Here I would like to thank to Prof. Dr. Rohini Kuner for a chance to perform this Diploma Thesis in her laboratory as an Erasmus exchange student at the University of Heidelberg. I would like to thank her and to Kiran Kumar Bali for an expert advices they gave me. Then I would like to thank to my supervisor PharmDr. Tomáš Šimůnek, PhD. for a professional advice. Last thanks is intended for my parents for great support during my studies.

Here I declare that this thesis is my author work and that I have written it on my own. All the literature and other used sources are listed in chapter References and properly cited in the text.

……..

Dagmar Kopecká

1 Introduction

Chronic pain is a major health problem. It is the main reason why people seek medical care. Unfortunately current therapies are inadequate for certain types of pain or cause adverse side effects. If we want to discover more successful pain treatment we need to understand underlying molecular mechanisms of the pain generation. In this thesis we focus on molecular approaches in the analysis of chronic pain in mice.

1.1 The goal of Diploma Thesis

In this project we focus on the expression of mTOR (serine/threonine kinase) and Rac (Rho GTPase) in chronic pain states. We want to check the temporal modulation of Rac1 and mTOR in the spinal cord following paw injection of Complete Freund's Adjuvant (CFA) to reveal whether these target proteins (molecules) could have the role in synaptic potentiation and structural plasticity in chronic pain states. It has long been recognized that structural plasticity plays an important role in the generation of chronic pain.

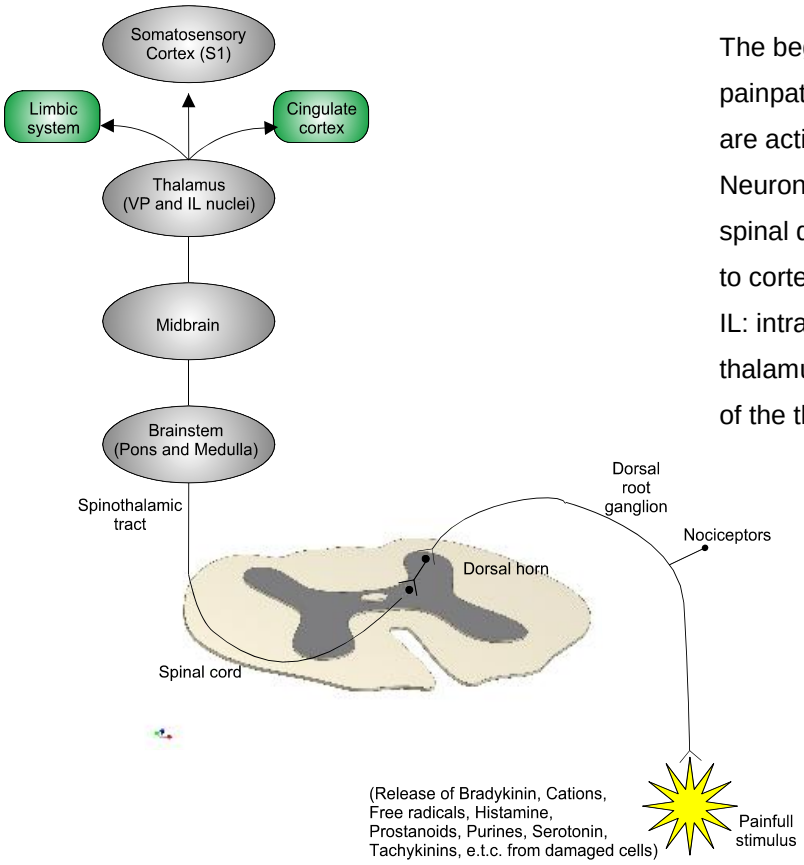
2 Theory

2.1 Pain pathway

Pain transduction and perception is extensive and complex, involving fundamental biologic events at multiple levels of the nervous system.

First components of pain pathway are primary sensory neurons (nociceptors). These neurons can be activated by ‘noxious‘ stimuli (stimuli that can produce tissue damage, e.g. inflammation). Nociceptors are very heterogenous. They differ in the neurotransmitters which they contain, in receptors and ion channels which they express, in their response properties to noxius stimuli and in their capacity to be sensitized during inflammation, injury and disease.

These neurons project to the outermost region of the spinal dorsal horn (lamina I and outer lamina II) and terminate largely on spinal neurons that project to higher-order pain centers in brain (via the thalamus to the cortex). This mechanism leads to sensation of pain (see Fig. 2.1)(Stucky et al., 2001).



The beginning of ascending painpathway is in nociceptors which are activated by noxious stimuli. Neuronal signal is transferred via spinal dorsal horn, brainstem, thalamus to cortex (centers of pain).
IL: intralaminar nucleus of the thalamus, VP: ventroposterior nucleus of the thalamus.

Fig. 2.1. Ascending pain pathway (adapted from Sigma-Aldrich).

2.2 Pain and neuronal plasticity

Neuronal plasticity is fundamental to many neurobiological functions, including pain. Changes in neuronal structure, connections between neurons and alterations in the quantity and properties of neurotransmitters, receptors, and ion channels can ultimately result in increased functional activity of neurons in the pain pathway. On the contrary plasticity can decrease the body's own pain inhibitory systems, resulting ultimately in increased pain (Stucky et al., 2001).

2.2.1 Mammalian Target of Rapamycin (mTOR)

The mammalian target of Rapamycin (mTOR) is a highly conserved serine/threonine protein kinase that regulates a number of diverse biological processes important for the cell growth and proliferation including ribosomal biogenesis and protein translation (Sandsmark et al., 2007).

2.2.1.1 Upstream regulation of mTOR

mTOR was first identified in 1991. It was revealed that it is the target of immunosupressive macrolide Rapamycin. Rapamycin forms complex with FKBP12 and blocks the protein-protein interactions which are critical for mTOR kinase activity (see Fig.2.2) (Heitman et al., 1991).

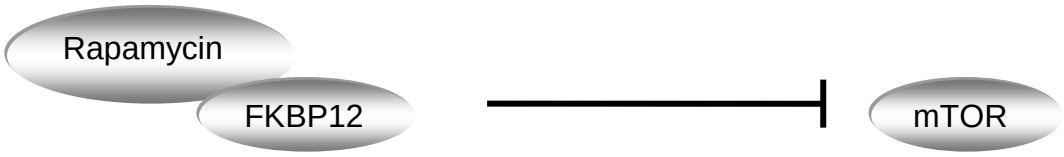


Fig.2.2. Inhibition of mTOR by Rapamycin. Rapamycin, macrolide produced by *Streptomyces hygroscopicus* binds to FK506 binbing protein (FKBP12) and inhibits mTOR kinase (adapted from Heitman et al., 1991).

In fact mTOR forms two distinct multiprotein signalling complexes but only one is sensitive to rapamycin-FKBP12.

Another upstream regulation of mTOR is via **PI3K** signalling pathway. When activated by receptor tyrosine kinase, PI3K phosphorylates membrane phospholipids, generating lipid second messengers. These second messengers recruit and activate a second kinase, **Akt**. Nowadays is supposed that Akt activates mTOR by direct phosphorylation (Nave et al., 1999) or by inactivating the protein complex encoded by the TSC1 and TSC2 genes. Their inactivation cause subsequent activation of Rheb by an as yet unknown mechanism (see Fig.2.3). Rheb then activates mTOR (Dan et al., 2002).

mTOR is regulated also by nutrients, energy and accessability of oxygen.

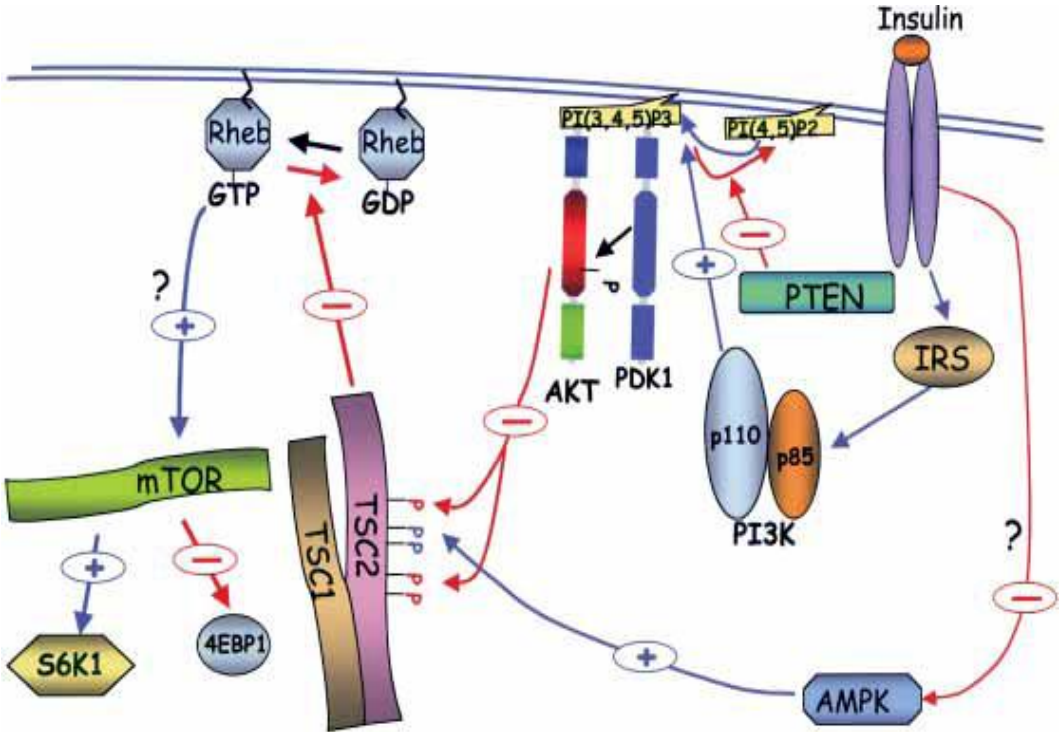


Fig.2.3. Function and regulation of mTOR. Regulation: Insulin or insulin-like factors initiates signalling at the insulin receptor, leading to activation of IRS and PI3K. The lipid products of PI3K, PIP3 (i.e. phosphatidylinositol 3,4,5 trisphosphate and phosphatidylinositol 3,4 bisphosphate), recruit both PDK1 and Akt to the plasma membrane. Akt phosphorylates TSC1/2 complex. This leads to the activation of Rheb which activates mTOR. Function: mTOR activates ribosomal S6 kinase1 (S6K1) and supress elongation factor 4E binding protein1 (4EBP1). Both mechanisms result in protein translation (reproduced from Hay et Sonenberg, 2004).

2.2.1.2 Downstream regulation of mTOR

mTOR regulates protein translation via two different mechanisms. First by activation of S6 kinase1 (S6K1) which leads to phosphorylation 40S ribosomal protein S6 (Jefferies et al., 1994, 1997). The second way is by supression of 4EBP1 (elongation factor 4E binding protein1) what prevents the blocking of elongation factor (Pause et al., 1994).

mTOR participates also in the regulation of ribosomal biogenesis e.g. regulates export of newly synthetized ribosomal subunits from the nucleus to the cytoplasm.

2.2.1.3 mTOR and synaptic plasticity

Synaptic plasticity is characterized by the change in the strenght of synapses in response to the noxious stimuli. An important feature of synaptic plasticity is the long-term potentiation (**LTP**) and long-term depression (LTD) of synaptic connections. LTP is the persistent increase in synaptic strength following high-frequency stimulation (e.g. noxious stimuli) of a chemical synapse, conversely is LTD the persistent decrease. Both, LTP and LTD have an early- and late-phases. Early phase is mostly dependent on the activity of kinases and phosphatases that modulate the delivery and removal of neurotransmitter receptors from the synapse. Late phase is dependent on the synthesis of new proteins in both LTP and LTD (Kelleher et al., 2004).

Strong noxious stimulation induces LTP in a subset dorsal horn neurons that project areas of the brain that are critical for the pain sensitization. It is proposed that central sensitization of the pain pathway is in fact largely explained by LTP (Sandkuhler, 2007).This mechanism is very specific because the synthesis of molecules which allow LTP to take place in the dendrites near the certain synapse instead of a soma of a neuron (Martin et al., 2000; Wang et al., 2004).
As mentioned before mTOR plays an important role in control of translation. Here in dendrites mTOR regulates translation via the controling of the initiation of cap-dependent translation (see Fig. 2.4) 4EBP1 binds the cap-binding factor eIF4E and, when it is hypo-phosphorylated, inhibits the formation of the eIF4E/eIF4G elongation complex preventing translation. When 4EBP1 is hyper-phosphorylated, 4EBP1

dissociates from eIF4E allowing eIF4G binding and the initiation of cap-dependent translation (Gingras et al., 1999).

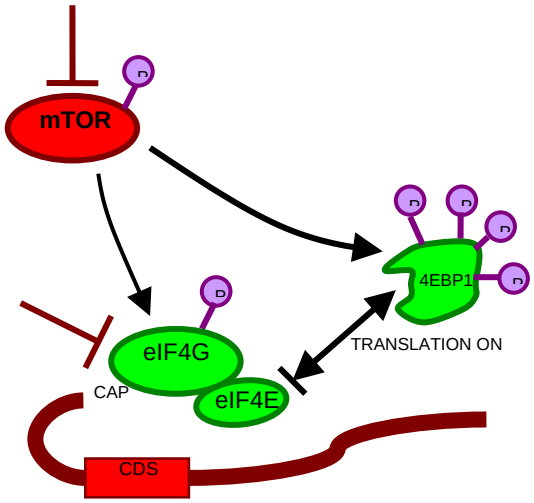


Fig.2.4. mTOR controls the protein translation. mTOR kinase is activated by upstream kinases in activity-dependent neurons. mTOR phosphorylates a. 4EBP1 causing dissociation from eIF4E; b. eIF4G allowing association with eIF4E. The dissociation of 4EBP1 from eIF4E and the formation of the eIF4E/eIF4G complex turns on CAP-dependent translation. mTOR activity is blocked by rapamycin eIF4G binding to eIF4E is blocked by 4EGI-1 (adapted from Gingras et al., 2004).

2.2.2 Rho GTPases and development of neuronal system

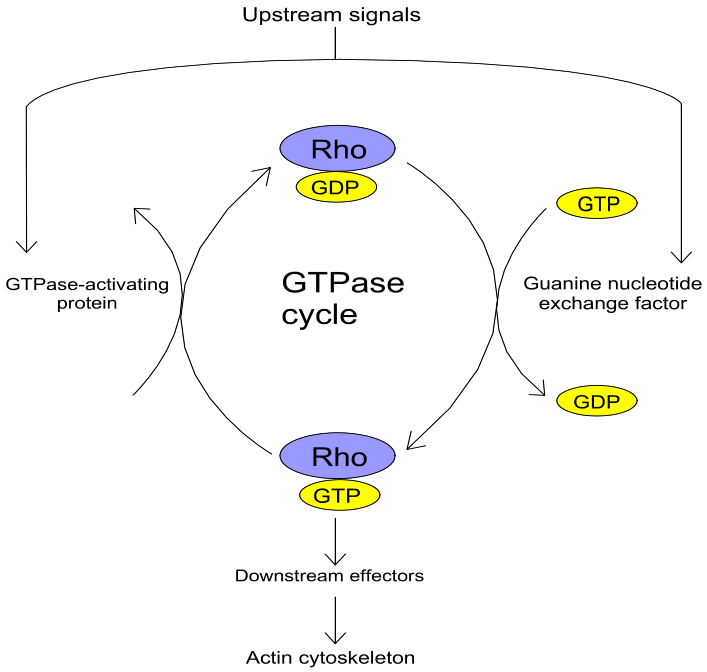
Development of neuronal system is a very complex process. Many molecules are involved on this process and Rho GTPases belong to them.

Once neuron is born, it might migrate a long way before it finds its destination and begins to differentiate (Hatten, 1999). It then sends two classes of processes: a single axon to carry its output and several dendrites to collect input (Craig and Banker, 1999). Once neuronal polarity is established, the axon navigates through a complex enviroment to find its target (Tessier-Lavigne,Goodman, 1996) and dendrites can undergo extensive growth and branching. The last step in constructing a rough scaffold of the nervous system is the establishment of synaptic connections between different neurons (Sanes et Lichman, 1999).

Rho GTPases are involved in all the above mentioned processes and in certain of them they act as a key regulators. Hence, they play a role in regulation of neuronal morphogenesis, including migration, polarity, axon growth and guidance, dendrite elaboration and plasticity and synapse formation (Luo, 2000).

Rho GTPases are small (~200 amino acids long) proteins. They constitute a subfamily of the Ras superfamily. They funtion as binary molecular switches by cycling between an active GTP-bound state and an inactive GDP-bound state (see Fig. 2.5). Their activity is determined by the ratio of GTP to GDP in the cell and can be influenced by different regulatory molecules (Van Aelst et D'Souza-Schorey, 1997). These upstream molecules which regulate their function can be:

- positive regulators such as guanine-nucleotide exchange factors (GEFs) facilitating the exchange of GDP for GTP and thereby switching Rho GTPases on
- negative regulators such as GTPase activating protein (GAPs) which switch them off
- guanine nucleotide dissociation inhibitors



Upstream signals to Rho GTPases are regulated via two distinct regulators. The positive regulators (GEFs) which facilitate switching on Rho GTPases and negative reulators (GAPs) which switch them off. Active Rho GTPases (GTP-bound state) active their downstream effectors.

Fig. 2.5. Rho GTPases as molecular switches (adapted from Luo, 2000).

The best studied Rho GTPases are RhoA (Ras homologous member A), Rac1 (Ras-related C3 botulinum toxin substrate1) and Cdc42 (cell division cycle 42) (Luo, 2000).

2.2.2.1 Ras related C3 botulinum toxin sustrate 1

Rac1 is 22 kD G-protein in Rho family that regulates neuron motility in response to extracellular signals (Henle et al., 2006). Lack of Rac1 from very early development results in embryonic death in mice, because it is very essential protein (Sugihara et al., 1998).

Rac is involved in:

- reorganization of actin cytoskeleton . Rac1 regulates the formation of lamellipodia (Nobes et Hall, 1995).
- the establishment of neuronal polarity. Neuronal polarity is a process which results in the formation of one axon and multiple dendrites required for neuronal circuitry. Rac1 act as a regulator of development both types of neurites (axon and dendrites) (Craig and Banker, 1994).
- the spine enlargement
- activity-dependent dendrite growth in hippocampal neurons which is depending on the type extracellular enviroment (Curtis, 2006)
- synaptogenesis. Here has Rac1 two distinct functions. First is the initiation of spinogenesis and the second is the regulation of the function and morfology of pre-existing spines. Rac induces clustering of AMPA receptors which mediates fast synaptic transmission in CNS. AMPA receptors (non-NMDA ionotropic transmembrane receptors for glutamate) are integrated in LTP (Mayer, 2005).

2.3 The principles of used methods

2.3.1 Protein assay according to Bradford

Bradford assay is a spectroscopic analytical procedure used for measuring the protein concentration in a solution. It was first performed in 1976 by Dr. Marion Bradford. She used for detection and quantification of total protein Coomassie G-250 in a coloric reagent.

Principle of this reaction is the change of the absorbance level of Coomassie G-250 and Comassie G-250 in complex with protein. Coomassie G-250 has an absorbance maximum at 465 nm (reddish/brown colour). Proteins containing basic aminoacids (especially histidine, arginine, lysine) bind via Van der Waals forces and hydrophobic interactions to the dye. This results in the change of absorbance maximum from 465 nm to 610 nm. Dye binds to protein proportionally to the number of positive charges. Hence free aminoacids, peptides and low concentrated protein solutions don't give a reaction with dye. Blue colour can be measured at any wavelength between 575 nm and 615 nm but the most significant difference Coomassie dye - protein complex from from Coomassie dye is at 595 nm (Bradford, 1976).

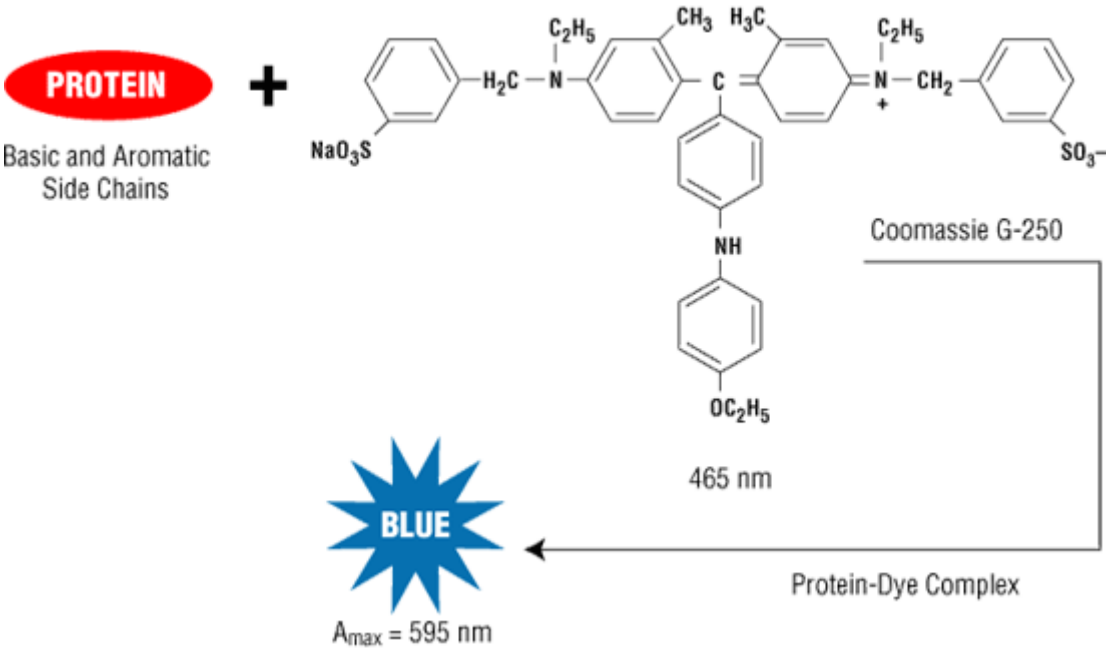


Fig.2.7. Principle of Bradford method. Schematic reaction between protein and Coomassie G-250 (reproduced from Thermo Scientific).

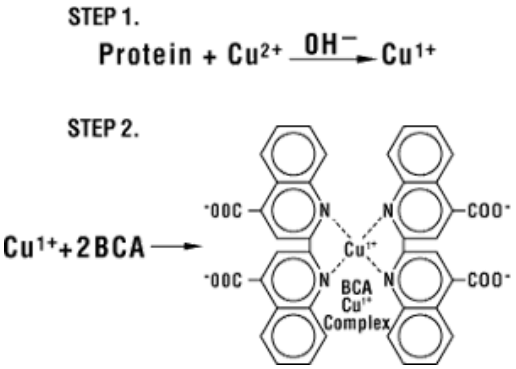
2.3.2 BCA protein assay

Bicinchoninic acid (BCA) based protein assay is another protein assay used for colorimetric detection and quantification of total protein. It was introduced by Paul K. Smith et al. in 1985.

The **principle** of this method is the reduction of Cu^{2+} to Cu^{1+} by protein in alkaline medium and subsequently selective colorimetric detection of Cu^{1+} by bicinchoninic acid (Smith et al., 1985).

First step, reduction of Cu^{2+} to Cu^{1+} by protein, is based on well-known biuret reaction. In this reaction, urea is heated forming organic compound biuret. Biruet subsequently reacts with Cu^{2+} in alkaline conditions resulting in light blue copper complex. One cupric ion forms a colored coordination complex with four to six nearby peptides bonds. The intensity of the color produced is proportional to the number of peptide bonds participating in the reaction. Single aminoacids and dipeptides don't give a biuret reaction.

The second step is the reaction of cuprous cation with bicinchoninic acid resulting in purple coloured complex (see Fig. 2.8). This complex is formed by the chelatation of two molecules of bicinchoninic acid with cuprous ion. The BCA/copper complex is water-soluble and exhibits a strong linear absorbance at 562 nm with increasing protein concentrations. Absorbance may be measured at any wavelenght between 550 and 570 nm with minimal less of signal. The reaction that leads to BCA color formation as a result of the reduction of Cu^{2+} is also strongly influenced by the presence of particular aminoacids (cysteine, cystine, tryptophan and tyrosine) (Wiechelman et al., 1988).



Reduction of Cu^{2+} to Cu^{1+} by protein in alkaline conditions (step 1.); Chelatation of cuprous ion with two molecules of bicinchoninic acid (step 2.).

Fig. 2.8. Principle of BCA assay (reproduced from Smith et al., 1985).

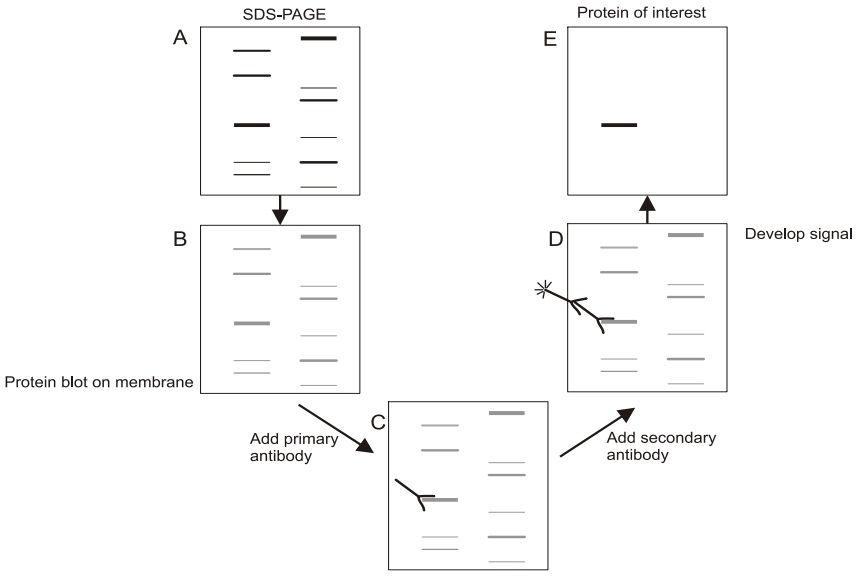
2.3.3 Western blotting

Western blotting is a powerful and unique procedure for immunodetection of proteins following electrophoresis.

Western blotting evolved from DNA (Southern blotting) and RNA (Nothern blotting) (Alwine et al., 1977). Towbin et al. was the first who invented the basis of western blotting was in 1975. The term ‘Western blotting ‘ was coined (Burnette, 1981) to describe a procedure that was slightly modified from that of Towbin et al. to retain geographic naming tradition initiated by Southern (Southern, 1975).

A procedure preceding the protein transfer is sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE). This procedure allows the separation of proteins in electric field according to their molecular weight. Western blotting allows the transfer of proteins from a sodium dodecyl sulfate polyacrylamide gel to an adsorbent membrane which ‘blots‘ the proteins. The blotted proteins form an exact replica of the gel and the protein of interest is subsequently detected by antibodies (Laemmli, 1970) (see Fig.2.9).

Western blotting is a powerful tool to detect and characterize a multitude of proteins, especially those proteins that are of low abundance.



A. Polyacrylamide gel with seperated proteins; B. Exact replica of SDS-PAGEobtained as a blot followinf transfer; C. Primary antibody binding to specific band on the blot; D. Secondary antibody conjugated to an enzyme (alkaline phosphatase or horse radish peroxidase; E. Development of specific band reveals the protein of interest.

Fig. 2.9. Scheme of western blotting and the detection procedure (adapted from Kurien et Scofield, 2006).

Western blot offer these specific advantages:

- wet membranes are pliable and easy to handle
- the proteins immobilized on the membrane are readily and equally accessible to different ligands
- only a small amount of reagents are required for transfer analysis
- multiple replicas of a gel is possible
- prolonged storage of transferred patterns
- the same protein transfer can be used for multiple successive analyses (Gershoni et Palade, 1982; Gershoni, 1988)

3 Experimental part

3.1 Material

3.1.1 Animals

Used mice were adult male wild-type (8-12 weeks old) C57Bl/6 mice from Charles River Laboratory, Germany.

3.1.2 Chemicals

General

Diethylether (J.T. Baker, Deventer, Holland)
Dodecyl sulfate Na-salt (Applichem, Darmstadt, Germany)
EDTA (Roth, Karlsruhe, Germany)
Ethanol (EtOH) 98% (J.T. Baker, Deventer, Holland)
Glycerol (J.T. Baker, Deventer, Holland)
Glycin (Applichem, Darmstadt, Germany)
Hydrochloric acid (HCl) 37% (Applichem, Darmstadt, Germany)
NaCl (J.T. Baker, Deventer, Holland)
Sodium deoxycholate (Merck Chemicals, Darmstadt, Germany)
Tris (Roth, Karlsruhe, Germany)
Tween 20 (Applichem, Darmstadt, Germany)
Acetic acid, 100% (J.T. Baker, Deventer, Holland)

Injecting animals

Freund's Adjuvant Complete (Sigma-Aldrich; Steinheim, Germany)

Lysate preparation

Protease Inhibitor Coctail Tablets, EDTA-free (Roche, Mannheim, Germany)

Protein estimation

BCA Protein Assay Kit (Pierce Biotechnology, Rockford, USA)

Natriumdodecylsulfate (SDS)-Polyacrylamid-Gelelectrophoresis (PAGE) and Western Blot

Ammoniumperoxidsulfate (APS), (Applichem, Darmstadt, Germany)

Bromo-phenol blue (Waldbeck GmBH, Münster, Germany)
ECL Plus (Amersham Biosciences, Little Chalfont, United Kingdom)
Methanol (J.T.Baker, Deventer, Holland)
Milch powder (Roth, Karlsruhe, Germany)
Natriumazid (Na_3N) (AppliChem, Darmstadt, Germany)
N,N,N',N'-Tetramethylethylendiamin p.A (TEMED) (Applichem, Darmstadt, Germany)
NP-40 (Merck Chemicals, Darmstadt, Germany)
PageRuler™ Prestained Protein Ladder Plus (Fermentas, Germany)
Ponceau S (Applichem, Darmstadt, Germany)
Rotiphorese Gel 30 (Karlsruhe, Germany)
β-Mercaptoethanol (Sigma-Aldrich, Steinheim, Germany)
Isopropanol (J.T.Baker, Deventer, Holland)

3.1.3 Instruments

General

Balance (Kern, Balingen, Germany)
Centrifuge Biofuge fresco/pico (M&S Labortechnic, Wiesloch, Germany)
Eppendorf thermomixer compact (Eppendorf, Hamburg, Germany)
Eppendorf tubes (1.5;2 ml) (Eppendorf, Hamburg, Germany)
Falcon tubes (15;50 ml) (Becton Dickinson, Heidelberg, Germany)
Fridge (Liebherr, Germany)
Latex gloves (VWR international, Leuven, Belgium)
Magnetic stirrer (IKA-Combimag RCO, Namur, Belgium)
pH-meter (Mettler-Toledo, Giesen, Germany)
Pippetes (20 μl; 200μl; 1000μl) (Gilson, France)
Rotary mixer (A. Hartenstein, Germany)
Vortexer (IKA vortex, Genius 3, Germany)
Water bath Julabo (Labortechnic GmBH, Seelbach, Germany)
Other instruments: graduated cylinders, beakers, filter-papers, pipette tips, excitator.

Needles BD Microlance™ (Becton Dickinson, GmBH, Germany)
Syringes (1ml) BD Plastipak (Becton Dickinson, Madrid, Spain)

Lysate preparation

150 Watt Ultrasonic disintegrator, MSE, FTZ-Nr.: B-134/72

Protein estimation

Incubator (Memmert, Munich, Germany)

Multiscan Ascent (Labsystems, Dreieich, Germany)

96-well plates (Wiesbaden, Germany)

SDS-PAGE and Western Blot

Optimax X-Ray film Processor (GmBH&Co.KG, Oberstenfeld, Germany)

Protran® nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany)

Shaker DOS-10L (NeoLab Migge, Heidelberg, Germany)

Voltage suppply Model No:PowerPac 300 (Biorad, California, USA)

Microliter ™ Syringes (Hamilton Bonaduz AG, Switzerland)

Mini-Protean® II/III Electrophoresis Cell (Bio-Rad, Munich, Germany)

Mini Trans Blot® Electrophoretic Transfer Cell (Bio-Rad, Munich, Germany)

Kodak® X-Omat AR (Sigma, Deisenhofen, Germany)

Fuji Medical X-Ray Film (A. Hartenstein, Germany)

3.1.4 Buffers and solutions

Name and composition	**Amount**
RIPA (1X)	
2 M Tris pH 7.4	1.25 ml
0.5 M EDTA	500.00µl
5 M NaCl	1.50 ml
NP-40	500.00 µl
20% SDS	250.00 µl
10% Deoxycholate (Na)	2.50 ml

Stacking buffer	
Tris	30.4 g
20% SDS	10.0 ml

Name and composition	Amount
Ultrapure water	up to 1 l
pH set to 6.8	

Separating buffer	
Tris	90.8 g
20% SDS	10.0 ml
Ultrapure water	up to 1 l

pH set to 8.8

Loading buffer (4X)	
1 M Tris pH 6.8	40.00 ml
20% SDS	40.00 ml
0.5 M EDTA	3.25 ml
Ultrapure water	6.00 ml
100% Glycerol	40.00 ml
Bromo-phenol blue	40.00 ml
Beta-mercaptoethanol	8.00 ml

Running buffer (10X)	
Tris	30.28 g
Glycin	144.20 g
SDS	10.00 g
Ultrapure water	up to 1 l

Transfer buffer (10X)	
Tris	60.56 g
Glycin	288.00 g
Ultrapure water	up to 2 l

pH set to 8.5

Transfer buffer (1X)	

Name and composition	Amount
Transfer buffer (10X)	100 ml
Methanol	200 ml
Ultrapure water	700 ml

PBS-T (10X)	
Tris	24.22 g
NaCl	204.40 g
Tween 20	5.00 ml
Ultrapure water	up to 1 l

pH set to 7.5

Ponceau S solution	
Ponceau S	0.2%
Acetic acid	1.0%

Blocking solution	
Milch powder	50 g
PBS-T (1X)	up to 1 l

Stripping buffer	
Glycin	15 g
SDS	1 g
Tween 20	10 ml
Ultrapure water	up to 1 l
pH set to 2.2	

3.1.4.1 Antibodies

Table 3.1. Review of used antibodies

Type	Specifity	Dilution	Producer
Primary antibodies	Rabbit anti-mTOR	1:750	Cell Signal.Tech, Germany
	Rabbit anti-pmTOR	1:750	Cell Signal. Tech, Germany
	Mouse anti-Rac1	1:500	BD Trannsduction Laboratories, Germany
	Rabbit anti-tubulin	1:1000 1:10000	Sigma-Aldrich, Germany
Secondary antibodies	Anti-mouse-HRP	1:2000	GE Healthcare, Germany
	Anti-rabbit-HRP	1:2000	Sigma-Aldrich, Germany

3.1.5 Software

Ascent software Multiskan Ascent, version 2.4.2.

ImageJ 1.38x

Microsoft Windows / Office 2003/XP

Sigmastat 3.5.

3.2 Methods

For each experiment three animals were used. One for preparation of control sample, one for 2 hours and one for 6 hours time points samples.

3.2.1 Injecting animals

Two animals were anesthetized in a chamber saturated with diethylether. Afterwards they were injected with 20 μl of Complete Freund's Adjuvant (CFA) into each back limb from ventral side, so 40μl of CFA per animal.

3.2.2 Tissue dissection

Exactly in two hours after injection one animal was killed under $C0_2$ atmosphere. 4th and 5th lumbal segments (L4+L5) of spinal cord was dissected using autoclaved instruments (scissors, scalpel and forceps). This tissue was placed immediately in Eppendorf tube and immersed in liquid nitrogen. This flash freezing kept all proteins in intact state untill they were used for lysate preparation.

At six hour time point after injection again L4+L5 part of spinal cord was dissected from the second animal and put the tissue in Eppendorf tube into liquid nitrogen for 10 minutes.

The same process was done with control mouse (without injection). Afterwards to each Eppendorf tube containing tissue was added 150μl of RIPA buffer with freshly added protease inhibitors and tubes were kept on ice. This prevents or tremendously decreases proteolysis, dephosphorylation and denaturation of proteins. This part was done by Kimar Kumar Bali.

3.2.3 Lysate preparation

Samples immediately were placed into 37°C water bath for 15 minutes. Then they were kept on ice. Tissues were roughly disintegrated into a smaller pieces by a pestle suitable for Eppendorf tubes.

The samples were then sonicated. It was done two times for 10 seconds and in between the proteins were kept on ice to prevent heating of the sample.

Sonication is a method which breaks the cells using high frequency sound waves. These sound waves cause disruption to the cell wall. Thanks to this we can afterwards pellet the insoluble material by centrifugation.

For the better lysis of the cells tubes with samples were placed for 30 minutes on orbital rotator at 4°C.

Samples were centrifuged for 10 minutes at 4°C at 13.000 rpm. Supernatant was aspirated and placed in fresh tube kept on ice. The pellet was discarded.

3.2.4 Protein estimation

Protein estimation was performed for each lysate. For this purpose Bradford Assay or BCA assay was used. Bovine serum albumine (BSA) was used in both methods as a protein standard. At first the protein estimation was performed using Bradford assay but we didn't get equal loading control bands. This was probably because of incompatibility of Bradford's reagent with RIPA buffer. Hence we changed to BCA protein assay.

3.2.4.1 Bradford assay

- Calibration standarts were prepared according to the Table 3.2.

Table 3.2. Concentrations of BSA standard

BSA (μg/ml)	μl out of BSA-solution	H_2O_{dd} μl
0	-	110
20	40μl out of 100μl/ml	160
30	45μl out of 100μl/ml	105
40	80μl out of 100μl/ml	120
50	60μl out of 100μl/ml	60
60	120μl out of 100μl/ml	80
80	160μl out of 100μl/ml	40
100	100μl out of 100μl/ml	-

- Each sample was diluted with deionized water into the concentrations of 1:25, 1:50, 1:100, 1:200, 1:500, 1:1000
- Calibration standards were pipetted (50 μl of each) in double of each into the wells of the culture plate
- 50 μl of each sample concentration was pipetted into the wells of a cultured plate

- 2 volumes of Roti®-Quant (5x) were diluted in 5,5 volumes of H_2O_{dd} and 250 µl of this solution was pipetted to the standards and to the samples on the plate.
- The culture plate was incubated for 5 minutes at the room temperature. OD_{590} was measured and the amount of total protein in sample was read on calibration curve.

3.2.4.2 BCA protein assay

We performed this method using the BCA protein assay kit.

- Calibration standarts were prepared according to the schedule Nr8. and 25µl of each was in double pipetted into a microplate well
- Each sample was diluted with deionized water into the concentrations of 1:25, 1:50, 1:100, 1:200, 1:500, 1:1000 and 25µl of each was pipetted into a well of culture plate
- BCA working reagent were prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B (50:1, reagent A:B)
- 200 µl of BCA working reagent was added to each well
- Plate was incubated at 37°C for 30 minutes
- Plate was cooled to room temperature
- Absorbance was measured at 590 nm and the amount of sample was read on calibration curve.

3.2.5 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

3.2.5.1 Protein denaturation

Proteins are naturally in tertiary conformation. If we wanted to detect them with antibody which recognize only small, specific portion of the protein (referred as epitope) we wouldn't be succesful because it may reside within the tertiary conformation of the protein. By the protein denaturation tertiary conformation changes into primary and we can easily detect the protein on membrane (see later). Hence samples were denatured by adding loading buffer (4x) and boiling at 95°C for 5 minutes. 4x loading buffer was mixed with sample in ratio 1:4.

Loading buffer contains:

- SDS (Sodium dodecyl sulfate) which cause denaturation. SDS anions binds to proteins and change them into negatively charged.
- β-mercaptoethanol which reduces disulphide bridges.
- Glycerol, which makes to sample more concentrated, hence maintain the sample on the bottom of the well.
- Bromo-phenol blue, which enables the visualization of the protein migration. It acts as a front of separated proteins, because it is very small and anionic substance, which migrates fastest of all sample components.

3.2.5.2 Gel preparation

At first we prepared 6% for mTOR and 10% gel for Rac1 analysis. The percentage of gel depends on the molecular size of protein which we want to detect. mTOR size is ~250 kD and Rac1 size is ~22 kD. The higher the size of protein is, the larger pore size in gel we need. Later we set 8% gel for mTOR, for better separation.

Gel for gelelectrophoresis is composed from separating gel and stacking gel. The composition of gels is written in schedule below (see Table 3.3). 30% Acrylamide contains acrylamide and N,N-methylenebisacrylamide. The polymerisation is initiated by the addition of ammonium persulfate (APS) along with Temed. Temed was added every time as the last one.

Table 3.3. Composition of separating and stacking gel

Separating gel				Stacking gel	
pecentage	6%	8%	10%	Pecentage	6%
Acrylamide 30%	1.02 ml	3.3 ml	1.7 ml	Acrylamide 30%	0.5 ml
Separating buffer	2.5 ml	6.25 ml	2.5 ml	Stacking buffer	1.25 ml
H_20	1.08 ml	2.88 ml	0.9 ml	H_20	0.75 ml
100% Temed	4 μl	10 μl	4 μl	100% Temed	3 μl
12.5% APS (120mg/ml)	40□μl	100 μl	40 μl	12.5% APS (120mg/ml)	30 μl

Gel electrophoresis was performed using the Mini-Protean III system (BioRad, München). Gel was allowed to polymerize (45 minutes) and covered with isopropanol at room temperature. After the polymerization, isopropanol was removed and stacking gel was poured onto separating gel. Immediately comb forming the wells was inserted. After complete polymerisation (10 more minutes), gels were assembled in the vertical electrophoresis chamber filled up with 1x running buffer.

3.2.5.3 Loading samples and running gels

Samples were loaded into narrow wells using Hamilton syringe.
In order to analyse Rac1 expression was loaded 40 μg of total protein (control, CFA 2 hours, CFA 6 hours lines) and in case of mTOR 80 μg of total protein (again three lines). Onto each gel was loaded 3-5μl of protein molecular weight standard (PageRuler^{TM}, Fermentas). Electrophoresis was conducted at constant voltage of 120 V until the dye front migrated to the bottom of the gel. This procedure allows to proteins become seperated according to their molecular weight.

3.2.5.4 Protein electrotransfer (western blotting)

For this propose it is necessary that proteins are negatively charged. Hence, they can be induced to travel through the gel in an electrical field, and afterwards they can be transferred in an electrical field from the gel onto a sturdy support, a membrane that ‘blots‘ the protein from the gel.
In order to perform this nitrocellulose membrane (Protran$^{®}$, 0.45 μm, Schleicher & Schuell, Dassel) and Mini-Trans-Blot system (BioRad, München) was used.
To performed a wet transfer a ‘sandwich‘ (sponge/filter paper/gel/filter paper/sponge) was built and assembled into a transfer chamber filled with 1x transfer buffer. Electrotransfer was conducted at constant amperage of 300 mA for 2 hours (see Fig. 3.1).

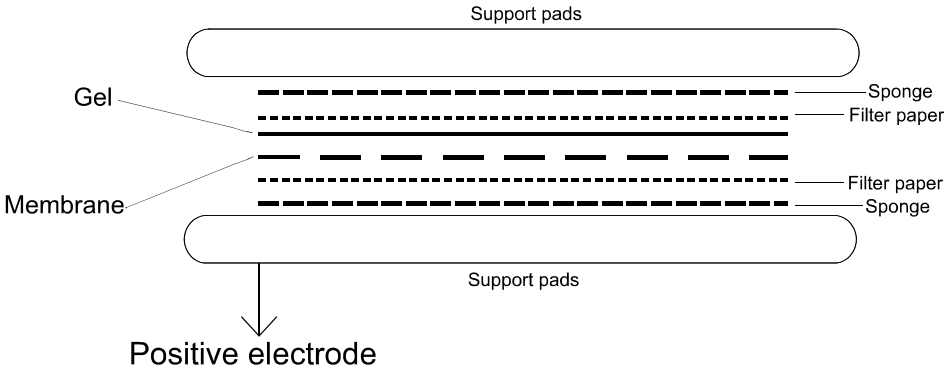


Fig.3.1. The Mini-Trans-blot gel-membrane sandwich. Transfer membrane is sandwiched between the gel,filter papers, sponges and support pads. Membrane faces the positive electrode. The cassette containing the assembled gel-membrane sandwich is inserted into a Trans-blot apparatus containing cold transfer buffer and negatively charged proteins in gel are transferred in the electric field into the membrane (adapted from Curien and Scofield, 2006).

3.2.5.5 Visualization of proteins in membrane: Ponceau staining

In order to ensure success of transfer and the equal loading, membranes were then stained with the Ponceau S staining solution for a few seconds, and washed with 1x TBS-T for Western blotting on the agitator.

3.2.5.6 Blocking the membrane

Blocking the membrane prevents non-specific background binding of the primary and/or secondary antibodies on the membrane. Membranes were put into blocking solution (5% non-fat milk in TBST) and incubated for one hour under agitation at room temperature. Filtrated milk solution was used, because non-filtrated milk would lead to presence of tiny dark grains and thereby contaminate the blot during development.

3.2.5.7 Incubation with primary antibody

Blots were placed in Falcon tubes with appropriate primary antibody solution in dilution 1:750 in the case of mTOR and phospho-mTOR and 1:500 in case of Rac1. Whilst anti-mTOR and anti-Rac1 antibodies were diluted in 5% non-fat milk, anti-phospho mTOR antibody was diluted in 5% BSA solution. The reason is that milk contains cassein which is a phospho-protein and it causes high background because the phospho-specific antibody detects also the cassein present in the milk. Membranes were incubated overnight at 4^{o}C with agitation.

3.2.5.8 Incubation with secondary antibody

Membranes were washed in 1X TBST three times for 15 minutes to remove residual primary antibody. Afterwards membranes were placed in falcon tube containing solution of secondary antibody and incubated for 2 hours at room temperature with agitation. In the case of mTOR and p-mTOR membranes were incubated with an anti-rabbit IgG horseradish peroxidase-linked antiserum in dilution 1:1000. In the case of Rac1 membranes were incubated with an anti-mouse IgG horseradish peroxidase-linked antibody.

3.2.5.9 Film developing

After washing with three changes of 1x TBST for 10 minutes each, immunoreactive bands were visualized through the enhanced chemiluminescence (ECL) detection system, where 1:1 mixture of Reagent-1 and Reagent-2 from the ECL Kit was applied on the membrane and the result was registered on the X-ray film (Kodak® X-Omat AR, Sigma, Deisenhofen; Fuji Medical X-Ray Film, A. Hartenstein). Subsequently, the film was developed and fixated using X-ray film processor (Optimax, Protec, Oberstenfeld).

3.2.5.10 Tubulin test

In order to check that the lanes in our gels have been evenly loaded with sample we performed loading control analysis. We checked the expression of tubulin, which is a protein present in each cell and the expression of tubulin isn't influenced by CFA injection.

Membranes were washed for 30 minutes and afterwards membranes were incubated 1 hour for blocking. Afterwards we followed the steps described in section 3.2.5.6 up to 3.2.5.9 using appropriate antibody dilution. In order to check the expression of tubulin in the case of Rac1 we used anti-tubulin primary antibody in dilution 1:1000 and secondary horseradish peroxidase-conjugated anti-mouse IgG in dilution 1:2000. In order to perform the analysis of tubulin expression in the cases of mTOR and p-mTOR we used first anti-tubulin antibody in dilution 1:1000 and secondary horseradish peroxidase-conjugated anti-mouse IgG in dilution 1:2000. Later we decrease the dilution of primary antibody to 1:10000 because developed bands were over-expressed.

3.2.5.11 Analysis of western blot bands

Bands were quantified by digital imaging and are expressed as integrated density values (band area × relative intensity) using ImageJ programme. At first we measured integrated density of each band in each experiment and substracted the values of background integrated density.

To compare the expression of control sample, 2 hours and 6 hours time point samples we normalised their values with corresponding tubulin, calculated percentage change of expression and these values put into graph. We checked for the significance using non-parametric statistical test Anova on ranks because our results have no normal distribution.

4 Results

4.1 Expression of total Rac1 following bilateral paw injection of CFA

We analysed 13 blots from 6 independent preparations. We observed the expression of 22 kD band corresponding to Rac1. Obtained data were normalized with corresponding tubulin (55 kD). Percentage values of the expression of total Rac1 are shown in Table 4.1. These results are shown in graph (see Fig. 4.1). We have performed Anova on ranks and found that **there is no significant change in the expression of Rac1 at both, 2 hours and 6 hours time points.** Representative western blot is shown in Fig. 4.2.

Table 4.1. Percentage values of expression of Total Rac1

	Percentage values of expression of total Rac1			
blot	**control**	**2 hours**	**6 hours**	**Sample preparation**
1	100,00	65,81	98,23	**I**
2	100,00	109,33	109,29	**III**
3	100,00	94,40	112,94	**V**
4	100,00	107,93	94,98	**VI**
5	100,00	69,86	40,51	**III**
6	100,00	125,95	39,85	
7	100,00	100,23	99,08	**X**
8	100,00	95,79	35,31	
9	100,00	159,78	199,31	
10	100,00	88,62	69,95	
11	100,00	88,02	184,11	**XI**
12	100,00	91,59	141,13	
13	100,00	106,43	293,52	
Average	100,00	100,29	116,79	
STDEV	0,00	23,99	73,56	
SEM	0,00	6,65	20,40	

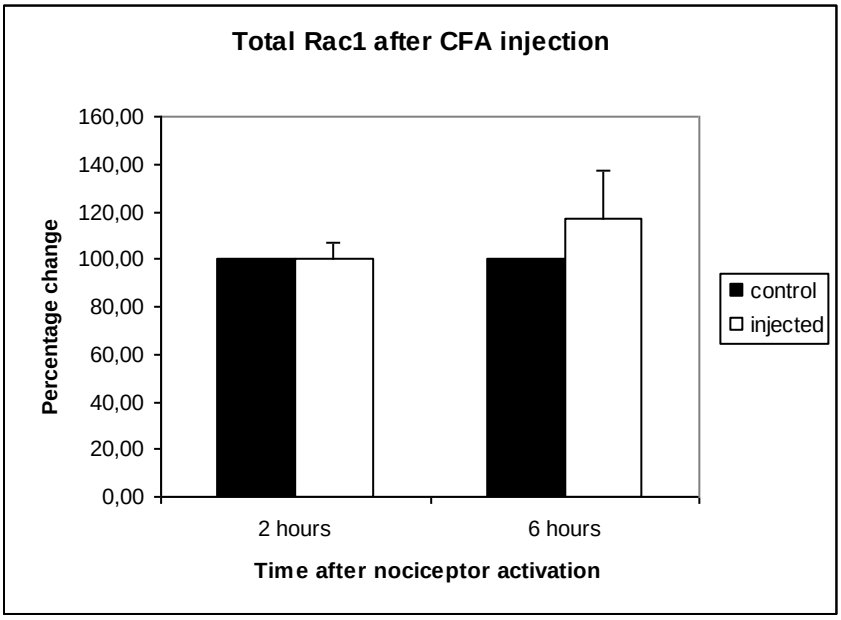


Fig. 4.1. Percentage change of the expression of total Rac1 at 2 and 6 hours time points following bilateral paw injection of CFA.

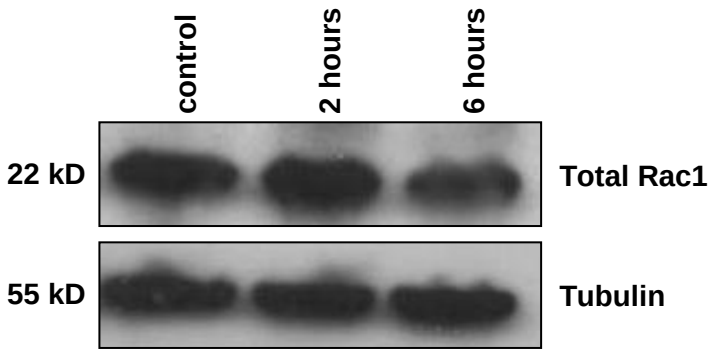


Fig. 4.2. Representative western blotting analysis of total Rac1 and corresponding tubulin in lysates of L4+L5 segments of mouse spinal cord following CFA injection.

4.2 Expression of mTOR following bilateral paw injection of CFA

4.2.1 Expression of total mTOR

We analysed 12 blots from 6 independent preparations. We detected 250 kD band corresponding to mTOR. The expression of total mTOR in different preparations was not stable. Some samples showed higher expression and some samples showed lower expression. We calculated percentage change in the expression of total mTOR with respect to control (Table 4.2). These values we put into the graph (Fig. 4.3). We have checked for the significance using Anova on ranks and found that there is **no significant difference at 2 hours and 6 hours time points.** But as it is shown in a graph (Fig. 4.3) **there is clear trend of increase in total mTOR levels at 6 hours time point**. Representative western blot of Total mTOR is shown in Fig. 4.4.

Table 4.2. Percentage change of expression of total mTOR

	Percentage values of expression of total Rac1			
Blot	**control**	**2 hours**	**6 hours**	**Sample preparation**
1	100,00	70,25	154,88	**I**
2	100,00	71,91	77,18	
3	100,00	52,65	57,28	**II**
4	100,00	33,86	70,00	**III**
5	100,00	66,89	107,56	
6	100,00	79,00	80,51	
7	100,00	90,11	141,17	**X**
8	100,00	112,96	67,09	
9	100,00	112,90	194,49	**XI**
10	100,00	27,59	113,06	**XI**
11	100,00	253,03	289,33	**XII**
12	100,00	224,97	266,53	**XII**
Average	100,00	99,68	134,92	
STDEV	0,00	70,41	78,34	
SEM	0,00	20,32	22,61	

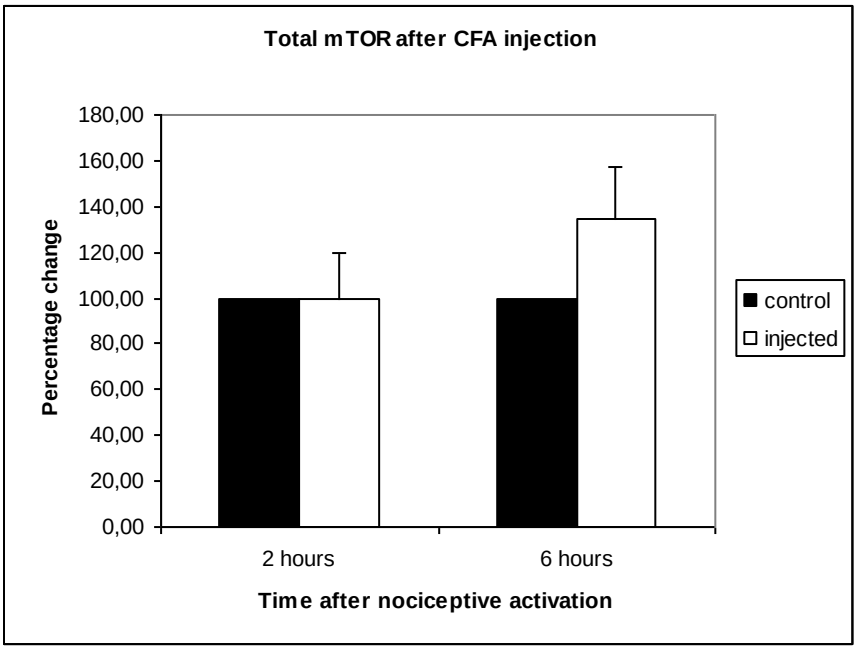


Fig. 4.3. Expression of total mTOR following bilateral paw CFA injection and its comparison with basal values.

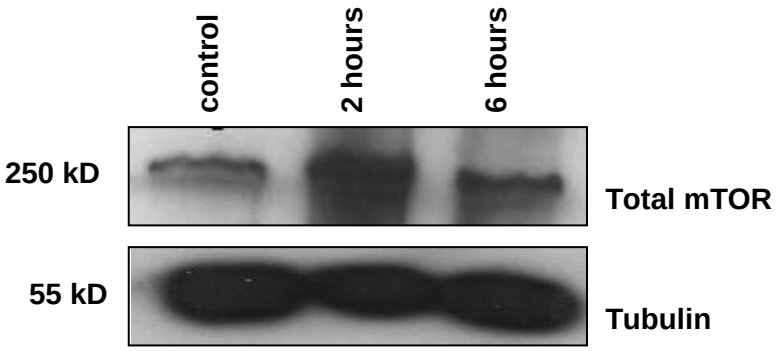


Fig. 4.4. Representative western blotting analysis of total mTOR and corresponding tubulin in lysates of L4+L5 segments of mouse spinal cord following CFA injection.

4.2.2 Expression of activated mTOR

It was very difficult to detect activated mTOR even in a control sample. This could be because of fast degradation of activated mTOR in sample. We observed that when sample was analysed immediately after its preparation the immunodetection of activated mTOR was more successful. But sometimes even in this case we weren not succesful. Another reason of its difficult detection could be because of sample precipitation. We observed in some samples the protein precipitation during the protein denaturation process preceding sodium dodecyl sulfate polycacrylamide gel electrophoresis.

We have analysed 6 blots from 3 independent preparations. We have established the expression of activated mTOR in control, 2 and 6 hours time points and normalized to total mTOR. Percentage values which we gain by measuring of integrated intensity of bands are shown in Table 4.4. These data we put into the graph (Fig. 4.5). Representative western blot of activated mTOR and corresponding tubulin is shown in Fig. 4.6.The Anova test showed that there was **no significant change at both 2 and 6 hours time points**. However, as seen in Fig. 4.5, there is a **clear trend of increase**. Lack of statistical significance could be probably because of low number of samples and more experiments are therefore necessary to either confirm or reject these preliminary findings.

Table 4.4. Percentage values of expression of activated mTOR.

	Percentage values of expression of activated mTOR			
Blot	**control**	**2 hours**	**6 hours**	**Sample preparation**
1	100,00	115,19	83,06	**I**
2	100,00	258,84	208,79	
3	100,00	338,06	485,32	**III**
4	100,00	198,96	326,79	
5	100,00	99,61	106,15	**X**
6	100,00	98,99	67,03	
Average	100,00	184,94	212,86	
STDEV	0,00	98,62	165,37	
SEM	0,00	40,26	67,51	

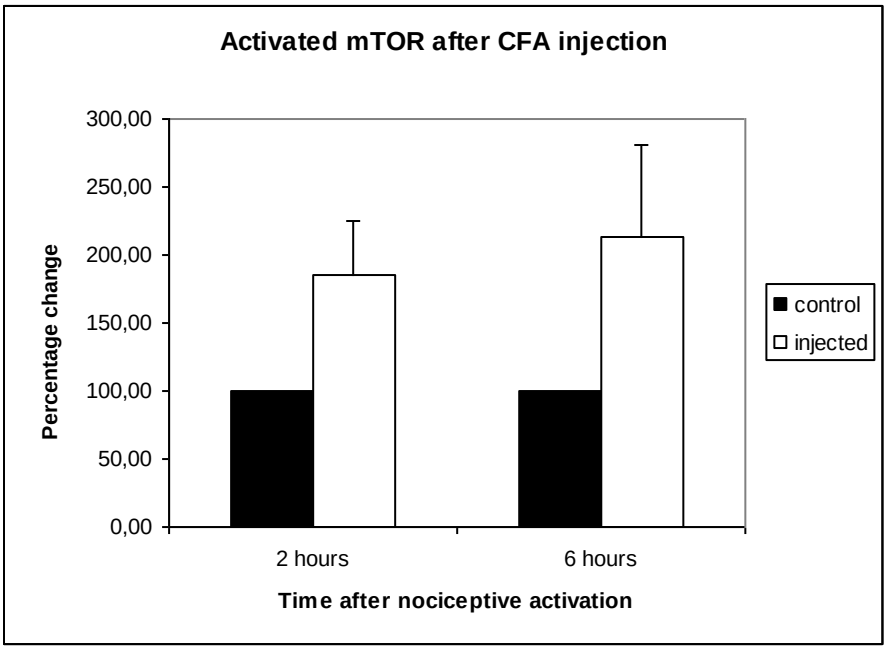


Fig. 4.5. Percentage change in the expression of activated mTOR following CFA injection

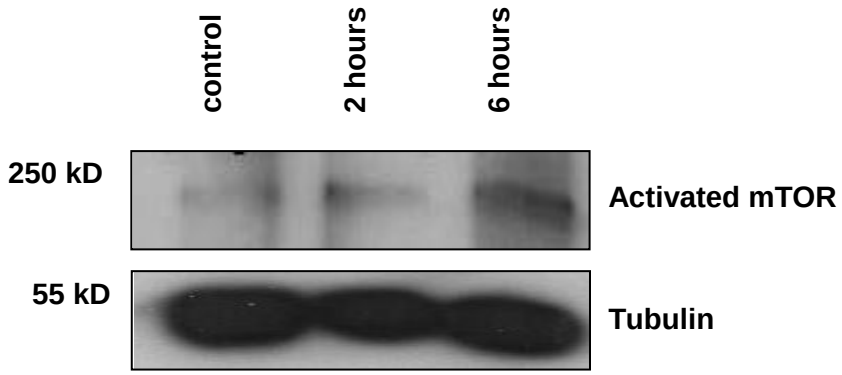


Fig. 4.6. Representative western blot of the expression of activated in control and both 2 and 6 hours time points with corresponding tubulin.

5 Discussion

We have checked the expression of total Rac1 following CFA injection. CFA is a water-in-oil emulsion containing as an antigen heat-killed mycobacterial cell wall components. Application of CFA into a body leads to stimulation of local innate immune response resulting in enhanced adaptive immunity. An essential component of this response is an intense inflammation in a site of deposition of the antigen. The reaction of the body is so strong that it can even lead to development of arthritic pain.

We have found that the difference between basal expression of total Rac1 and the expression of total Rac1 chronic pain state is not significant. There was no change at 2 hours time point. At 6 hours time point we have detected a trend of increase expression of total Rac1 but the difference between basal state and 6 hours time point following CFA injection was not significant. So we have to conclude that there is no difference in the expression of Rac1 in chronic pain state.

We've performed these experiments because we have addressed a question on the potential role of Rac1 in pain processing. The analysed samples were prepared from L4+L5 segments in 2 hours and 6 hours time points following CFA injection. We have injected CFA into both hind paws (20μL in each) of the mouse. CFA caused inflammation leading to intense activation of nociceptors (subpopulation of primary sensory neurons). We analysed lysate from L4+L5 spinal cord segment because we've supposed the most of signal coming from periphery via primary sensory neurons enters this part of spinal cord. Rac1 is a small GTPase playing an important role in communication of neurons. It is a key molecule of the formation of synapses between axon of presynaptic neuron and dendrites of postsynaptic neurons (Van Aelst and Cline, 2004). Therefore we have examined the expression of Rac1 in spinal neurons to see whether Rac1 could contribute to transfer of signal leading to sensation of pain.

To establish the expression of total mTOR we have used antibody raised against to total mTOR. We have found that there is no regulation of total mTOR levels at 2 hours time point following CFA injection but there is a trend of increase in total mTOR levels at 6 hours time point. To detect activated mTOR we have used antibody raised against to mTOR phosphorylated at Ser2448 via the PI3 kinase/Akt signaling pathway. In case of activated mTOR we have found that there is no

regulation at both 2 and 6 hours. But this cannot be the final conclusion because there was a clear trend of increase although not reaching the level of statistical significance. Further samples are needed to be added to get final conclusion about activated mTOR.

We wanted to check the regulation of the expression of mTOR in inflammatory (chronic) pain states. It is already known that mTOR is involved in LTP and LDP, the main features of the synaptic plasticity. It has long been recognized that synaptic plasticity plays an important role in sensitization to noxious stimuli and a critical role of the generation of chronic pain (Woolf et al., 2000). Therefore we can hypothesize that mTOR could be involved in activity-dependent plasticity in context of chronic pain because mTOR plays a key role in the protein translation in dendrites (Jaworski et al., 2006). Unfortunately, no definite conclusions can be drawn at this moment, because more experiments have to be done to confirm that regulation of mTOR in chronic pain state is actually significant. If the regulation is confirmed there will be a potential of a role of mTOR in the chronic pain processing.

This Diploma Thesis is involved in project ‘Inhibition of pain memory and chronic pain using RNAi’. The goal of this project is to address a role of novel signaling molecules in pain (mTOR, Rac1). In this Thesis we perfomed first two parts of this project. We checked the basal levels expression of this molecules and we checked the temporal expression of these proteins in the spinal cord following paw injection of Complete Freund’s Adjuvant (CFA). We examined the expression of these two proteins at 2 and 6 hours after application of intense noxious stimulus. The question is whether 2 hours time period is enough time for protein formation. We found that there is a potential in regulation in the case of mTOR. Whether our findings will be confirmed overexpression and downregulation of mTOR will follow (selectively in the spinal cord) and the effects will be checked on pain phenotype.

6 Conclusion

The aim of this Diploma Thesis was to study the expression of Rac1 and mTOR in chronic pain states. For this purpose we have used CFA mouse pain model and checked the temporal modulation of the expression (at 2 and 6 hours time points) via western blotting analysis. We have concluded that there was no regulation in the expression of total Rac1. In case of total mTOR we found no regulation at 2 hours time point but a trend of increase in total mTOR levels at 6 hours time point which was not significant. More experiments are needed to get final conclusion. If the mTOR regulation is confirmed, there is a potential for a role of mTOR in activity-dependent plasticity in context of chronic pain.

7 Summary

Functional and structural plasticity of central synapses in pain pathways is responsible for the allodynia, hyperalgesia and pain memory caused by different insults like inflammation, neuropathy or trauma. Unfortunately molecular mechanism underlying plasticity at different levels of pain transmission are not yet clearly understood. mTOR is serine-threonine kinase playing a significant role in Long Term Potentiation (LTP) and in local protein translation in Hippocampus and Rac1 (Rho-GTPase) is the key regulator of actin cytoskeleton. Therefore we addressed the potential role of mTOR and Rac1 in activity-dependent plasticity in the spinal cord in the context of chronic pain. Using the western blot analysis, we examined the temporal expression of these proteins in L4+L5 spinal segments using inflammatory pain model. In case of Rac1 we found that the expression is unchanged. In case of mTOR we showed that the change in the expression is not significant but there is a clear trend of increase. To confirm these findings more expesiments are needed to be added. If this is confirmed there is a potential role of mTOR in pain processing.

8 Souhrn

Funkční a strukturální plasticita synapsí nervových drah bolesti je zodpovědná za alodynii, hyperalgésii a paměť bolesti, které mohou být vyvolány zánětem, neuropatií nebo traumatem. Bohužel molekulární mechanizmy této plasticity nejsou na různých úrovních transmise bolesti ještě zcela poznány. mTOR je serin/threonin kináza hrající důležitou roli v indukci dlouhodobé poteciace (LTP) a translace proteinů v hipokampu. Rac1(Rho-GTPáza) je klíčovým regulátorem aktinu při utváření buněčné kostry. Proto se zde zaměřujeme na mTOR a Rac1, abychom zjistili, jestli hrají roli v utváření spinální plasticity v souvislosti s chronickou bolestí. Pomocí Western blot analýzy jsme zjišťovali časovou změnu exprese těchto proteinů ve čtvrtém a pátém segmentu spinální míchy za použití zánětlivého modelu bolesti. V případě proteinu Rac1 jsme zjistili, že se exprese nemění. V případě kinázy mTOR jsme ukázali, že změna exprese není výrazná, avšak pozorujeme jasný trend zvýšení exprese tohoto proteinu. Abychom mohli s jistotou konstatovat, že změna exprese skutečně nastává, je třeba provést více pokusů. Pokud se naše hypotéza potvrdí, je možné, že mTOR hraje určitou roli v utváření bolesti.

9 List of Figures

10 List of Tables

11 List of Abbreviations

4EBP1 - 4E binding protein 1

4EGI -1-α-[2-[4-(3,4-Dichlorophenyl)-2-thiazolyl]hydrazinylidene]-2-nitro-benzenepropanoic acid

BCA - Bicinchoninic acid

BSA - Bovine Serum Albumine

CDC42 - cell division cycle 42

CFA - Complete Freund's Adjuvant

ECL - enhanced luminescence

e.g. - exempli gratia, example

eIF4E - eucaryotic translation initiation factor 4E

eIF4G - eucaryotic translation initiation factor 4E

FKBP12 - FK506 binbing protein

GAPs - GTPase activating proteins

GDP - Guanosine diphosphate

GEFs - Guanine-Nucleotide Exchange factors

GTP - Guanosine triphosphate

IgG - immunoglobulin G

i.e. - id est, that is

IL - intralaminar

L4+L5 - 4^{th} and 5^{th} lumbal segment of spinal cord

LTD - Long Term Depression

LTP - Long Term Potentiation

mTOR - Mammalian Target of Rapamycin

NP40 - Nonidet-P40 buffer

PAGE - PolyacrylAmide Gel Electrophoresis

PBS - T- Phosphate Buffered Saline Tween-20

PIK3 - Phosphatidylinositol 3-kinase

Rac1 - Ras-related C3 botulinum toxin substrate1

Rho - Ras homologous member

RIPA buffer - Radio Immuno Precipitation Assay buffer

RNAi - RNA interference

S6K1 - S6 kinase 1

SDS - Sodium Dodecyl Sulfate

Tris - trishydroxymethylaminomethane

TSC1 - Tuberous sclerosis protein 1

TSC2 - Tuberous sclerosis protein 2

VP - ventroposterior

12 References

Alwine JC, Kemp DJ, Stark GR. Method for detection of specific RNAs in agarose gels by transfer to diazobenzyloxymethyl-paper and hybridization with DNA probes. *Proc Natl Acad Sci USA.* 1977; 74:5350–5354.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.*1976; 72:248-254.

Burnette WN. "Western blotting": electrophoretic transfer of proteins from sodium dodecyl sulfate - polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A, *Anal Biochem*.1981; 112:195-203.

Craig AM, Banker G. Neuronal polarity. *Annu Rev Neurosci.* 1994; 17:267–310.

Curtis I. Functions of Rac GTPases during neuronal development. *Dev Neurosci*. 2008; 30:47-58.

Dan HC, Sun M, Yang L, Feldman RI, Sui XM, Ou CC, Nellist M, Yeung RS, Halley DJ, Nicosia SV, !!. Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. *J Biol Chem.* 2002; 277:35364–35370.

Gershoni JM. Protein blotting: a manual. *Methods Biochem Anal*. 1988; 33:1–58,

Gershoni JM, Palade GE. Electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to a positively charged membrane filter. *Anal Biochem*. 1982; 124:396–405.

Gingras AC, Gygi SP, Raught B, Polakiewicz RD, Abraham RT, Hoekstra MF, Aebersold R, Sonenberg N. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. *Genes Dev*. 1999; 13:1422-1437.

Gingras AC, Raught B, Sonenberg N. mTOR signaling to translation. *Curr Top Microbiol Immunol.* 2004; 279:169-197.

Hatten ME. Central nervous system neuronal migration. *Annu Rev Neurosci.* 1999; 22:511–539.

Hay N, Sonenberg N. Upstream and downstream of mTOR. *Genes Dev.* 2004; 18:1926-1945.

Heitman J, Movva N, Hall MN. Targets for cell cycle arrest by the immunosuppresant rapamycin in yeast. *Science*. 1991; 253:905-909.

Henle F, Fischer C, Meyer DK, Leemhuis J. VIP and PACAP38 control NMDAinduced dendrite motility by modifying the activities of Rho GTPases and phosphatidylinositol 3-kinases. *J Biol Chem*. 2006; 281:24955–24969.

http://www.sigmaaldrich.com/Area_of_Interest/Life_Science/Cell_Signaling/Key_Resources/Pathway_Slides__Charts/Ascending_Pain_Pathway.html

Inoki K, Li Y, Zhu T, Wu J, Guan KL. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat Cell Biol Chem.* 2002; 4: 648-657.

Jaworski J, Sheng M. The growing role of mTOR in neuronal development and plasticity. *Mol Neurobiolog.* 2006; 34:205-219.

Jefferies H, Fumagalli S, Dennis PB, Reinhard C, Pearson RB and Thomas G. Rapamycin suppresses 5'TOP mRNA transaltion through inhibition od p70 S6K. *EMBO J*.1997; 16:3693-3704.

Jefferies H, Reinhard C, Kozma SC, Thomas G. Rapamycin selectively represses trasnlation of the polypyrimidine tract mRNA family. *Proc Naltl Acad Sci USA*. 1994; 91:4441-4445.

Kelleher RJ 3rd, Govindarajan A, Tonegawa S. Translational regulatory mechanisms in persistent forms of synaptic plasticity. *Neuron.* 2004; 44:59-73.

Kurien BT, Scofield RH. Western blotting. *Elsevier Methods*. 2006; 38:283-293.

Laemmli UK. Cleavage of structural proteins during assembly of the head bacteriophage T4. *Nature.*1970; 227:680–685.

Luo L. Rho GTPases in neuronal morphogenesis. *Macmillan Magazines Ltd*. 2000; 1:173-175.

Martin KC, Barad M, Kandel ER. Local protein synthesis and its role in synapse-specific plasticity. *Curr Opin Neurobiol.* 2000; 10:587-592.

Mayer ML. Glutamate receptor ion channels. *Current Opinion in Neurobiology*. 2005; 15:282-288.

Nave BT, Ouwens M, Withers DJ, Alessi DR, Shepherd PR. Mammalian target of rapamycin is a direct target for protein kinase B: identification of a convergence point for opposing effects of insulin and amino-aciddeficiency on protein translation. *Biochem J.* 1999; 344:427– 431.

Nobes CD, Hall A. Rho, Rac, and Cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipoida, and filopodia. *Cell.* 1995; 81:53–62.

Pause A, Belsham GJ, Gingras AC, Donze O, Lin TA, Lawrence JC Jr, Sonenberg N. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator function. *Nature.* 1994; 371:762-767.
Sandkuhler J. Understanding LTP in pain pathways. *Mol Pain*. 2007; 3: 9.

Sandsmark DK, Pelletier C, Weber JD, Gutmann DH. Mammalian target of rapamycin: Master regulator of cell growth in the nervous system. *Histol Histopathol.* 2007; 22: 895-903.

Sanes JR, Lichtman J W. Development of the vertebrate neuromuscular junction. *Annu Rev Neurosci*. 1999; 22: 389–442.

Smith PK, Krohn RI, Hermason GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. Measurement of protein using bicinchoninic acid. *Anal Biochem.* 1985; 150:76-85.

Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol.* 1975; 98:503-517.

Sugihara K, Nakatsuji N, Nakamura K, Nakao K, Hashimoto R, Otani H, Sakagami H, Kondo H, Nozawa S, Aiba A, Katsuki M. Rac1 is required for the formation of three germ layers during gastrulation. *Oncogene*. 1998; 17:3427–3433.

Stucky Cheryl L, Gold Michael S, Zhang X. Mechanisms of pain. PNAS. 2001; 98, 11845-11846.

Tessier-Lavigne M, Goodman CS. The molecular biology of axon guidance. *Science.* 1996; 274:1123–1133.

Thermo scientific: http://www.piercenet.com/products/browse.cfm?fldID=02020105.

Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA.* 1979; 76: 4350–4354.

Van Aelst L, Cline HT. Rho GTPases and activity-dependent dendrite development. *Curr Opin Neurobiol*. 2004; 14:297-304.

Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling networks. *Genes Dev*. 1997; 11:2295–2322.

Wang H, Tiedge H. Translational control at the synapse. *Neuroscientist*. 2004; 10:456-466.

Wiechelman K, Braun RD, Fitzpatrick JD. Investigation of the bicinchoninic acid protein assay: Identification of the groups responsible for color formation. *Anal Biochem.*1988; 175:231-7.

Woolf CJ, Salter MW. Neuronal plasticity: increasing the gain in pain. Science. 2000; 288:1765-1769.