti-phosphoERK (A), anti-phosphoJNK (B) and anti-phosphoP38 (C). The same membranes were deshibrided and also probed with anti-ERK, anti-JNK and anti-P38, which recognizes both active and non-active forms of MAP kinases (ERK1/2, JNK, P38). At least, three independent experiments were made, and representative blots are shown on the top and group data averages are shown on the bottom of the figure. *, ** denote significant differences from control values with **p< 0.01 and * p<0.05.

Second, we studied if MMP's had any effects on the Late MAPKs activation. For that, Schwannoma cells were pretreated with 10 µM of GM6001 for 30 minutes, and thereafter stimulated with 250 µM of UTP for 12h (Late MAPK activation peak). Cell lysates were collected and submitted to Western Blot against the three MAPKs (ERK ½, JNK and p38).

Inhibition of MMP's had a critical effect on the late MAPKs phosphorylation due to UTP (**Figure r29**). Schwannoma cells treated with UTP showed a significant increase in the phosphorylation levels of the three-studied MAPKs, as we had already seen before (**Figure r10**). In contrast, Schwannoma cells pretreated with GM6001 and stimulated with UTP suffered a drastic decrease in the MAPKs Late phosphorylation (Fourth lane in **Figure r29**).

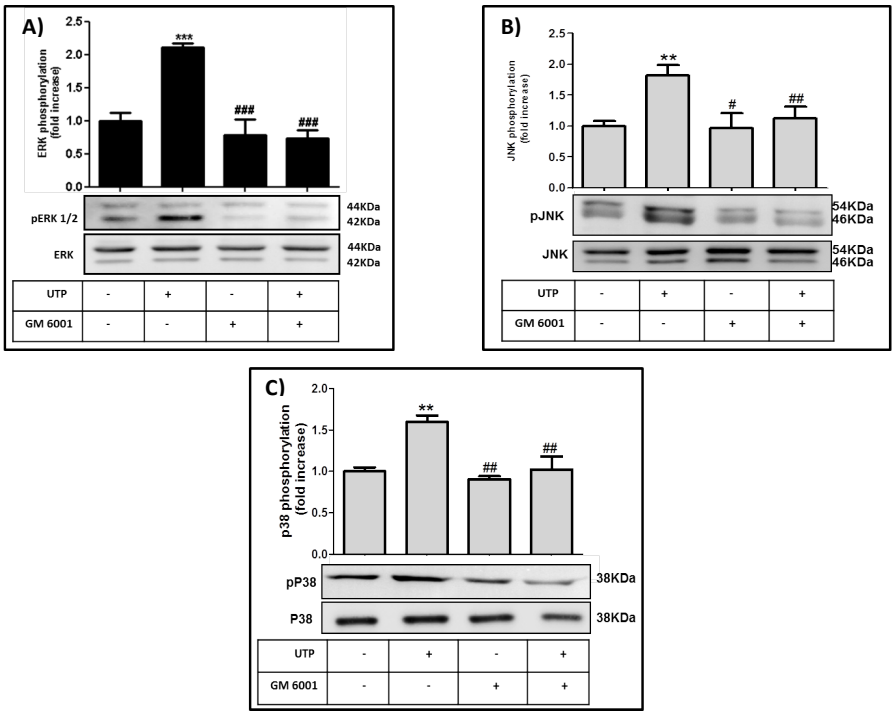


**Figure r29. MMP's inhibit Late MAPKs activation due to UTP.** Schwann cells were treated with GM6001 for 30 minutes and thereafter with UTP for 12 hours, cell extract were collected. A, B and C) cell lysates were collected, and equal amounts of protein (30 µg) were resolved in SDS-PAGE and blotted with anti-phosphoERK (A), anti-phosphoJNK (B) and anti-phosphoP38 (C). The same membranes were deshibrided and also probed with anti-ERK, anti-JNK and anti-P38, which recognizes both active and non-active forms of MAP kinases (ERK1/2, JNK, P38). At least, three independent experiments were made, and representative blots are shown on the top of the figure. **, *** denote significant differences from control values with **p< 0.01 and *** p<0.001, and $^{\#, \#\#, \#\#\#}$ denote significant differences from UTP values with $^{\#}$p<0.05, $^{\#\#}$p< 0.01 and $^{\#\#\#}$p<0.001.

These results suggest that MMP's, in some way, are involved in the MAPKs late activation induced by UTP, because inhibition of MMP's blocked the latest MAPK activation.

Finally, we determined if MMP-2 activation was directly involved in the MAPKs late activation induced by UTP. For that, Schwannoma cells were transfected with the shMMP2 or the shRandom vectors and treated with UTP (250µM). Cell lysates were collected at different times (5 min, 45 min, 2 h, 6h, 12h, 24h and 48h) and submitted to western blot against the three phosphorylated MAPKs (ERK1/2, p38 and JNK).

Results showed that Schwannoma cells transfected with the shMMP2 vector and stimulated with UTP had similar phosphorylation levels than those observed previously only with UTP treatment in the early MAPKs activation (compare **Figure r30** with **Figure r10**). In contrast, the second activation peak of MAPKs (Late Activation) was not present in MMP-2 silenced cells even stimulated with UTP (**Figure r30 A**). These results are in agreement with those obtained before (**Figure r28 and r29**), in which we observed an inhibition of the late MAPKs phosphorylation peak after GM6001 treatment.

Our results strongly suggest that UTP-induced late activation of MAPKs is MMP-2 dependent.

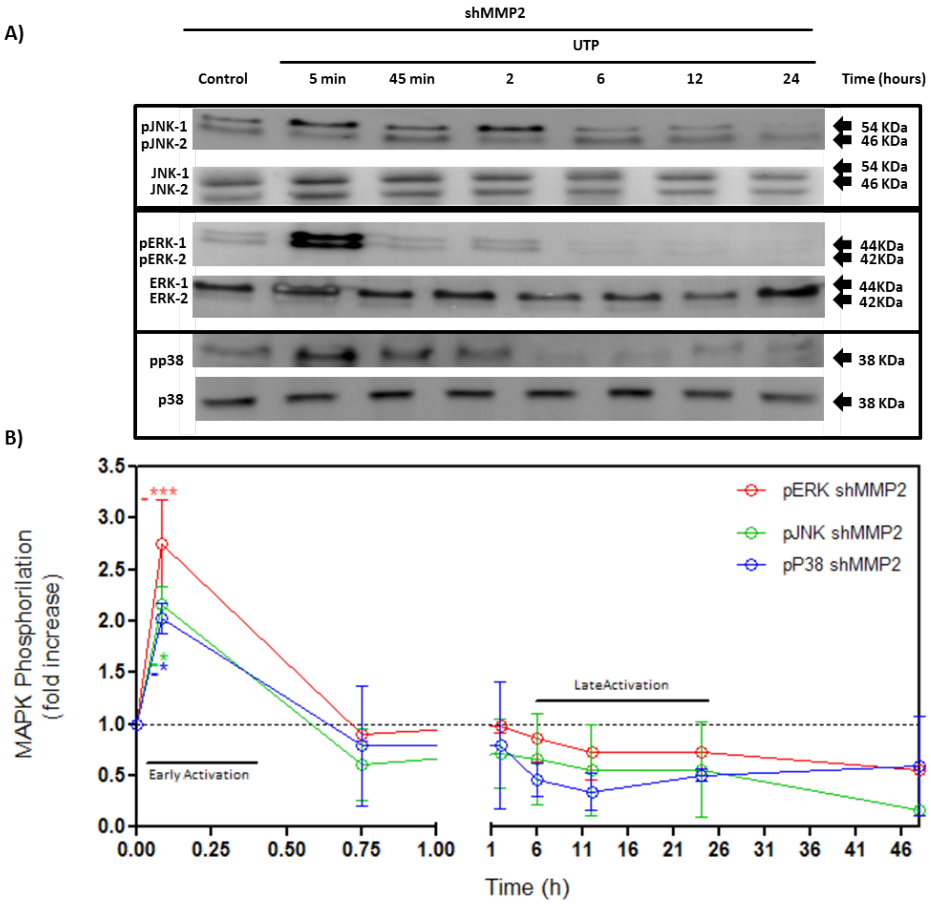


**Figure r30. MMP-2 is implicated in late activation of MAPK due to UTP.** Schwann cells were transfected with shMMP-2 and treated with UTP for different times. Cell lysates were collected, and equal amounts of protein (30 µg) were resolved in SDS-PAGE and blotted with anti-phosphoERK, anti-phosphoJNK and anti-phosphoP38. The same membranes were deshibrided and also probed with anti-ERK, anti-JNK and anti-P38, which recognizes both active and non-active forms of MAP kinases (ERK1/2, JNK, P38).

# *XI. DISCUSSION*

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## 1. GENERAL DISCUSSION

Neuropathic pain is one of the major factors causing impaired quality of life in millions of people worldwide. Unfortunately, this type of pain is frequently resistant to the painkillers currently in use. We are now beginning to understand that neuropathic pain is not only a symptom of disease but also a consequence of a functional disorder in the entire nervous system (Woolf, Mannion 1999). Nerve damage can have dramatic consequences on sensory and motor functions in the immediate aftermath of an injury.

The cellular and molecular changes that occur within the nerve following injury are components of a complex program that ultimately aims to repair and regenerate (Makwana, Raivich 2005). Evidence taken from animal models of neuropathic pain demonstrates that nerve damage leads to a dramatic change in microglia by converting these cells into an activated phenotype through a step-by-step process (Tsuda, Inoue et al. 2001). Recent evidence shows that patients suffering from different peripheral neuropathies significantly improve after treatment with the drug Nucleo Núcleo CMP Forte, which is comprised of various nucleotides: CMP, UMP, UDP, and UTP.

In 1972 Burnstock was the first to propose new roles for nucleotides as neurotransmitters. Recently obtained evidence suggests that nucleotides released from non-excitable cells or neurons play important roles in cell-to-cell communication in both physiological and pathological conditions (Burnstock, Satchell et al. 1972). Furthermore, nucleotides are also important signaling molecules in the nervous system (Pintor, Bautista et al. 2004, Zimmerman, Magasanik 1964, Burnstock, Kennedy 1985). Several studies have elucidated the signaling pathways developed after nucleotide treatment, above all ATP treatment, in glial cells (Tozaki-Saitoh, Tsuda et al. 2011, Köles, Leichsenring et al. 2011). The effects of nucleotides are mainly due to the activation of both ionotropic (P2X) and metabotropic purinergic receptors (P2Y) (Burnstock, Kennedy 1985), the latter of which are known to be present in both the central and the peripheral nervous systems (Burnstock 2007).

Previous results obtained by our research group have demonstrated that Núcleo CMP Forte, and principally UTP, induces purinergic receptor activation and a

significant increase in Schwann cell adhesion, mainly through the overexpression of N-cadherin. We demonstrated that this overexpression is mediated by P2Y receptor activation located at the cell surface of Schwannoma cells. This P2Y activation also induces a cytoskeletal reorganization of these cells (Martiañez, Carrascal et al. 2012, Martiáñez, Lamarca et al. 2012).

Cell migration plays a critical role in a diverse array of physiological processes, including inflammation, angiogenesis, wound healing, and development (Barreiro, Martin et al. 2010, Birmingham, Busik et al. 2009, Aman, Piotrowski 2010, Velnar, Bailey et al. 2009). Many different cell types migrate in response to extracellular cues, and migration is one of the principal processes that take place after nerve injury, with Schwann cells being the main glial population involved (Fenrich, Gordon 2004). When axons are degenerated, Schwann cells differentiate to assume a phenotype similar to that observed in immature cells and migrate to repair the injury.

Evidence in recent years has highlighted the importance of extracellular nucleotides and adenosine in the regulation of homeostatic mechanisms and the migration of different cell types, such as fibroblasts, keratinocytes, or neurons. Nucleotides are known to act through purinergic receptor activation, otherwise P2Y signaling pathways influence cell migration, and therefore they could be therapeutic targets for diseases in which migration is defective (Burnstock 2009). In addition, metabotropic P2Y receptor agonists are promising therapeutic molecules for the study of peripheral neuropathies; a precise understanding of the mechanisms of nucleotides in glial cells is therefore critical because of their potentially important role in the treatment of peripheral nerve disorders (Junger 2011).

With this in mind, the present work aims to elucidate the effects of nucleotides (principally UTP) in Schwann cell wound healing and migration, as well as decipher the exact molecular pathways involved in all these processes.

# 2. CHANGES IN SCHWANNOMA CELL MIGRATION DUE TO UTP STIMULATION. IMPLICATIONS OF THE P2Y-MAPK PATHWAY

Microglia is known to exist in 2 different and fundamental states: a resting surveillance state and an activated state. Conversion to the activated state depends mainly on various extracellular signals, such as those produced by nucleotides (Kettenmann, Hanisch et al. 2011). This activation is heavily involved in the pathogenesis of virtually all known pathologies of the nervous system, including brain and spinal cord injury, stroke, and degenerative diseases. Both neurons and Schwannoma cells keep and control a broad range of intracellular signalling pathways and regulate the main functions and structures in the PNS. Schwann cells have been shown to play fundamental roles in PNS regeneration, with 1 of the principal mechanisms involved being cell migration. Recent studies have made important progress in identifying the molecular pathways that initiate Schwann cell differentiation, although the molecular nature of the axonal signals that coordinate Schwann cell migration has not yet been elucidated (Britsch, Goerich et al. 2001).

Of the many molecules known to play important roles in the intracellular signals that take place after cellular injury, neuregulins, nucleotides, and growth factors are the most important. Nucleotides can be released into the extracellular milieu from aggregating platelets, degranulation macrophages, excitatory neurons, and injured cells (Zimmerman, Magasanik 1964). A growing body of evidence suggests that the nucleotides released upon injury stimulate P2 receptors and serve as endogenous signals to induce a rapid wound healing response in glial cells (Fields 2006, Weinger, Klepeis et al. 2005). Glial cells are known to express functionally active P2 receptors in cell cultures and *in situ* (Ferrari, Villalba et al. 1996). These data suggest that after injury released nucleotides could mediate astrocyte-microglia communication through purinergic receptor activation. Nevertheless, the exact implications of UTP nucleotides as selective P2Y agonists in injured Schwann cells remains unknown. Elucidating the role that purinergic receptors play in Schwann cells will provide useful information about their role in nerve regeneration. Our results strongly demonstrate that cell migration and wound repair significantly increases in Schwann cells after Nucleo Núcleo CMP Forte treatment. Of the nucleotides present in the drug, UTP shows the maximum rate of induction for wound healing and migration. These results are concurrent with previous studies describing the contribution of nucleotides to epithelial repair in

intestinal and corneal epithelial cultures (Dignass, Becker et al. 1998, Klepeis, Weinger et al. 2004). Moreover, nucleotides are known to act through purinergic receptors (Burnstock 2009). In addition to our group, other authors have described the presence of distinct and functional P2Y receptors in Schwann cells (Pintor, Bautista et al. 2004, Martiañez, Carrascal et al. 2012). The next logical question is whether these receptors are involved in the increased Schwannoma cell wound repair observed after UTP stimulation. First, we used Suramin as a broad range spectrum inhibitor of the purinergic receptors (Nakamura, Iwanaga et al. 2011) to study Schwann cell wound healing. Our results showed that these receptors are scarcely involved in cell migration induced by UTP. Pretreatment of Schwannoma cells with this inhibitor drastically decreased the wound healing ability observed after stimulation. In addition, the use of a specific short hairpin RNA (shRNA) against $P2Y_2$ revealed that the $P2Y_2$ receptor is precisely the purinergic receptor implicated in Schwannoma cell wound repair induced by UTP. $P2Y_2$ receptors have been previously shown to cause proliferation and/or migration in human epidermal keratinocytes, lung epithelial tumor cells, glioma cells, and smooth muscle cells, among other types of cells (Wilden, et al., 1998). $P2Y_2$ and $P2Y_4$ also induce corneal epithelial cell migration through the activation of a broad range of intracellular pathways (Pintor, Bautista et al. 2004). However, the role of the $P2Y_2$ receptor in Schwann cell wound healing has not been previously described. Our results reveal a novel role for $P2Y_2$ receptors in directly regulating Schwann cell migration and wound healing after UTP treatment. We also provide further evidence of a functional role of $P2Y_2$ receptors during cell injury. These findings are consistent with our previous results obtained using a proteomic approximation: we saw that UTP treatment in Schwann cells induces a reorganization of the actin cytoskeleton and an increase in Arp3 protein expression, and both processes are regulated through purinergic receptor activation (Martiañez, Carrascal et al. 2012). Arp3 plays a major role in the regulation of the actin cytoskeleton, which is important for many processes such as cell locomotion, phagocytosis, and intracellular motility of the lipid vesicles (Lai et al. 2008). Thus, an increase in Arp3 protein levels may be necessary for correct cell motility and migration.

The second set of messengers involved in the transduction pathways after purinergic receptor activation due to UTP ($P2Y_2$, $P2Y_4$ and $P2Y_6$) normally involves phospholipase C (PLC) activation, which in turn produces intracellular calcium release and activates PKC, Akt, and different intracellular signaling pathways mediated by MAPKs. In primary murine astrocytes, $P2Y_2$ receptors have been shown to mediate the activation of calcium-dependent and calcium-independent PKCs and ERK ½, both of which can activate the cytosolic phospholipase $A_2$

(Ballerini, Di Iorio et al. 2006). Stimulation of $P2Y_2$ receptors by UTP or ATP induces pronounced vasodilatation and promotes nucleotide-induced activation of PKC, cyclo-oxygenase, and MAPKs (Brambilla, Neary et al. 2002). In the present study, we report that UTP stimulation results in a biphasic activation of ERK ½ and 2 protein kinases involved in cellular stress: JNK and P38. This result is in agreement with previous studies that describe biphasic MAPK activation in response to many growth factors and cytokines in smooth muscle cells (Gurjar, Deleon et al. 2001, Lien, Usami et al. 2006). Nevertheless, no previous studies have described the involvement of UTP in biphasic MAPK activation in glial cells, a kinetic profile that has recently been proposed as a model that allows a common signaling system to play specific regulatory roles depending on the temporal duration of the activation peaks (Murphy et al., 2002).

Our results not only show the involvement of UTP in biphasic MAPK activation, but also directly correlate MAPK activation with the Schwann cell wound healing observed in response to UTP. Inhibition of any MAPK with specific pharmacological inhibitors (MEK ½ inhibitor U1206n, P38 inhibitor SB203580, and JNK inhibitor SP600125) drastically decreased the wound healing capacity observed in Schwannoma cells after UTP treatment. The role of MAPKs in cellular migration has already been extensively reviewed in some cell types, such as epithelial corneal cells, hematopoietic stem cells, or keratinocytes, among others (Rossi, Manfredini et al. 2007, Kawasaki, Smith et al. 2003, Yang, Cranson et al. 2004). Our results are in agreement with these previous studies and suggest that MAPK activation is necessary to induce Schwann cell wound repair after injury in response to UTP stimulation.

Additionally we show that MAPK phosphorylation after 12 hours of UTP treatment is completely blocked in the presence of the purinergic receptor inhibitor suramin, indicating that these proteins are downstream from the purinergic receptor activation in Schwannoma cells. Many studies report that MAPKs are activated through purinergic receptor stimulation in response to nucleotides in different cell types, including microglia (Potucek, Crain et al. 2006, Shigemoto-Mogami, Koizumi et al. 2001). In addition, P2Y activation by UTP is involved in early MAPK phosphorylation, as we and others have previously demonstrated (Martiáñez, Lamarca et al. 2012, Muscella, Giovanna et al. 2003, Nishida, Gotoh 1993, Potucek, Crain et al. 2006), but the present work describes for the first time the role of UTP in late MAPK phosphorylation in Schwann cells.

# 3. MMP'S ACTIVATION DUE TO UTP. IMPLICATION OF MMP'S IN SCHWANNOMA CELL MIGRATION

Matrix metalloproteinases (MMPs) are known to play complex roles in different neurological diseases, and right now we are beginning to appreciate their significant involvement after spinal cord injury (Faulkner, Herrmann et al. 2004). Matrix metalloproteinases are present in neurons, glial cells and the endothelium. They can selectively degrade various components of the extracellular matrix (ECM) and release the growth factors and cytokines present in the ECM. Through proteolytic activity, MMPs play a critical role in cellular migration and invasion, in addition to regulating the signaling pathways involved in these processes (Kelin, Bischoff 2011, Roy, Yang et al. 2009, Giannelli, Falk-Marzillier et al. 1997, Taraboletti G, Sonzogni L, Vergani V, Hosseini G, Ceruti R, Ghilardi C, Bastone A, Toschi E, Borsotti P, Scanziani E, Giavazzi R, Pepper MS, Stetler-Stevenson WG, Bani MR. 2000).

In a first approximation, the presence of specific MMPs was analyzed in both mRNA and activity levels. MMP-2 mRNA levels were detected in Schwann cells and, surprisingly, their activation was found to be induced by UTP. The presence and activation of MMP-2 in glial cells has been previously described in denervated nerves, but never in response to nucleotides (Ferguson, Muir 2000). In contrast, we were not able to detect MMP-9 in Schwannoma cells in either mRNA or any of the zymographies, and although other authors have observed MMP-9 in Schwann cells, their levels are very low in normal nerves (Shubayev, Myers 2002, Shubayev, Myers 2000). No previous evidence has reported MMP-9 activation in response to purinergic receptors in glial cells, and we hypothesize that the activation of these receptors through UTP is insufficient to induce MMP-9 expression in either Schwannoma cells or primary Schwann cell culture. MMP-7 was detected at mRNA levels but we could not detect it in casein zymography, suggesting that although MMP-7 is present in Schwann cells, it is nonexistent or less active in the presence of UTP. Finally, substrates for MMP-14 and MMP-28 have not yet been defined, and therefore even when detected at mRNA levels in Schwann cells, they could not be observed in any of the performed zymographies.

As MMPs are secreted as inactive pro-enzymes, their activation is a critical step in nerve injury repair. Interestingly, no previous studies report that nucleotides can activate the latent proforms of MMP-7, MMP-14, and MMP-28. Despite the mRNA

levels observed in these MMPs, it is possible that after UTP stimulation in Schwann cells they are not processed to their active forms. Some authors have reported the presence of MMP-7, MMP-14, and MMP-28 in glial cells (Hughes, P.M., Wells, G.M. et al. 2002), but further studies are required to elucidate the role that these MMPs play in Schwann cells

Our results show that UTP stimulation in Schwann cells induces an overexpression of the mRNA levels of MMP-2 as well as MMP-2 activation. In the PNS, MMP-2 has been previously reported to increase after nerve injury in developing dorsal root ganglia and in Schwann cells (Malin, Sonnenberg et al. 2009), although no studies have reported MMP-2 activation in response to UTP. MMP-2 is secreted from the cells in an active form (pro-MMP-2). Like other MMPs, the activation of pro-MMP-2 has been shown to occur at the cell surface, a fundamental step for the regulation of its activity and its role in matrix degradation (Corcoran, Hewitt et al. 1996). Our results suggest that a change occurs in the intracellular distribution of MMP-2 in response to UTP treatment. This stimulation induces migration in the intracellular MMP-2 protein, which is at first located at the periphery of the nucleus and then moves around the cytoplasm and the filopodia of Schwann cells upon UTP stimulation. Some studies have described changes in MMP-2 localization. In malignant adenocarcinoma cells, stained MMP-2 appears in a mostly cytoplasmic pattern, but in benign cells MMP-2 staining shows concentrations at the apical surface (Boag, Young 1993). To be secreted, MMP-2 must move through the cytoplasm of the cell to localize MMP-14 at the cell membrane. MMP-14 is one of the enzymes involved in proteolysis of the prodomain of MMP-2, which is indispensable for the final step of activation. MMP-14 is localized at the surface of the cell membranes in different carcinoma cell types (Bauvois 2012, Bourboulia, Stetler-Stevenson 2010, Stetler-Stevenson W.G., Aznavoorian et al. 1993, Chandler, Coates et al. 1995). Thus, after UTP stimulation MMP-2 moves to the cell membrane to be proteolyzed by MMP-14, an indispensable step in MMP-2 secretion and activation.

In addition to the results explained above, we demonstrate that MMPs, and MMP-2 in particular, govern Schwann cell migration *in vitro* thanks to the use of specific shRNA interference against MMP-2. Previous studies have shown that cell migration and invasion, both *in vitro* and *in vivo*, require the proteolytic activity of MMPs (D'Alessio, Ferrari et al. 2008). In the peripheral nervous system, both during development or after an injury, Schwann cells migrate toward axonal signals, where they can differentiate by ensheathing and/or myelinating axons and form a framework for nerve regeneration (Bunge, Bunge 1986). In spinal cord injury,

Schwann cells also migrate to the lesion and assist in sustaining axons (Blesch, Tuszynski 2003). Many factors that promote glial survival, such as neuregulins (NRG-1), also function in Schwann cell migration (Yamauchi, Cahn et al. 2004). Our results demonstrate for the first time that MMP-2, induced by UTP, is the most important metalloproteinase involved in Schwann cell migration.

ECM degradation is an important and necessary process that allows cellular migration and tissue remodelling. However, little information is known about the role of nucleotides in cell migration. We have found that UTP induces MMP-2 expression and activation and that wound healing potentiates the UTP-induced increase in MMP-2 activation. These results are consistent with previous studies, which indicate that MMP-2 levels increase during wound healing after spinal cord lesions. (Hsu, McKeon et al. 2006). Subsequently, we hypothesize that the ability of UTP to enhance MMP-2 activation may reflect an important mechanism though which this extracellular nucleotide contributes to Schwann cell wound repair and migration under injury conditions, as shown in **Figure d1**. These findings suggest MMP-2 may promote cell migration. However, it is possible that other MMPs also contribute to the Schwann cell wound healing observed after UTP stimulation. Notably, Schwann cell migration was not completely blocked with either a general MMP inhibitor or the specific shRNA against MMP-2, suggesting that MMPs are not the sole modulators of Schwann migration. With these findings in mind, we may postulate that UTP secreted from injured nerves induces MMP-2 activation, which in turn may help to induce the Schwann cell migration required for correct nerve regeneration.

# 4. HYPOTHETICAL PATHWAY INDUCED BY UTP. P2Y RECEPTORS, MAPK AND MMP-2

Previous studies on the specificity of the MAPK inhibitors used in this work describe the effectiveness of their use (Davies, Kopetz 2013). We observed that the inhibitor MEK ½ is able to block not only ERK ½ but also P38 and JNK. In addition, the p38 inhibitor (SB203580) also blocks the observed phosphorylation of the 3 studied MAPKs in response to UTP treatment in Schwannoma cells. SP600125 (a pJNK inhibitor) inhibits not only pJNK phosphorylation but also p38 phosphorylation; in contrast, this drug cannot block ERK ½ phosphorylation. In fact, MAPK pathways have always been characterized as lineal independent pathways, and cross-talk between them are little or nonexistent (Gallo, Johnson 2002, Garrington, Johnson 1999). In addition, many authors postulate that the relations between them are exclusive, and that the inhibition of 1 of the MAPK members entails an overexpression of the others. Thus, P38 inhibition (SB203580) produces an overphosphorylation of ERK and JNK. In contrast, ERK ½ inhibition induces an overexpression of p38 in fibroblasts and osteosarcoma cells (Xiao et al. 2002, Yi Qun Xiao, 2002, Shimo et al. 2007). In addition, ERK ½ activation through $P2Y_2$ receptor has been shown to induce the phosphorylation of the stress-activated kinases JNK and p38 in microglial cells (Gendron, Chalimoniuk et al. 2003). This receptor activation is also known to induce p38 and ERK ½-dependent upregulation of genes, which is involved in cell survival and migration in human astrocytoma cells, and neurite outgrowth in PC-12 cells (Chroma et al, 2004). Therefore, we cannot confirm whether the inhibition of the phosphorylation of specific MAPKs with 1 specific inhibitor is due to nonspecificity of the inhibitor, or instead cross-talk between MAPKs. Further studies will elucidate this possible cross-talk.

To determine whether any of the MAPKs were involved in the MMP-2 activation observed after UTP stimulation, 3 specific inhibitors of MAPKs were used. Surprisingly, inhibition of any of the MAPKs studied resulted in a complete block of MMP-2 activation in response to UTP stimulation. Our results also demonstrate that this MMP-2 activation is specifically dependent on $P2Y_2$ receptor activation through UTP. These data suggest, as schematized in **Figure d1**, that MMP-2 activation is a downstream event that takes place after MAPK activation through P2Y receptors stimulated by UTP. This hypothesis is supported by some early data: authors report that JNK activation is required for the MMP-2 release induced by ATP in human aortic smooth muscle cells and that the ERK ½ pathway mediates

adenosine stimulation of MMP-2 secretion by trabecular network cells of the eye (Robinson, Douillet et al. 2005).

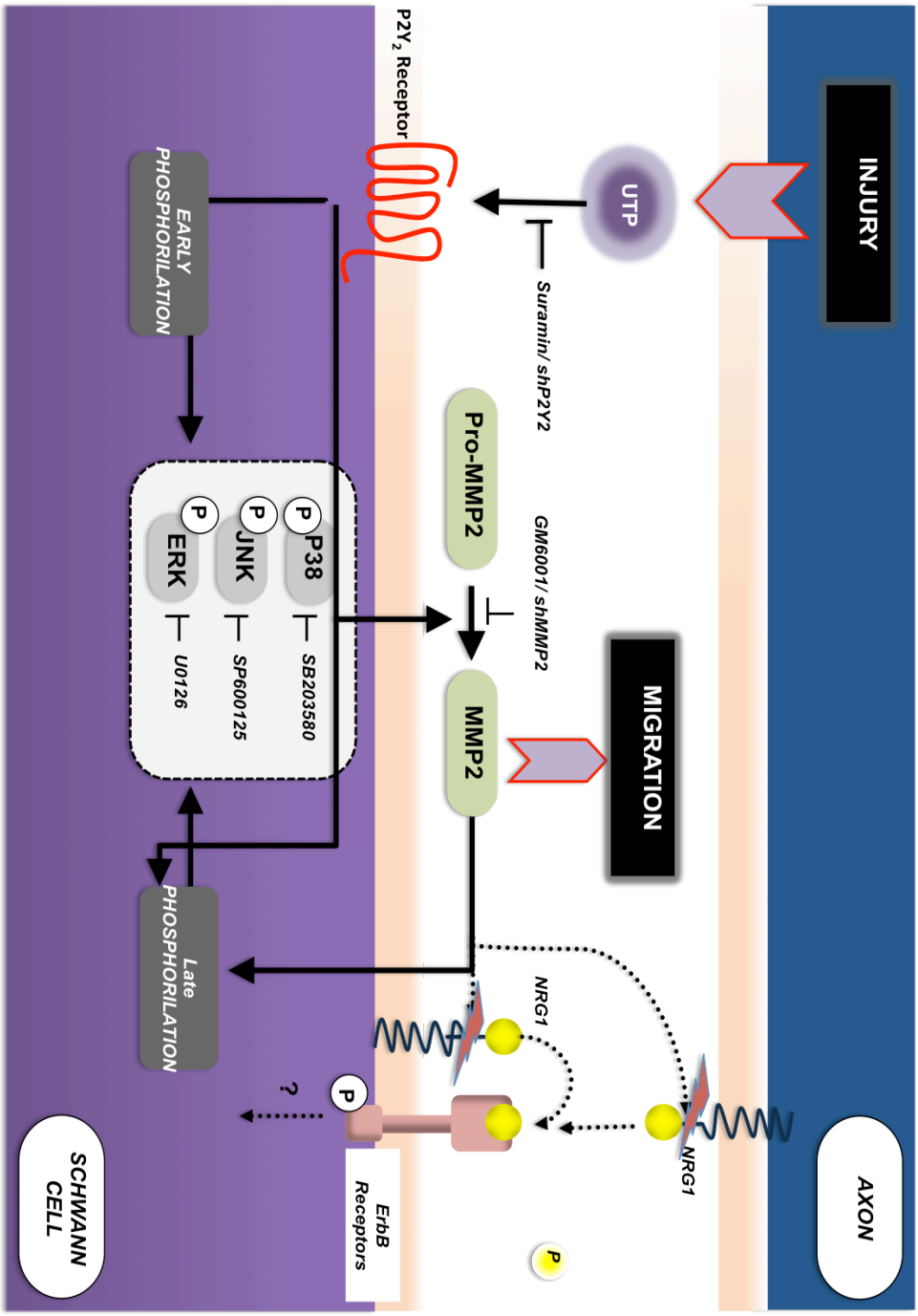
After nerve injury, an increase in the activity of multiple pathways, including ERK/MAPK/JNK/c-jun and JAK/STAT, has been detected in Schwann cells (Woodhoo, Sommer 2008). Our data also suggests that MAPK activation in Schwann cells is mediated by P2Y receptors, and this finding correlates strongly with previous studies made in both our labs and those of other groups (Yang, Cranson et al. 2004, Martiañez, Carrascal et al. 2012). This MAPK activation leads to the activation of several transcription factors, as previously described (Martiáñez, Lamarca et al. 2012), and also somehow regulates MMP-2, which is activated and secreted from Schwann cells in response to UTP, as we have seen in this study.

In contrast, the late MAPK activation peak, according to our results, is mediated by the MMP-2 activation induced by UTP. One possible explanation for the late MAPK peak, as schematized in **Figure d1,** is that active MMP-2 in the extracellular space could cleave a membrane-anchored growth factor, which may be present in Schwann cells or in the axon of neurons. Our hypothesis correlates with previous studies that suggest that neuregulin 1 (Nrg1) signals transduced through ErbB receptors function throughout Schwann cell development, influencing fate specification, motility, proliferation, survival, and myelination in the cell (Newbern, Li et al. 2011). ErbB receptor tyrosine kinases are continuously required to direct migration of Schwann cells. Nrg1 Type III stimulates Schwann cell proliferation and motility, and serves as an attractant that directs migration. Other authors have demonstrated that MMPs can activate the Ras/Raf/MEK-ERK½ signal transduction pathway through ErbB, IGF-1, and PDGF tyrosine kinase receptors in nerve sections (Cho, Jeong et al. 2010, Fromigue, Hamidouche et al. 2008). The exact mechanisms whereby MMP-2 can release NRG-1 need to be clarified and further studies are required, but there is some evidence of proteolytic MMP functions in the activation of trophic systems. Release of trophic factors from their regulatory proteins depend on the catalytic activity of MMPs, such as IGF-1 release from IGF- binding proteins in the central nervous system (Page-McCaw, Ewald et al. 2007). On the other hand, it is known that IGF-1 can stimulate the release of the EGF ligand (HB-EGF) through MMPs, leading to a cumulative transactivation of itself and its receptors and ending in Ras/Raf/MEK signaling (El-Shewy, Kelly et al. 2004, Roudabush, Pierce et al. 2000). It is also known that Schwann cells express Erb2, Erb3, and Erb4, and that Erb2 is indispensable in Schwann cell survival after nerve injury (Roelle, Grosse et al. 2003, Corfas, VElardez et al. 2004, Chattopadhyay S, Shubayev 2009).

Our results suggest that UTP needs to be present constantly to induce MAPK late peak, MMP-2 activation, and Schwann cell migration. Previous studies support this hypothesis: the treatment of microglial cells with apyrase, an enzyme that rapidly hydrolyzes extracellular nucleotides, completely blocks cell migration, suggesting that extracellular nucleotides released after injury are constantly required to induce cell migration (Hsu, McKeon et al. 2006). We hypothesize that the activation of P2Y receptors, MAPK, and MMP-2 are interdependent and necessary to induce Schwann cell wound repair in response to UTP. This nucleotide is needed for both early and late MAPK activation; if UTP were removed from the cell media, late MAPK phosphorylation would not occur and therefore MMP-2 could not be activated. Thus, we did not observe the presence of either active MMP-2 or late MAPK activation.

In our final hypothesis, as schematized in **Figure d1,** injury brings about UTP release to the extracellular media and through its interaction with P2Y receptors present in the surface of Schwann cells early MAPK phosphorylation is induced. This interaction also induces a late MAPK activation. Both activation peaks are needed to induce MMP-2 release and activation. In turn, activated MMP-2 may release NRG-1 anchored in the Schwann cell membrane, which would activate ErbB receptors present in Schwann cells, leading to the second MAPK activation peak observed after 12 hours of UTP treatment. Clearly, further studies are required to validate the second part of this hypothesis in terms of the role of MMP-2 in the release of NRG-1 from Schwann cells. In light of these findings, we conclude that extracellular UTP, which mimics nerve injury, stimulates Schwann cell migration and wound repair through an MMP-2-dependent mechanism via P2Y receptors and the activation of MAPK pathways. Moreover, our results showing that $P2Y_2$ receptors, MAPKs, and MMP-2 mediate the migratory response of Schwann cells evoked by PNS injury indicate that selective P2Y receptor agonists might provide new therapeutic opportunities for Schwann cell migration and peripheral nerve regeneration.

**Figure d1. Schematic overview of the activation of P2Y-MAPK-MMP2 pathway involved in Schwann cell migration and wound repair.**



# *XII. CONCLUSIONS*

# XII. CONCLUSIONS

- Schwann cell treatment with Núcleo CMP Forte induces Schwann cell migration and wound repair.

- The main nucleotide involved in migration and wound repair in Schwann cells is UTP, which acts through the $P2Y_2$ receptor.

- UTP treatment induces biphasic MAPK activation, with 1 peak after 5 min and second peak after 12 h of stimulation. This biphasic activation is indispensable for Schwann cell migration and wound healing.

- UTP induces the secretion and activation of MMP-2. Purinergic receptors and posterior MAPK activation are indispensable for this MMP-2 activation.

- MMP-2 activation is an indispensable step for the induction of wound repair by UTP in Schwann cells.

- MMP-2 activation induced by UTP is necessary for the late activation peak of MAPK in Schwann cells.

# XIII. ANNEX

# XIII. ANNEX

## Manuscript submitted to Glia (May 2013).

"UTP promotes Schwann cell migration through Matrix Metalloproteinase 2 activation.

**Aloa Lamarca**, Alejandro Gella, Joana Figueiro, Tània Martiáñez, Mònica Segura, Ramón Trullas, Núria Casals.

# ARTICLE 1.

"N-Cadherina expression is regulated by UTP in Schwannoma cells"

Tània Martiáñez, **Aloa Lamarca**, Núria Casals, Alejandro Gella,.

Purinergic Signalling, 2013, 9, 259-270

# ARTICLE 2.

"UTP affects the Schwannoma cell line proteome through P2Y receptors leading to cytoskeletal reorganization"

Tània Martiáñez, Montse Carrascal, **Aloa Lamarca**, Mònica Segura, Núria Durany, Roser Masgrau, Joaquín Abian, Alejandro Gella,.

Proteomics, 2012, 1, 45-56.

# ARTICLE 3.

"A nucleotide-based drug protects against glutamate and MMP+- induced neurotoxicity".

Alejandro Gella, Tània Martiáñez, **Aloa Lamarca**, Cristina Gutiérrez, Núria Durany, Núria Casals.

Neuroscience and Medicine, 2011, 2, 154-160

# *XIV. Resum en català*

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# XIV. RESUM EN CATALÀ

## 1. Hipòtesis i objectius de la tesis

El Nucli CMP Forte és un medicament que és prescriu a pacients que pateixen de diferents neuropaties. Es creu que els efectes beneficiosos d'aquest fàrmac, observats després de la seva administració, són deguts principalment a la capacitat que té el fàrmac sobre la regeneració i la protecció del dany axonal. Tot i això, els mecanismes moleculars exactes pels quals el fàrmac actua, són encara desconeguts.

Es coneix que els nucleòtids extracel·lulars, secretats per les cèl·lules danyades, interaccionen amb els receptors purinèrgics i condueix a la posterior activació de la via de les MAPKs. Aquesta via ha estat ampliament implicada en processos de proliferació, migració i supervivència cel·lular. A més a més, les MAPKs també s'han descrit com a necessàries per a la remodelació de la matriu extracel·lular i per a la migració cel·lular. Per tant, es creu que el tractament de les cèl·lules de Schwann amb els nucleòtids presents en el Nucli CMP Forte podrien activar els receptors purinèrgics presents a la superfície d' aquestes cèl·lules, i induir la secreció de metal·loproteases i la migració cel·lular, tots dos passos crítics per a la regeneració del nervi.

Amb tot això, l'objectiu principal d'aquesta tesis doctoral és identificar els mecanismes moleculars induïts pel fàrmac Nucli CMP forte i UTP en les cèl·lules de glia mielinitzants del sistema nerviós perifèric (cèl·lules de Schwann). Els objectius més concrets d'aquest treball són:

- Analitzar el paper del Nucli CMP forte i del UTP en la migració i reparació de les cèl·lules de Schwann.
- Estudiar els efectes del UTP sobre l'expressió i activació de la MMP-2.
- Elucidar les rutes intracel·lulars implicades en l'activació de la MMP-2 i la migració de les cèl·lules de Schwann.

# 2. Introducció

S'entén per neuropaties perifèriques qualsevol patologia que indueixi alteracions en el sistema nerviós perifèric. Les causes exactes d'aquest tipus de patologies no són molt conegudes, però sí se sap que poden donar-se com a resposta a una lesió del nervi, a tumors, a toxines, a una resposta autoimmune, a dèficits nutricionals, a trastorns vasculars o metabòlics i fins i tot com a resposta a un alcoholisme perllongat. Aquests tipus de patologies afecten actualment a un 6% de la població a tot el món, però fins al moment, encara no existeix un tractament eficaç per a elles. Queda clar doncs, que un bon coneixement de les causes que intervenen en aquestes diferents neuropaties són la base per a una satisfactòria regeneració dels axons malmesos. Centrant-nos en els tractaments farmacològics administrats en aquestes neuropaties perifèriques, ens trobem que la majoria de fàrmacs que es prescriuen són tractaments pal·liatius del dolor. En aquest marc, Nucleo CMP Forte ® que és un fàrmac compost per diferents nucleòtids: citidina monofosfat (CMP), uridina trifosfat (UTP), uridina difosfat (UDP) i uridina monofosfat (UMP); és un dels únics fàrmacs prescrits per neuropaties perifèriques del sistema nerviós (neuritis i neuropaties amb origen osteoarticular, metabòliques o infeccioses). L'eficiència dels nucleòtids extracel·lulars en el tractament de neuropaties ja es coneix des de fa anys, i es creu que els efectes beneficiosos d'aquest fàrmac es troben en la seva capacitat de regeneració i protecció sobre el dany axonal (Muller 2002). Diversos treballs clínics han demostrat els efectes fisiològics beneficiosos d'aquest fàrmac, tanmateix, els mecanismes moleculars exactes així com les vies moleculars implicades en les neurones i les cèl·lules mielinitzants del sistema nerviós perifèric, després del seu tractament, són de moment desconegudes.

Els nucleòtids exerceixen els seus efectes, a nivell de segons missatgers, a través de la seva interacció amb els receptors purinèrgics. Aquests receptors van ser descrits per primera vegada per Burnstock a l'any 1972. En un primer moment es van classificar en dos gran famílies, segons el tipus de lligand que interaccionava amb ells, els receptors P1 sensibles a nucleosides i els P2 sensibles a nucleòtids. Uns anys més tard, els receptors P2 es van subdividir de nou en dos subtipus, els P2X que són canals iònics i

els P2Y que són receptors acoblats a proteïnes G (Burnstock, Satchell et al. 1972, Burnstock, Kennedy 1985). Avui dia es coneix que aquest tipus de receptors juguen papers molt importants en les interaccions que es donen entre les neurones i la glia. Les diferents vies que s'activen després de la estimulació d'aquests receptors, depenen tant del subtipus de receptor activat com del tipus cel·lular que expressi aquest receptor (Ralevic, Burnstock 1998). De tots els subtipus de receptors purinergics, només tres es coneixen per ser sensibles a nucleòtids de uridina: $P2Y_{2,4\ i\ 6}$. Se sap que l' activació dels receptors $P2Y_{2,4\ i\ 6}$. resulta en l'activació de tot un seguit de cascades de senyalització, d'entre les quals la de les MAPKs és una de les més conegudes (Boeynaems, Communi et al. 2012, Graham, et al 1996).

La família de les MAPKs  és una superfamília que es pot dividir en tres subgrups. Una quinasa regulada per senyals extracel·lulars (ERK ½), i dues quinases implicades en estrès cel·lular: p38 i JNK. Totes elles estan activades per fosforilacions en diferents residus de treonines i tirosines (Kim, Bar-Sagi 2004).  Aquesta superfamília de proteïnes  s'ha descrit àmpliament que estan implicades en la migració de diferents tipus cel·lulars. Per exemple, JNK s'ha descrit com a promotor de la migració de fibroblasts, neurones i fins i tots cèl·lules de Schwann (Huang, Rajfur et al. 2003, Huang, Jacobson et al. 2004, Javelaud, Laboureau et al. 2003). Per altra banda, p38 s'ha estudiat que està implicada en la migració de cèl·lules epitelials o fibroblasts, entre d'altres (Hedges, Dechert et al. 1999, Sharma, He et al. ). Finalment, ERK ½ que és el membre més àmpliament estudiat de la família de les MAPK s'ha relacionat també amb la migració de múltiples tipus cel·lulars com fibroblasts o cèl·lules de carcinoma (Huang, Jacobson et al. 2004, Huang, Rajfur et al. 2003). Tot i això, el paper exacte que les MAPKs tenen en la migració de les cèl·lules glials és de moment poc conegut.

Per altra banda, moltes altres molècules a part de les MAPKs estan implicades en els processos de migracó cel·lular. No és difícil pensar que tant la degradació com la remodelació de la matriu extracellular són processos fonamentals que s'han de donar per a la correcta migració i invasió cel·lular. Es coneixen principalment dos enzims proteolítics implicats en aquests processos, el sistema activador de plasminogen (uPA) i les metalloproteinases de matriu (MMPs). Les MMPs són una superfamília d'enzims, de les quals se'n coneixen 23 tipus, que mantenen entre elles una

estructura molt conservada (Kessenbrock, Plaks et al. 2010). Aquesta superfamília, es pot dividir en funció de si són metalloproteinases anclades a membrana o bé secretades. En ambdós casos, se sap que la seva expressió, secreció i activació, són processos molt controlats en la cèl·lula.

Els mecanismes de secreció de les metalloproteinases no són encara gaire coneguts, pero se sap que aquests enzims són secretats de la cèl·lula en forma latent com a zimògens (Pro-MMPs). A continuació, per a poder ser activades aquestes MMPs pateixen primer una ruptura dels ponts de zinc i cisteïna de la seva estructura (**Figura I23**) i a continuació la pèrdua del prodomini amino-terminal. En la majoria dels casos, aquesta proteòlisis es dona en la membrana cel·lular a través d'altres MMPs. De les metalloproteinases secretades, les mes conegudes són les gelatinases, que reben aquest nom perquè tenen com a substrat principal la gelatina. Se'n coneixen principalment dues, la MMP-2 i la MMP-9. Aquests dos tipus de enzims s'han vist implicats en diferents papers funcionals del sistema nerviós. Per altra banda, el paper d'aquestes metalloproteinases en la migració cel·lular són també àmpliament conegudes (Fu, P.: Jiang, X.: Arcasoy, M.O. 2009, Dufour, Sampson et al. 2008).

# 3. Resultats I discussió

Resultats previs del nostre grup de recerca indiquen que tant el Núcleo CMP Forte, com principalment l'UTP, indueixen una àmplia varietat de canvis moleculars i estructurals en les cèl·lules de Schwann. L'estimulació d'aquestes cèl·lules amb UTP condueix a una reorganització del citoesquelet i a un augment en l'adhesió cel·lular. Aquests processos s'han vist que estan regulats tant per l'activació de les MAPK, com a causa d'una sobreexpressió de la proteïna N-cadherina (Martiañez, Carrascal et al. 2012, Martiáñez, Lamarca et al. 2012).

El present treball pretén elucidar tant els canvis moleculars com cel·lulars, que tenen lloc en les cèl·lules de Schwann un cop induïdes amb el Nucli CMP Forte i el UTP, centrant-se principalment en les propietats de migració d'aquestes cèl·lules després del tractament.

## 3.1. Paper del UTP en la migració de les cèl.lules de Schwann. Rol dels receptors purinergics i de les MAPKs.

La micròglia es coneix que pot trobar-se en dos estats diferents: un estat de repòs i un estat actiu. El pas d'un estat a l'altre depèn principalment dels factors extracel·lulars. De tots ells, els nucleòtids són uns dels més estudiats i coneguts (Kettenmann, Hanisch et al. 2010). Per altra banda, l'activació de la micròglia està molt implicada en la patogènesis d'un gran nombre de alteracions del sistema nerviós, entre les que podem destacar lesions de la medul·la espinal o d'altres patologies neurodegeneratives. Dins de la micròglia, es coneix que tant les neurones com les cèl·lules de Schwann mantenen i controlen un ampli nombre de senyals i vies intracel·lulars, regulant així les principals funcions i estructures del sistema nerviós perifèric. Per altra banda, el paper de les cèl·lules de Schwann en els mecanismes de regeneració nerviós també han estat descrits, i sembla evident pensar que per tal que es pugi donar una correcta regeneració nerviosa, les cèl·lules de Schwann han de migrar fins a la zona ferida. Tot i que s'han fet avanços definint les rutes moleculars que regulen la diferenciació de les cèl·lules de

Schwann, els senyals que regulen la migració d'aquestes cèl·lules encara no han sigut del tot ben descrits (Britsch, Goerich et al. 2001).

Se sap que moltes molècules juguen papers importants en els senyals intracel·lulars que tenen lloc després d'un dany cel·lular. De totes elles, la neuregulina, els nucleòtids i els factors de creixement són els més estudiats. Els nucleòtids poden alliberar-se al espai extracel·lular a partir de l'agregació de plaquetes, neurones excitatòries o cèl·lules danyades (Zimmerman, Magasanik 1964). Nombroses evidencies suggereixen que els nucleòtids alliberats després d'un dany cel·lular estimulen els receptors purinèrgics i són utilitzats com a senyals endògenes i indueixen una ràpida resposta per a la reparació, en cèl·lules glials, les quals se sap que expressen receptors P2 (Fields 2006, Weinger, Klepeis et al. 2005). Això ens suggereix la possibilitat que els nucleòtids alliberats després d'un dany podrien mediar la comunicació entre els astròcit i la microglia. Tanmateix, les implicacions dels nucleòtids d'uridina (selectius de P2Y) en el dany de les cèl·lules de Schwann encara no es coneixen. Els primers estudis realitzats van mostrar clarament, que el tractament de les cèl·lules de Schwann (tant en línia cel·lular com en cultiu primari) amb el fàrmac i principalment amb l'UTP produïa un increment significatiu de la capacitat de migració i reparació en les cèl·lules de Schwann (**Figures R1, R2, R3, R4 R5 i R6**). Aquests resultats es correlacionen amb estudis previs, que demostren que la estimulació amb nucleòtids indueix migració en cèl·lules de tipus epitelial (Dignass, Becker et al. 1998, Klepeis, Weinger et al. 2004).

Es coneix que els nucleòtids realitzen les seves accions a través de la seva interacció amb els receptors purinèrgics. Per tant, el següent objectiu d'aquest treball va ser intentar elucidar quin era el receptor implicat en aquests processos de migració. Gràcies a la utilització, per una banda, de la Suramina com a inhibidor no selectiu dels receptors P2Y i per l'altra la utilització de un shRNA específic per el receptor $P2Y_2$, vam ser capaços de determinar que el receptor $P2Y_2$ és el receptor directament relacionat amb l'increment de migració observat en les cèl·lules de Schwann després del tractament amb el UTP (**Figures R7 i R9**). Els receptors $P2Y_{2\ i\ 4}$ ja s'havien descrit anteriorment que induïen la migració de les cèl·lules epitelials de cornea, a través de l'activació i regulació de un gran nombre de vies intracel·lulars (Pintor, Bautista et al. 2004). A més, el receptor $P2Y_2$ ja ha

sigut descrit prèviament, que està relacionat amb migració i proliferació de keratinocits, cèl·lules epitelials, cèl·lules de glioma o cèl·lules del múscul llis (Wilson, Hoeppner et al. 1997). Tanmateix, aquest és el primer estudi que relaciona aquest receptor amb la migració de cèl·lules de la glia, i els resultats descriuen per primer cop un paper funcional del $P2Y_2$ durant la reparació de un dany cel·lular.

Per altra banda, aquests resultats estan en concordança amb previs estudis del nostre grup de investigació, ja que havíem vist prèviament que el tractament de les cèl·lules de Schwann amb CMPF i UTP indueix una reorganització del citoesquelet d'actina i un increment de la proteïna Arp3, tot a través de l'activació dels receptors purinèrgics. Arp3 és una proteïna àmpliament implicada en la regulació del citoesquelet d'actina. Cal recorder que aquest és un procés imprescindible per la mobilitat, la fagocitosis i la locomoció cel·lular. Per tant un increment en Arp3 podria ser necessari per a una correcta mobilitat i migració cel·lular.

En relació amb els segons missatgers induïts per l'activació dels receptors purinèrgics a través del UTP ($P2Y_{2,4\ i\ 6}$), se sap que normalment inclouen l'activació de la PLC, que porta a l'alliberament del calci intracel·lular i a la posterior activació de la PKC, la Akt i finalment de les MAPK (Ballerini, Di Iorio et al. 2006). En cultius primaris d'astròcits, els receptors P2Y2 s'ha vist que medien l'activació de vies dependents i independents de calci i de PKC i ERK ½ que poden activar la fosfolipasa $A_2$ citosòlica (Ballerini, Di Iorio et al. 2006). En concordança amb aquests estudis previs, els nostres resultats demostren que l' estimulació amb UTP de les cèl·lules de Schwann, porta a una activació bifàsica de tres membres de la família de les MAPK (ERK ½, JNK i P38, **Figura R10**). Aquesta activació bifàsica de MAPK, ja havia sigut descrita anteriorment en resposta a diferents factors de creixement i citoquines (Gurjar, Deleon et al. 2001, Lien, Usami et al. 2006). Per altra banda no s'havia descrit mai en resposta al UTP. Els nostres estudis també demostren, tal com s'ha descrit anteriorment tant en el nostre com en d'altres grups de recerca, que l'activació de les MAPK en resposta al UTP, és mediada per l'estimulació dels receptors P2Y(Martiáñez, Lamarca et al. 2012, Muscella, Giovanna et al. 2003). La inhibició dels receptors amb Suramina (inhibidor no selectiu de P2Y) provoca un bloqueig de l'activació d'aquestes kinases (**Figura R12**).

Per altra banda, el paper del UTP en la primera activació de les MAPK ja havia sigut descrit anteriorment, mentre que no s'havia descrit encara el seu paper en l'activació tardana de les MAPKs. Aquest perfil cinètic ha estat proposat, recentment com un model que permet un sistema de senyalització comú per jugar papers reguladors diferents, en funció de la durada temporal dels diferents pics d'activació (Murphy et al. 2002).

A més a més, els nostres resultats demostren que l'activació de les MAPKs és crucial per a l'increment de la migració observat en les cèl·lules de Schwann després de l'estimulació amb UTP, al inhibir qualsevol de les tres MAPKs amb inhibidors específics, es bloqueja completament la inducció de la migració de les cèl·lules de Schwann (**Figura R11**). El paper de les MAPKs en la migració cel·lular ja ha sigut descrita anteriorment per a diferents tipus cel·lulars, tals com cèl·lules epitelials de la córnea o queratinocits entre d'altres (Rossi, Manfredini et al. 2007, Kawasaki, Smith et al. 2003, Yang, Cranson et al. 2004). I per tant, els nostres resultats es correlacionen amb aquests estudis previs i suggereixen que l'activació de les MAPKs en les cèl·lules de Schwann a través del UTP són necessàries per a la capacitat migratòria d'aquestes cèl·lules.

## 3.2. Activació de les MMPs en respota al UTP, i el seu paper en la migració de les cèl.lules de Schwann.

Les metalloproteinases de matriu es coneix que juguen papers fonamentals en diferents patologies neuronals, i tot just ara estem començant a entendre la seva importància en el dany de la medul·la espinal. La seva presència ha sigut descrita en neurones, i en cèl·lules de la glia i del endoteli, i sabem que la seva funció principal és la de degradar diferents components de la matriu extracel·lular, i alliberar així els factors de creixements i citoquines presents en aquesta matriu (Faulkner, Herrmann et al. 2004). A través de la seva activitat proteolítica, les MMPs jugen papers crítics durant la migració i invasió cel·lulars, tot regulant les vies intracel·lulars implicades en aquests processos (Kelin, Bischoff 2011, Roy, Yang et al. 2009, Giannelli, Falk-Marzillier et al. 1997, Taraboletti G, Sonzogni L, et al. 2000). Nosaltres hem descrit en aquest estudi que les cèl·lules de Schwann mostren la presencia de diferents MMPs, a nivell de mRNA, com la MMP-2, la MMP-7, la MMP-14 i la MMP-28 (**Figura R13**). Per altra banda la MMP-9 no es va detectar ni a nivell de mRNA, ni a nivell de activació. Estudis previs a aquest, han descrit la presencia de la MMP-9 en cèl·lules de Schwann de nervis sans, però sempre en nivells molt baixos (Shubayev, Angert et al. 2006). Per tant, la nostra hipòtesis és que l' activació dels receptors purinèrgics no és suficient per induir l'expressió d'aquesta metalloproteinasa.

De totes elles, mitjançant la tècnica del zimograma, hem observat que la MMP-2 és activada en presència del UTP. Encara més sorprenents són els resultats obtinguts de l'activació d'aquest enzim en presència de UTP i ferida. L' activació de la MMP-2 es troba més de 100 cops sobreexpressada en presència de ferida i UTP (**Figura R19**). Això ens indica que l'activació de la MMP-2 juga un paper primordial en els mecanismes de reparació induïts per UTP.

Per corroborar aquests resultats es van inhibir les MMPs amb un inhibidor general de les MMPs (GM6001) i també mitjançant un shRNA específic contra la MMP-2, que vàrem dissenyar personalment. Es va observar que el silenciament d'aquests enzims provoquen un bloqueig de la migració induïda per UTP, a més a més, de totes les MMPs, la MMP-2

sembla ser la principal implicada en aquesta migració induïda per UTP, ja que al inhibir específicament aquest enzim mitjançant el shMMP2 es va bloquejar completament la migració induïda per UTP (**Figura R24**). Aquests resultats suggereixen un paper important de la MMP-2 en els mecanismes de reparació induïts per UTP en cèl·lules de Schwann. Per altra banda, aquests resultats concorden amb estudis previs que descriuen increments en l'activació de la MMP-2 després de danys en la medul·la espinal (Hsu, McKeon et al. 2006). De totes maneres, també cal comentar que després del silenciament específic de la MMP-2, la migració de les cèl·lules de Schwann no es veu del tot bloquejada, indicant que probablement la MMP-2 no és l' única metalloproteinasa implicada en aquesta migració.

Finalment, els nostres resultats també demostren que després de la estimulació amb UTP, la MMP-2 sofreix un canvi en la seva localització intracel·lular, la qual és principalment perinuclear en condicions basals, i en canvi és principalment citoplasmàtica després del tractament de les cèl·lules de Schwann amb UTP (**Figura R21**). Aquest canvi de localització pot ser degut al fet que la MMP-2 per a ser activada cal que es secreti. Aquestes MMPs són sempre secretades com a pro-enzims abans de la seva activació final. Se sap d'estudis previs que per a la seva activació, la MMP-2 travessa la membrana citoplasmàtica on la MMP-14, que és una metalloproteinases de membrana la talla per el propèptid i l'activa. Per tant, el canvi de localització observat després del tractament amb UTP en les cèl·lules de Schwann s'explicaria pel fet que la MMP-2 en forma latent va a localitzar a la MMP-14, que hem vist en aquest estudi que està present en les cèl·lules de Schwann, la qual es localitza a la membrana de les cèl·lules per tal que aquesta l'activi i pugui,d'aquesta manera, ser secretada com a enzim actiu (Boag, Young 1993, Bauvois 2012, Stetler-Stevenson W.G., Aznavoorian et al. 1993).

Amb tots aquests resultats, la nostra conclusió és que l'habilitat del UTP per induir l' activació de la MMP-2 reflecteix un important mecanisme pel qual el UTP contribueix a la migració cel·lular en les cèl·lules de Schwann després d' una ferida.

## 3.3. Ruta intracel·lular hipotètica. Receptors P2Y, MAPK i MMP-2

Es va realitzar una aproximació farmacològica per tal d'intentar elucidar si les MAPK estaven implicades en la activació de la MMP-2 observada després de la estimulació amb UTP de les cèl·lules de Schwann. Els resultats obtinguts al inhibir específicament cadascuna de les MAPKs són sorprenents, ja que indiquen que la inhibició de qualsevol de les MAPKs provoca un bloqueig de l'activació de la MMP-2. Alhora també observem que la inhibició dels receptors purinèrgics amb l'inhibidor no selectiu Suramina i amb el shRNA específic contra el P2Y2, produeixen també un bloqueig de l'activació de la MMP-2 (**Figura R25**). Aquests resultats ens indiquen que les MAPKs estan directament implicades en l' activació de la MMP-2 com a resposta a l'UTP. També ens demostren, que l'activació de la MMP-2 és un pas que té lloc després de l'activació de les MAPKs a través dels receptors purinèrgics, tal com s'esquematitza a la **Figura C1**. Per altra banda, hi ha estudis previs que recolzen aquestes hipòtesis, ja que ja s'havia vist que en cèl·lules aòrtiques del múscul llis, l'activació de JNK es necessària per a la estimulació i posterior secreció de la MMP-2 (Robinson, Douillet et al. 2005).

L'especificitat dels inhibidors utilitzats en aquest estudi ha estat qüestionada anteriorment, i de fet diferents estudis han estudiat la seva especificitat. En el nostre cas, els nostres resultats indiquen que les vies de les MAPKs no són independents entre elles i que la inhibició d' una de les MAPKs involucra la inhibició de les altres, indiquen que és possible que hi hagi alguna interacció creuada entre elles (**Figura R12**). De totes maneres, caldrien més estudis per tal de determinar si realment hi ha una relació creuada entre elles o si els resultats obtinguts són efecte d'una inespecificitat dels inhibidors utilitzats.

Per altra banda, els nostres resultats també demostren, tal com ja s'ha vist anteriorment en múltiples ocasions, que la primera activació de les MAPKs és una resposta a l'activació dels receptors purinèrgics. La inhibició d'aquests receptors mitjançant la suramina provoca un bloqueig de l'activació de les MAPKs (**Figura R12**). Aquest efecte ha sigut ampliament observat tant en el nostre com en altres grups de recerca anteriorment a aquest estudi (Martiañez, Carrascal et al. 2012, Yang, Cranson et al. 2004).

En canvi, el segon pic d'activació de MAPKs no havia sigut descrit anteriorment en resposta al UTP. No només això, sinó que els nostres resultats també suggereixen que aquest segon pic d'activació està regulat directament per la MMP-2. Un cop silenciem específicament la MMP-2 amb el shRNA dissenyat, el segon pic de MAPK no s'observa per cap de les tres quinases estudiades (**Figura R30**). En vistes d'aquest sorprenent resultat, la nostra hipòtesis és que la MMP-2 activa alliberada a l'espai extracel·lular, podria alliberar un factor de creixement anclat a la membrana de les Schwann . En concret, creiem que el factor de creixement alliberat és la NRG-1 (Neuregulina 1).

Per altra banda, els resultats també ens demostren la importància del UTP en tot aquest procés, ja que hem vist que si retriem l'UTP del medi, un cop ja s'ha donat el primer pic d'activació de les MAPKs, és a dir, si després de 15 minuts de tractament, reemplacem el medi amb UTP per medi fresc sense UTP, el segon pic de activació de les MAPKs no s'observa, com tampoc s'observa l'activació de la MMP-2 (**Figura R27**). Tot això ens demostra que el UTP es indispensable per al correcte tancament de la ferida. Per tant podem postular, que quan hi ha una ferida en condicions fisiològiques, l' UTP seria alliberat de les cèl·lules i aquesta alliberació provocaria l' activació dels receptors purinèrgics. Un cop els receptors s'han activat, s'indueix l'activació de les rutes intracel·lulars, en aquest cas de les MAPKs. I aquesta activació facilitaria un desplaçament de la MMP-2 des de la perifèria del nucli fins a la membrana cel·lular, on gràcies a la MMP-14 seria activada i alliberada. La MMP-2 alliberada, podria ajudar a l'alliberament de' un factor de creixement anclat a la membrana de la cèl·lula, com podria ser la neuregulina-1 (NRG-1) i l'alliberament d'aquest factor provocaria l'activació d'uns altres receptors, probablement els receptors de ErbB induint així la segona activació de les MAPK. Tot aquest procés es veu esquematitzat a la **Figura C1**.

# 4. Conclusions

Les conclusions que podem extreure d'aquest estudi son les següents:

- Les cèl·lules de Schwann expressen receptors purinèrgics funcionals, sensibles a UTP.

- El tractament amb Núcleo CMP forte en cèl·lules de Schwann indueix la migració i la capacitat de reparació d'aquestes cèl·lules.

- El principal nucleòtids implicant en la migració i reparació d'aquestes cèl·lules de Schwann és l'UTP, que actua principalment a través del receptor $P2Y_2$.

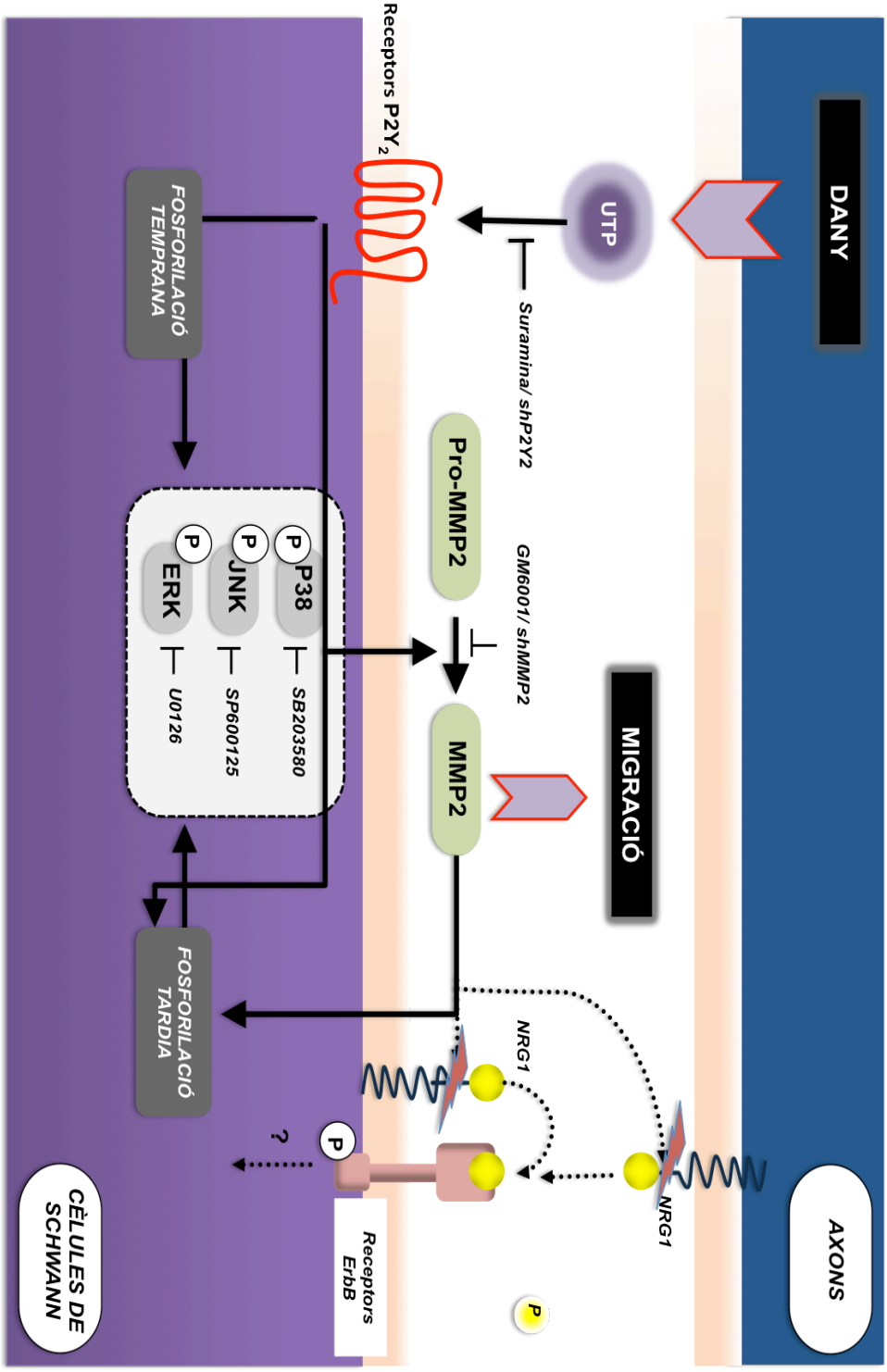
- L'activació dels receptors purinèrgics en cèl·lules de Schwann mitjaçant el UTP indueix l'activació temprana de les MAPK.

- L'activació dels receptors purinèrgics i posteriorment de les MAPK són indispensables per l'activació de la MMP-2 i per la seva secreció induïda per UTP.

- L'activació de la MMP-2 a través de l' UTP, indueix l'activació tardia de les MAPK en les cèl·lules de Schwann.

- Ambdós activacions, la del receptor purinèrgic i la de les MAPKs són indispensables per a la migració i reparació induïda per UTP en les cèl·lules de Schwann.

- L'activació de la MMP-2 és indispensable per a la inducció de la migració a través d' UTP en les cèl·lules de Schwann.


AXONS
NRG1
NRG1
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Receptors ErbB
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?
MIGRACIÓ
MMP2
GM6001/ shMMP2
Pro-MMP2
Suramina/ shP2Y2
UTP
DANY
Receptors P2Y2
FOSFORILACIÓ TARDIA
P38
P
SB203580
JNK
P
SP600125
ERK
P
U0126
FOSFORILACIÓ TEMPRANA
CÈLULES DE SCHWANN

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THE END
NO, IT ISN'T