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NANOTECHNOLOGIES AND PHAGE DISPLAY:

SELECTION OF PEPTIDES MIMICKING BDNF

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ABSTRACT

Brain derived neurotrophic factor is a neurotrophin of vital importance for neuron survival.

It may therefore be of great help to people affected by sensorineural hearing loss and cochlear implant users, as it has already been demonstrated that it maintains neuronal cells alive and this proved to be necessary for the device to work.

The issue with its clinical application is related to its large size that makes it difficult to deliver.

In this thesis I took on one of the tasks of a European project.

The objective of the Nanoear project is the creation of multifunctional nanoparticles that could be used as drug carriers for a target delivery in the inner ear.

The final aim is to find a substitute for BDNF protein, able to mimic its action but small enough to be attached to the nanoparticles and carry them to their target in the inner ear.

The best method to obtain small peptides with these features is the phage display. This technique allows selection of a pool of specific peptides binding specifically to an immobilized target.

In collaboration with an English company involved in the project as well, we worked to set all the parameters in order to make the phage display for this project feasible.

As our goal was attaining BDNF mimetic peptides our target had to be its functional receptor, TrkB.

Starting from a polyA+ RNA library from adult human brain we could get the genes coding for TrkB and BDNF and follow all the process up to the expression of the two proteins in bacteria and in eukaryotic cells, as well.

In order to include into nanoparticles the most specific peptides many approaches for the phage display were taken into consideration and experimented on.

This technique was in fact performed in vivo on guinea pigs and all the parameters were set for different trials in vitro either on eukaryotic cells or on the specific binding domain purified from bacteria.

Therefore the results and the data obtained in this study can lead to a new generation of drug with great clinical potential.

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1 INTRODUCTION

Hearing impairment is a full or partial decrease in the ability to detect or understand sounds which can be caused by a wide range of factors.

It has a huge impact on the population as it affects more than 560 million people worldwide (80 million just in Europe) and about one sixth of the population of Italy.

According to the World Health Organization it is very likely that by 2050, hearing loss will rise to 900 million worldwide.

The incapability of hearing has disastrous effects on people's social and working life because it eventually leads to the isolation of the individual by limiting his skill to communicate with the rest of the world.

Those born with a severe hearing loss have very limited chance of learning to speak and develop oral language because of the complete absence of sound input and this could eventually lead to poorer educational outcomes.

As for the adults who develop a severe hearing loss, losing vocational skills and social interaction proves to be traumatic.

Furthermore, individuals with a severe to profound hearing loss often exhibit signs of paranoia and depression (Maillet et al, 1995).

Subjects with hearing impairment are likely to be more vulnerable to depression but also physical and mental illnesses (Van Oyen et al, 2001).

Moreover hearing impairment will represent an increasing challenge to public health as lifespan increases and the general population ages.

There are different types of hearing loss according to the part that is actually damaged.

The ear consists of three parts: the outer ear, the middle ear and the inner ear.

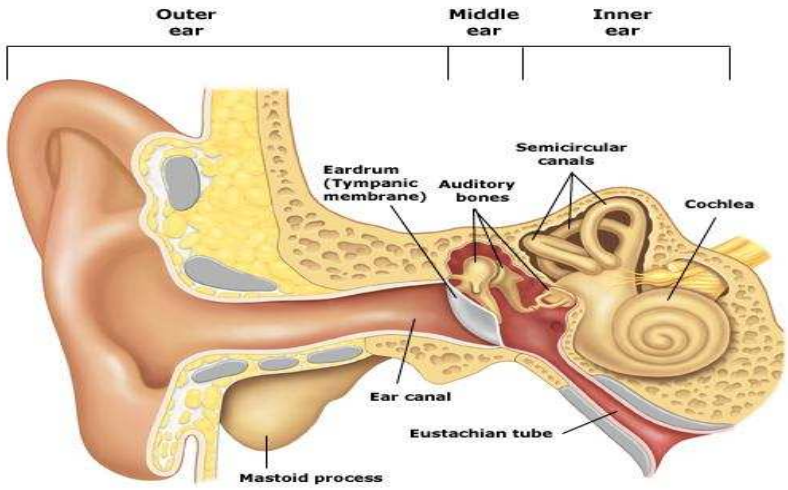


Figure 1.1 *scheme of the ear (image from www.utdol.com)*

Based on the area affected we can then refer to conductive or sensorineural hearing loss.

Conductive hearing loss is caused by a mechanical problem in the ear canal or the middle ear that blocks the conduction of sound. It can be easily corrected with hearing aids.

Sensorineural hearing loss is caused by damage to the inner ear, auditory nerve or auditory nerve pathways in the brain.

Cochlear hearing loss occurs when the structures inside the cochlea are damaged. This can occur in several different ways, such as exposure to ototoxic chemicals or intense sounds, infection, metabolic disfunctions, allergies, or as a result of genetic factors (Waters C, 1999).

The damage may extend beyond the cochlea to include higher centres in the auditory pathway. The more general term of sensorineural hearing loss

is used to describe a hearing loss where damage occurs to either the cochlea or neural structures.

About the 90% of the people with hearing impairments present damage to the cochlear structures and one out of a thousand children is affected by a congenital sensorineural deafness.

In contrast with other species which can produce or regenerate cells (Stone et al, 2007) involved in hearing throughout their life, mammalians hair cell when deteriorated are lost thus creating an irreversible situation.

1.1 The cochlear implant

While patients with a partial hearing loss could be helped with hearing aids, which just amplify the loudness of sounds, in cases where the auditory system is more profoundly damaged and the functionality of the organ is compromised the only possible treatment is the cochlear implant: regardless of how amplified the sounds are they will not be perceived.

Usually in patients with a severe hearing loss a portion of the auditory nerve cells is still functional, and cochlear implants are meant to electrically stimulate those neuronal cells, thus bypassing the damaged parts of the auditory system.

In cochlear implants, also called bionic ear, the damaged structures are replaced with a wire that is implanted in the cochlea.

The hearing process is stimulated when sound waves are perceived by a microphone that hangs over the back of the ear, then sounds are passed via a thin wire to a speech processor that amplifies and filters sounds before converting it into digital signals.

These are sent back via the same wire to a transmitter which in turn sends radio signals to a receiving unit embedded under the scalp. This stimulates the wire implanted in the cochlea enabling it to send clear signals to the auditory nerve.

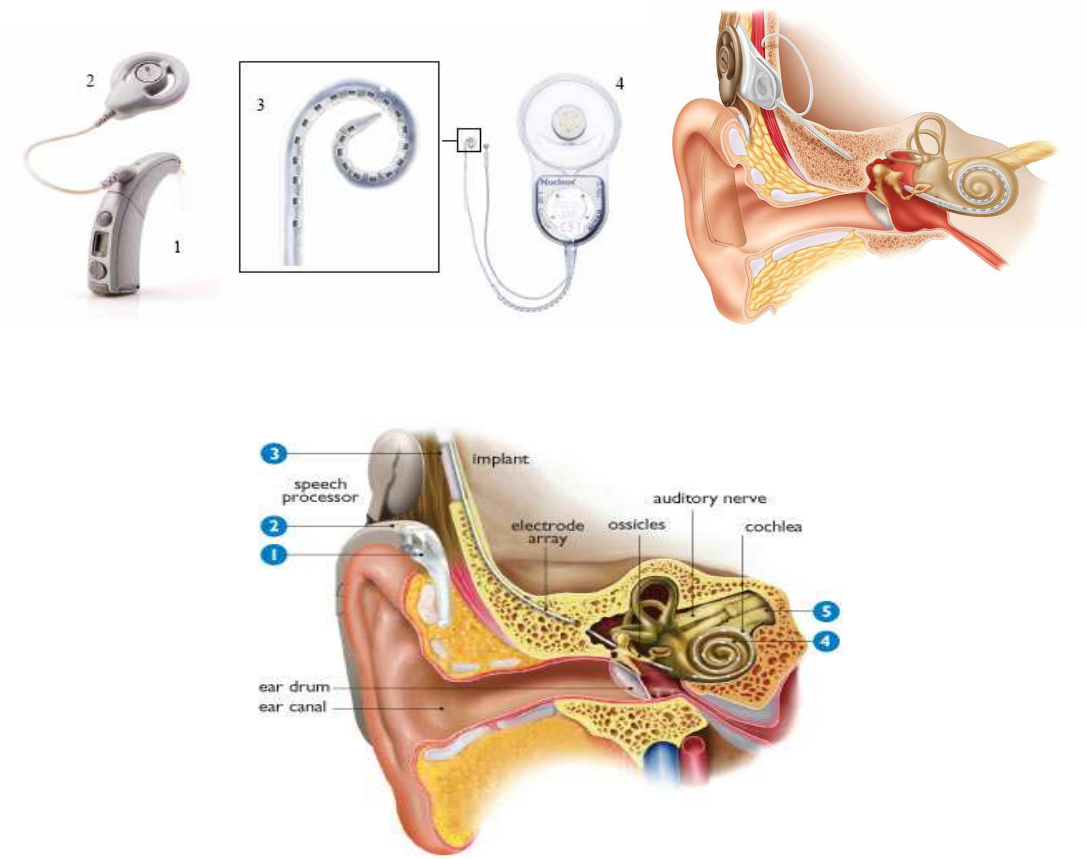


Figure 1.2 *The cochlear implant (images taken from Cochlear and Medel websites)*

Although the surgery permanently damages the cochlea the implants can greatly improve hearing, even in people who are profoundly deaf and nowadays many patients can benefit from this system and hear again.

Implant recipients have shown great improvements after getting the bionic ear, as many studies demonstrated (Oh et al., 2003).

Moreover Blamey et al in 1996 could prove ongoing improvements in patients performances were related to the duration of implant use.

Therefore the use of cochlear implants really is a great benefit for deaf people as they can hear again, their tinnitus usually decreases and they can get their confidence back which helps improving on their conditions.

Children implanted have especially shown great achievements in particular when it comes to learning to speak and so in their conversational fluency (Rubinstein, 2002; Tye-Murray, 2003) which helped them get better educational and vocational chances.

The prosthesis however, has sometimes proven unsatisfactory because of the poor perception/discrimination of words or for aesthetic reasons.

Many factors can determine great differences in individual outcomes, though, such as the age at onset of deafness, the age at implantation, the duration of this condition, the extent of residual hearing and the experience with the implant (Tong et al, 1988).

This means that many improvements can still be done on the bionic ear in order to enhance its performance and allow deaf people to have better hearing capabilities (Wilson et al 2003).

Several studies were brought on to enhance the efficacy of the cochlear implant either on the electronic device or on the possibility of using a combined pharmacological strategy to halt the ongoing and irreversible degeneration in the damaged auditory system.

Indeed prosthesis function depends on survival and electrical excitability of the cochlear neurons (Nadol JB et al, 1989) but trauma and loss of cochlear inner hair cells causes a series of events that result first in the retraction of the peripheral processes of the auditory nerve, scar formation in the organ of Corti, and over the course of months to years the loss of auditory nerve cell bodies (spiral ganglion cells).

Degeneration of the auditory nerve occurs after lesions of its peripheral target field (organ of Corti), specifically, including loss of inner hair cells (IHCs).

But a local treatment of the cochlea may enhance survival of auditory neurons.

Several strategies have been taken into consideration in trying to ameliorate or prevent this pathological condition.

The mechanism of cell death in the cochlea has been studied showing its two possible pathways: necrotic cell death mediated by very loud noise, or apoptosis, mediated by the activation of the caspases, a cysteine protease family within the cells.

A pharmacological intervention on each of these two mechanisms: necrosis and apoptosis could provide the possibility to reduce and, in some cases, to prevent cochlear cell death.

Among the prevention strategies considered the use of antioxidants, glutamate receptor antagonists, neurotrophins (Shinohara et al, 2002; Gillespie et al, 2004) and NOS inhibitors were evaluated (Fetoni, 2005).

Many investigated the role of antioxidant agents in different models of peripheral hearing disorders and it resulted that antioxidants protect the cochlea from noise-induced trauma, as well as cisplatin and aminoglycoside exposure.

Protection of auditory sensory cells from one of the major ototoxic drugs, that is cisplatin, is carried out at the molecular level by three mechanisms: prevention of ROS formation; neutralization of toxic products, and blockage of apoptotic pathways(Ciorba et al, 2008; Gabaizadeh et al 1997).

Nowadays even cell regeneration through genic therapy appears as a feasible approach (Edge AS, 2008).

Cochlear hair cells and supporting cells derive from the same precursor cells during the embryogenesis but then differentiate thanks to many genes and factors.

Recent experiments have given promising results concerning the processes related to inner ear damage. In the mammalian vestibular system, Kawamoto shown that hair cell regeneration can occur under certain circumstances (Kawamoto et al, 2009).

In the auditory system there is evidence of hair cell regeneration in newborn mice given explants from cochlea and in replacing the damaged hair cells by converting the supporting cells using the gene Math 1 (Kawamoto et al. 2003).

Math 1 gene has been shown to act as a "pro-hair cell gene" and it has a crucial role in the differentiation of hair cells from multipotent progenitors.

In 2005 Izumikawa et al. demonstrated that by using gene therapy the lost hair cells in mammals can regenerate and that hearing may be returned to the profoundly deaf mammalian ear.

New hopes were raised after observing a spontaneous recovery of hearing after noise trauma in humans which implies that restoring the hearing function may be possible.

Despite the fact that the mechanisms lying beneath the recovery have not yet been fully delineated, there is noteworthy evidence that cochlear damage induced by noise can be prevented by the application of different pharmacologically active substances.

A feasible way of delivering drugs to the inner ear is under investigation because not all the approaches known are clinically and technically possible.

Drugs can reach the cochlea either locally, from the middle ear over the round window membrane (RWM) through permeation, or directly injected through the RWM or the oval window or with an osmotic pump (passing through the lateral wall of the cochlea) or by systemic application (orally, intravenously or via the cerebrospinal fluid) .

A new approach that has been considered is the one involving the use of nanoparticles which can carry drugs and reach even the cells in the inner ear.

I joined a European project whose goal is to develop novel multifunctional nanoparticles (MFNPs), which are targetable to selected cell populations, biodegradable, and with a high efficacy of transfection by biological surface modification.

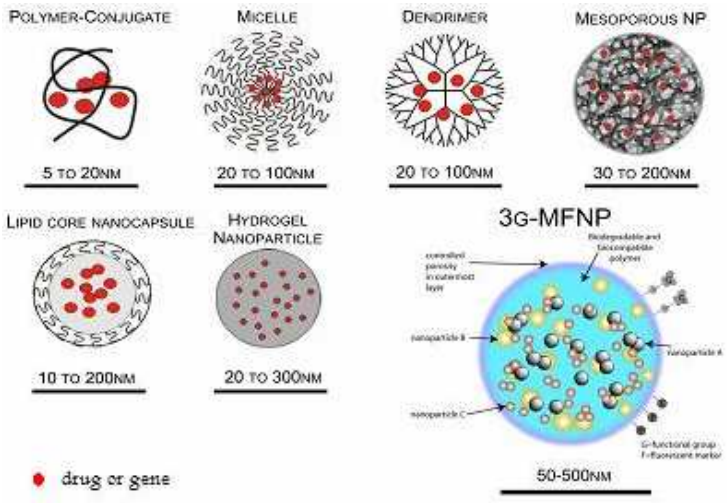


Figure 1.3 *Nanoparticles comparison (from Nanoear website)*

One of the “challenges” of this project is the specific cell targeting of the nanoparticles.

My particular aim is therefore to find targeting peptides mimicking the endogenous ligands, which can bind to specific receptors present on spiral ganglion cells, the TrkB receptors.

Peptides will be identified with the phage display screening. Such selection technique is also known as biopanning, and results in highly selective peptides binding to specified receptors, thus allowing accurate targeting.

1.2 Anatomy of the inner ear

The human auditory system comprises the ears and their connections to and within the central nervous system.

The auditory system can be divided into the outer, middle, and inner ears, the auditory nerve, and the central auditory pathways.

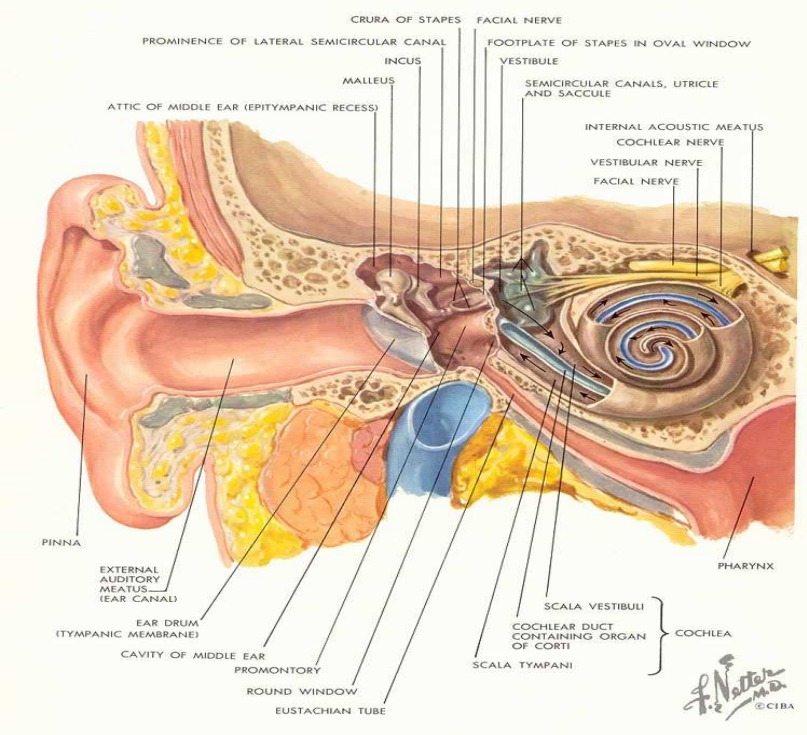


Figure 1.4 *Anatomy of the inner ear (picture from Netter)*

The oval window separates the middle from the inner ear.

This consists of sensory organs of hearing (the cochlea) and of balance (the semicircular canals, utricle, and saccule) whose name is vestibular system.

The cochlea forms a cone-shaped spiral with about 2 and ¾ turns. It is widest at the base and it narrows towards the apex.

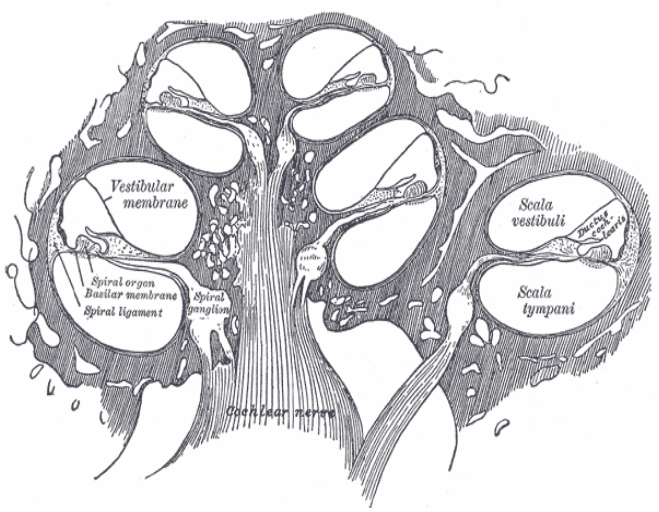


Figure 1.5 *The cochlea (image taken from Gray)*

Two membranes, Reissner's membrane and the basilar membrane, divide the cochlea along its length into three different chambers: scala media, scala vestibuli, and scala tympani.

The Organ of Corti, which is located on the basilar membrane in the scala media consists of a single row of inner hair cells (IHCs), three to five rows of outer hair cells (OHCs), different types of supporting cells, and the pillar cells forming the tunnel of Corti which divides the OHCs from the IHCs.

Most sensory hearing loss is due to poor hair cell function due to age, disease, damage or deformity of the cells.

Either cochlear or vestibular sensory cells are called hair cells because they are characterised by having a cuticular plate on top with a variable number of stereocilia bathing in the surrounding endolymph.

The cell body itself is instead located in the perilymph compartment.

These two types of cells, inner hair cells and outer hair cells, differ by their position, shape and pattern of the stereocilia.

There are approximately 3500 IHCs and 12 000 OHCs in the cochlea (Simpson, 2007).

IHCs are supported by phalangeal cells which hold their rounded base.

The outer hair cells are longer and smaller and are supported by Dieter Cells. Inner and outer hair cells differ from each other structurally, as well as functionally. The flask-shaped IHCs contain a huge system of tubulovessicular endoplasmic reticulum, Golgi apparatus and mitochondria (Lim, 1986).

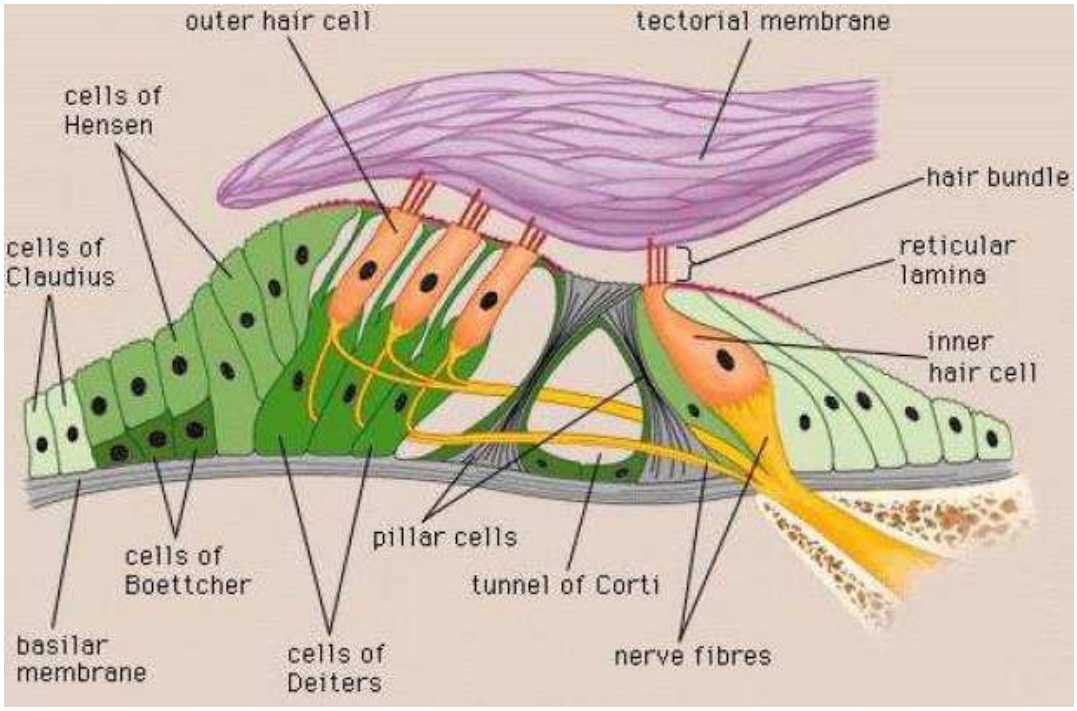


Figure 1.6 *The organ of Corti (taken from ourworld.compuserve.com)*

This is probably a hint of a high level of metabolic activity, which may be related to the transduction of mechanical to electrochemical energy.

Outer hair cells have a much more developed endoplasmic reticulum system and also contain contractile proteins as stereocilia and cuticular plates which enable them to actively contract and elongate (Slepecky et al, 1988).

On top of the cuticular plate present at the surface of both inner and outer hair cells there are the stereocilia whose number varies based on the tonotopy.

Each OHC contains as many as 150 stereocilia at the base and their number decrease to about 60 at the apex. Inner hair cells have circa 50-70 stereocilia each that differ from the OCHs' for the fact they do not change in number and they are thicker.

Another difference is that whereas OHC stereocilia are in contact with the overlying tectorial membrane, IHC stereocilia seem to have not to have contact with it.

1.3 Perception of sound in the auditory system

The tympanic membrane vibrates when it receives the sound waves travelling along the auditory canal.

Its transmitted vibrations then pass through the middle ear and the three ossicles, the malleus, incus and stapes, to reach the oval window at the base of the cochlea.

The vibrations delivered to the oval window cause the basilar membrane to move.

This movement in turn stimulates a sideways motion of the tectorial membrane and of the stereocilia in contact with it.

OCHs cilia bend because of a direct contact while the IHCs' will follow the fluid movement that comes with the tectorial membrane's vibration.

When stereocilia bend transduction pores on their surface open allowing access to a channel through which ions can pass.

Immediately after in the space between the hair cell and the afferent nerve a neurotransmitter is released causing an excitatory response along the auditory nerve.

This process transforms mechanical energy into electrical energy.

The signal is then carried through the central auditory system to the auditory cortex in the brain.

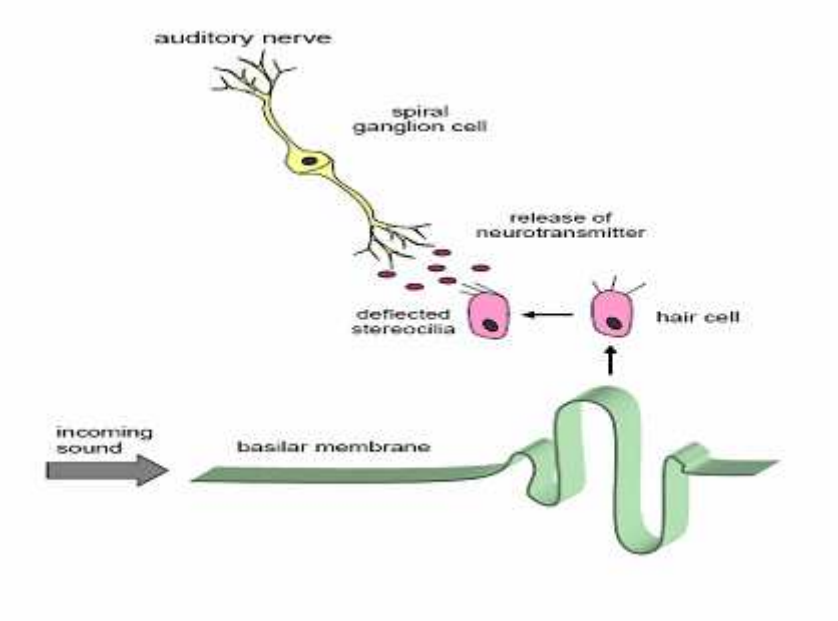


Figure 1.7 *Schematic representation of the way acoustic signals are passed from the basilar membrane to the auditory nerve (taken from Simpson A, 2007)*

Inner hair cells therefore are important as they translate mechanical movements in the cochlea into electrical stimuli and neural activity. They play a crucial role in conveying information about sound from the cochlea to higher auditory pathways via the afferent neurons.

Outer hair cells instead influence the response of the basilar membrane changing their length, shape and stiffness in response to sound, so they are just a part of an "active mechanism" in the cochlea.

Each hair cell has several contacts with neurons, finally ending in the spiral ganglion inside the modiolus.

Both types of hair cells are innervated by specific afferent and efferent systems forming a loop to and from the brainstem.

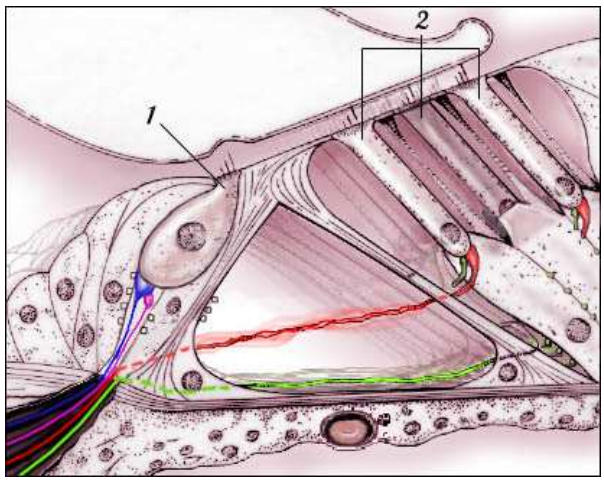


Figure 1.8 *Innervation of the hair cells (from Promenade 'round the cochlea website)*

The radial afferents (blue) and the lateral efferents (pink) innervate the inner hair cells; the spiral afferents (green) and the medial efferents (red) innervate the outer hair cells (Spoendlin, 1979, 1985 and 1988).

IHCs are synaptically connected to all type I spiral ganglion neurons forming the radial afferent system (blue) going to the cochlear nuclei.

The lateral efferent system (pink) coming from the small neurons in the ipsilateral lateral superior olivary complex creates a feedback control to the IHC/type I afferent synapse (Altschuler and Fex, 1996).

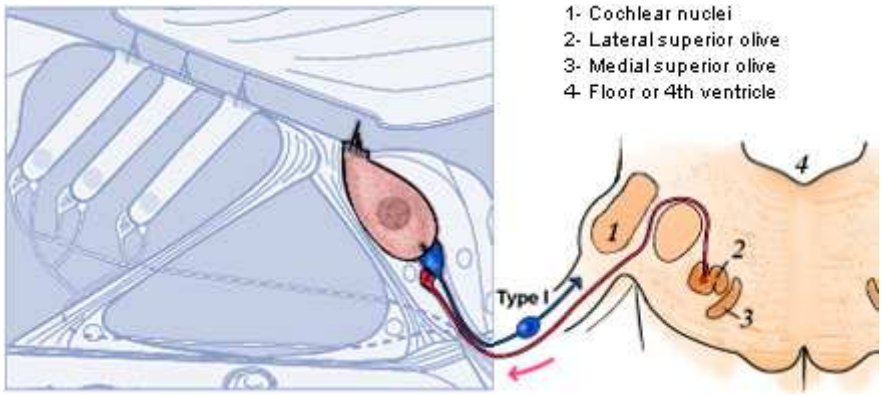


Figure 1.9*IHC innervation (from Promenade 'round the cochlea website)*

On average, one IHC is innervated by ten synaptic complexes from afferent dendrites.

These are made up of a radial afferent bouton (blue) and a lateral efferent ending (pink).

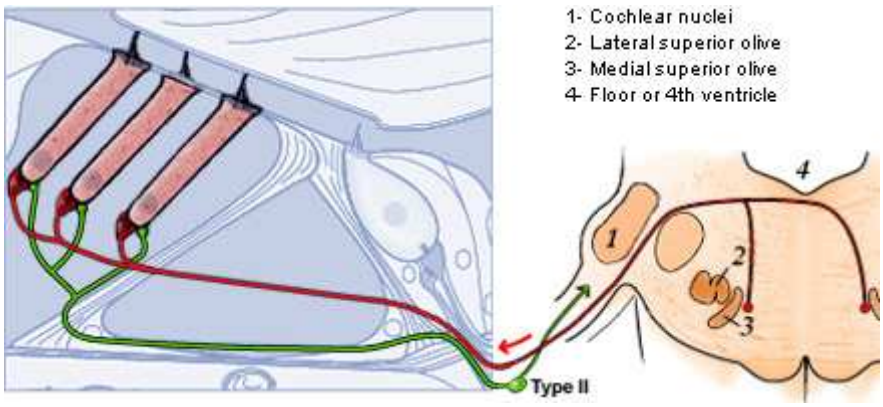


Figure 1.10 *OHC innervations (from Promenade 'round the cochlea website)*

The OHCs only synapse with a small number of small endings from type II spiral ganglion neurons, making up the spiral afferent system (green).

Large neurons of the medial efferent system (red), from both sides of the medial superior olivary complex, form axo-somatic synapses with the OHC (Pujol, 1994).

At the synaptic pole of the outer hair cells there are an afferent bouton (green) from a type II ganglion neuron and a medial efferent ending (red).

The spiral ganglion derives from the primitive otocyst. It differentiates very early, before the organ of Corti (Torres and Giraldez, 1998)

In man, it is composed of 30 to 35,000 bipolar neurons of two main types.

Large and myelinated type I neurons (around the 95% of the total) are connected to inner hair cells; small and unmyelinated type II neurons are

connected to outer hair cells. Both types have central axons delivering signals to the cochlear nuclei.

In mammals type I spiral ganglion neurons (95% of the ganglion neurons) have a single ending radially connected to IHCs (Berglund, Ryugo, 1987)

Type II small, unmyelinated neurons penetrate the organ of Corti and spiral before branching to contact about ten OHCs, generally in the same row (Brown et al, 1988).

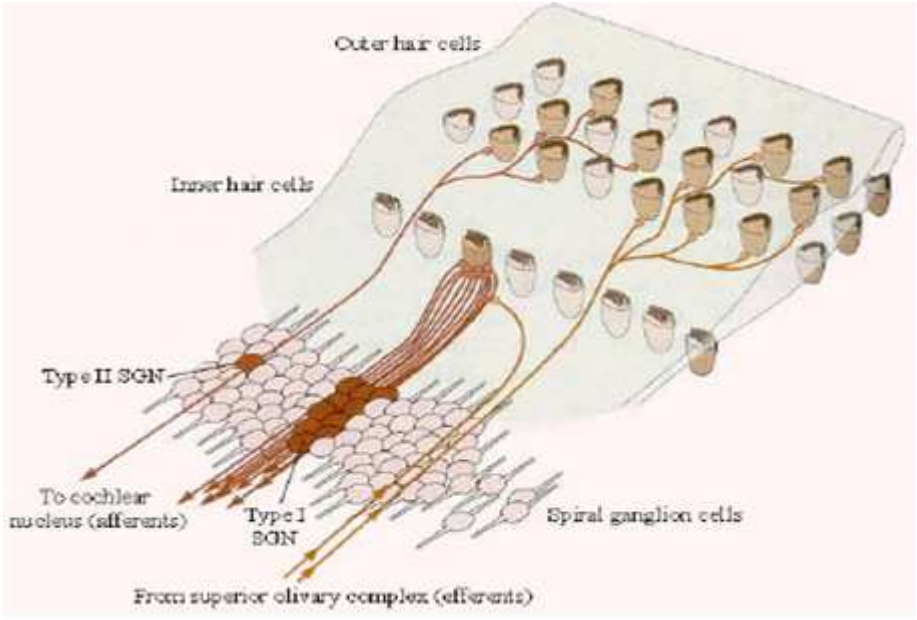


Figure 1.11 *Innervation of the cochlea (taken from Gillespie L, 2004)*

Cochlear implants can restore people's hearing bypassing the damaged areas of the auditory system but their efficacy depends totally upon SGN survival (Marzella and Clark, 1999)

Response to electrical stimulation by neurons is highly affected by loss of dendrites, demyelination of axons and soma and shrinkage of neurons themselves (Leake and Hradek, 1988).

Patients could benefit from the cochlear prostheses just if the SGNs and the auditory nerve are alive and functional and the success of the device depends almost entirely upon the survival of these structures.

Many studies on animals have shown that the ongoing degeneration of the neural cells close to the implant, could severely compromise the effects of the hearing device.

Indeed Shepherd and Javel, 1997 and Hardie and Shepherd, 1999 revealed that in implanted animals ongoing SGN degeneration made perception of sound get worse.

Despite this fact the same effect on humans carrying a cochlear implant has not yet been demonstrated.

Ylikoski in the nineties remarked that SGNs could survive only if they maintain communication through synapses with their target and are supported by neurothrophins (generally expressed by hair cells).

As the neuronal degeneration in the inner ear apart from being irreversible is also progressive in time, a way to improve the prosthesis performances could be preventing this process from starting.

This could be achieved developing a pharmacological strategy able to halt or at least slow down neuronal degeneration in the inner ear, thus improving cochlear implants' performances too.

Moreover, while conflicting results have been reported about the survival effects of chronic electrical stimulation on SGNs, it has been proven that BDNF delivery in conjunction with electrical stimulation of the nerve through a cochlear implant in deaf animals may result in enhanced SGN survival (Serruto et al, 2003).

This could mean that allowing spiral ganglion neurons to live longer trying to promote their survival or blocking their degeneration may greatly

improve the hearing possibilities of cochlear implants recipients and thus have a great potential both clinically and commercially.

1.4 BDNF and the neurotrophic factors

Neurotrophins are a family of homologous protein that tightly regulate the development and maintenance of the vertebrate neural system (Fritzsch et al, 1997)

The neurotrophins, best known through the nerve growth factor (NGF) discovered by Levi-Montalcini in 1952, have six family members among vertebrates with a well-characterized molecular structure and an emerging evolutionary history.

They are: the Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3; also hippocampal-derived neurotrophic factor or HDNF), neurotrophin-4/5 (NT-4/5), neurotrophin-6 (NT-6) and neurotrophin-7 (NT-7) (Lewin and Barde, 1996).

They are small, basic homodimers consisting of two monomers, each of ~120 residues, their dimeric form cause the receptor dimerization during the interaction.

Neurotrophins share similar biochemical characteristics and are all synthesized as soluble precursor proteins, then modified by cleavage to mature forms, and secreted.

Neurotrophins have a quite similar genetic pattern as they all have a single 3’ coding exon and many upstream 5’ non coding exons separated by large introns (Liu et al, 2006).

Different transcription processes result in the generation of many distinct neurotrophins transcripts which eventually evolve to identical protein (fig.1.12).

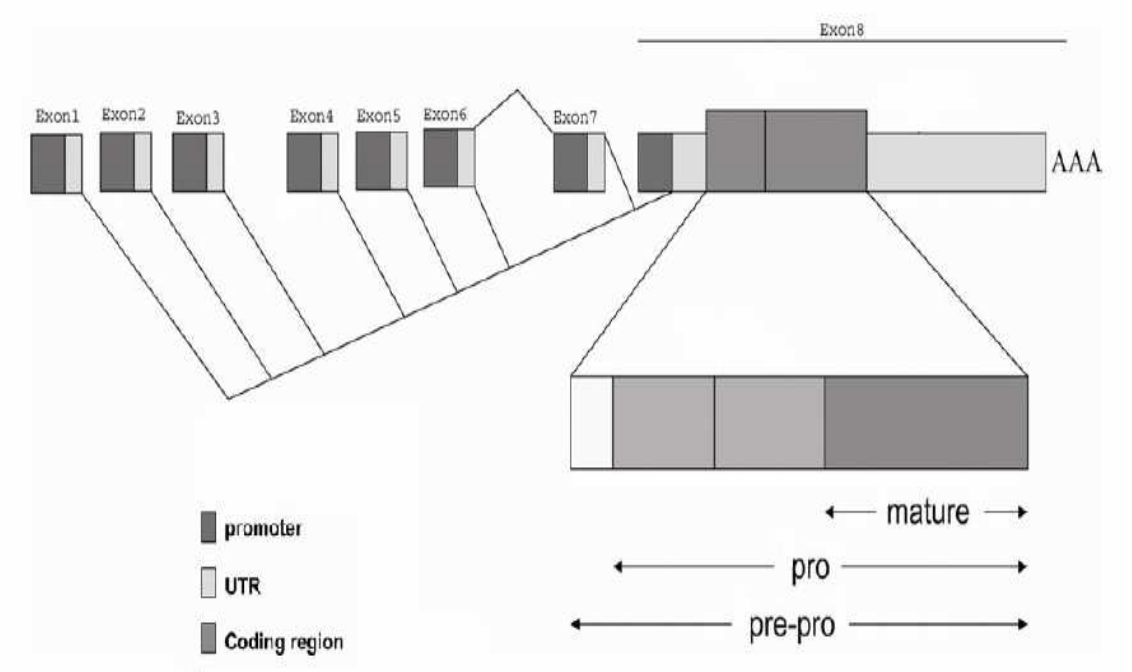


Figure 1.12 *Schematic picture showing BDNF gene and protein (adapted from Rantamaki T, 2006)*

These trophic factors are synthesized as 30-35 kDa pro-NTs (Seidah et al., 1996a; Seidah et al.1996b), which are then directed through the *trans*-Golgi apparatus to either constitutive (non-Ca2+-dependent) or regulatory (Ca2+-dependent) secretory vesicles (Lessmann et al,2003).

Pro-NTs are cleaved inside the cell by different types of enzymes as furin or proconvertases , by matrix metalloproteases (MMP; specifically MMP-3 and MMP-7) or by plasmin giving the mature proteins(Chao and Bothwell, 2002; Seidah et al, 1996 a,b).

Then they all form non-covalent-linked homodimers where each monomer is approximately 13-15 Kda.

About half of the amino acid residues in the mature protein are common to all neurotrophins and they all share six cysteine residues at identical positions.

This leads to the formation of the disulfide bridges and the similar tertiary structure of the molecules.

Neurotrophins are all basic proteins with isoelectric points above 9.0. Because of the characteristic formation of a double loop formed by two disulphide bonds, in which a third disulphide bond is inserted, they are classed as part of the Cysteine Knot Superfamily (Ultsch et al, 1999).

X-ray cristal structures revealed that the neurotrophins have a common structure: each monomer consists of seven beta strands (contributing to the three longitudinal antiparallel beta sheets) connected by three solvent-exposed hairpin loops (loops 1, 2 and 4) and a longer loop (number 3) and contains three disulfide bridges between six fully conserved cysteine residues arranged in a cysteine-knot motif, which is characteristic of this growth factor superfamily (Narhi, 1993; Zhongcai et al, 2003).

However in contrast to most of the other members of the superfamily, the neurotrophin monomers are arranged in the dimer in a parallel fashion, and are held together exclusively by non-covalent (mostly hydrophobic) interactions.

All of the neurotrophins show different distributions through the peripheral nervous system and central nervous system with different and overlapping specificity towards the populations of sensory neurons of both neural crest and neural placode origin (Shelton et al, 1995; Ernfors et al, 1992).

The expression of NTs in the brain is regulated during development.

Highest levels of BDNF mRNA, NGF mRNA and NT-3 mRNA in the adult brain are found in the hippocampus, with 50 times more BDNF mRNA being present than NGF mRNA.

NT-3 is most abundantly expressed in the perinatal brain whereas in the adult brain its expression is significantly reduced.

BDNF mRNA expression levels increase significantly in the first weeks after birth and even in the adult brain BDNF is highly expressed, in particular the presence of great quantities was noticed in the hippocampus and the cortex.

In the basal forebrain and hippocampus there are high levels of NGF but significant quantities can also be found in other areas of CNS, spinal cord included.

NT-4/5 is expressed most in the postnatal and adult hippocampus, neocortex and thalamic nuclei.

Overall, BDNF seems to be more widely expressed than the other neurotrophins in the adult CNS.

NT-3 is the most highly expressed in the immature CNS and its expression dramatically lowers down with maturation (Rantamaki T, 2006).

In the central nervous system, neurotrophins are generally expressed by neurons however glial cells express as well NT-3 and NT-4/5.

In situ hybridization has shown that two neurotrophins (brain-derived neurotrophic factor, BDNF; and neurotrophin 3, NT-3) and their specific high-affinity tyrosine kinase receptors (trkB and trkC) are expressed in the ear and its innervating sensory neurons (Pirvola et al, 1992).

Several studies on various neurotrophins and neurotrophin receptor mutants have established a crucial role for these two neurotrophins and their receptors for the embryonic survival of the afferent innervation of the ear (Schimmang et al, 1995).

Brain-derived neurotrophic factor (BDNF) was originally purified from pig brain by Barde et al. in 1982.

It is able to promote survival and differentiation of several populations of neurons, including mesencephalic dopaminergic neurons, motor neurons, and cholinergic neurons, and to protect them against neurotoxicity and ischemia (Binder and Scharfman, 2004).

BDNF plays an important role in the nervous system regulating neuron survival and differentiation during development and postnatally maintaining its functionality (Bibel and Barde, 2000).

Different BDNF mRNAs transcripts are localized and regulated differently indicating the biological significance of their existence.

BDNF and all the neurotrophins exert their action through interactions with neuron cell surface receptors, whose two families have been identified: the high affinity and specific Trk receptors ($K_D \sim 10^{-9}$), and a generic, lower affinity p75 receptor ($K_D \sim 10^{-11}$) (Barbacid, M.; 1994).

1.5 Neurotrophin receptors

Neurotrophins bind and activate two different types of transmembrane receptors: a high affinity one, which mediate their actions and a common low affinity one, whose function is still not completely clear, p75NTR.

The Trk (tropomyosin related kinase) family consist of high-affinity

(~10^{-11}M dissociation constant) ligand-selective receptors.

Despite the high similarity every neurotrophin is specific for a particular receptor: NGF binds primarily to TrkA, while BDNF and NT-4/5 to TrkB and NT-3 to TrkC.

Probably NT-4/5 and BDNF interact with TrkB in distinct domains which gives BDNF a higher affinity to TrkB than NT-4/5 (Klein et al, 1991-1992).

NT-3 can also bind and activate TrkA and TrkB receptors, but with a much lower efficacy (Ip et al, 1993; Ryden and Ibanez, 1996).

Both BDNF and neurotrophin NT4/5 induce TrkB autophosphorylation and so its activation (Ip et al, 1992; Fandl et al 1994).

Tropomyosin related kinase receptors exist in different isoforms: *trkA* gene codes for two tyrosine kinase containing TrkA isoforms with just a difference of 6 aminoacids.

trkB gene instead code for more than 8 different transcript products ranging in size from 0.7 to 9.0 kb which finally result in at least two types of receptors: full-length and truncated TrkB (Barbacid, 1994).

Their extracellular regions are identical but truncated receptors lack the entire catalytic tyrosine kinase domain.

Localizations of NTs and their receptor genes in human genome		
chromosome	protein	References
1q21-q22	TrkA	(Weier et al, 1995)
9q22.1	TrkB	(Nakagawara et al, 1995)
15q25	TrkC	(Valent et al, 1997)
1p22	NGF	(Francke et al, 1983)
11p13	BDNF	(Maisonpierre et al, 1991)
12p13	NT-3	(Maisonpierre et al, 1991)
19q13.3	NT-4/5	(Berkemeier et al, 1992)
17q21-q22	p75NTR	(Huebner et al, 1986)

Two truncated TrkB receptors have been found in rodents, TrkB.T1 and TrkB.T2, of which TrkB.T1 is largely expressed in their brain (Baxter et al, 1997), the same happens in human (Middlemas et al, 1991).

Another member, TrkB.Shc, has been subsequently described in the human brain (Stoilov et al., 2002), but the two species isoforms' are quite similar.

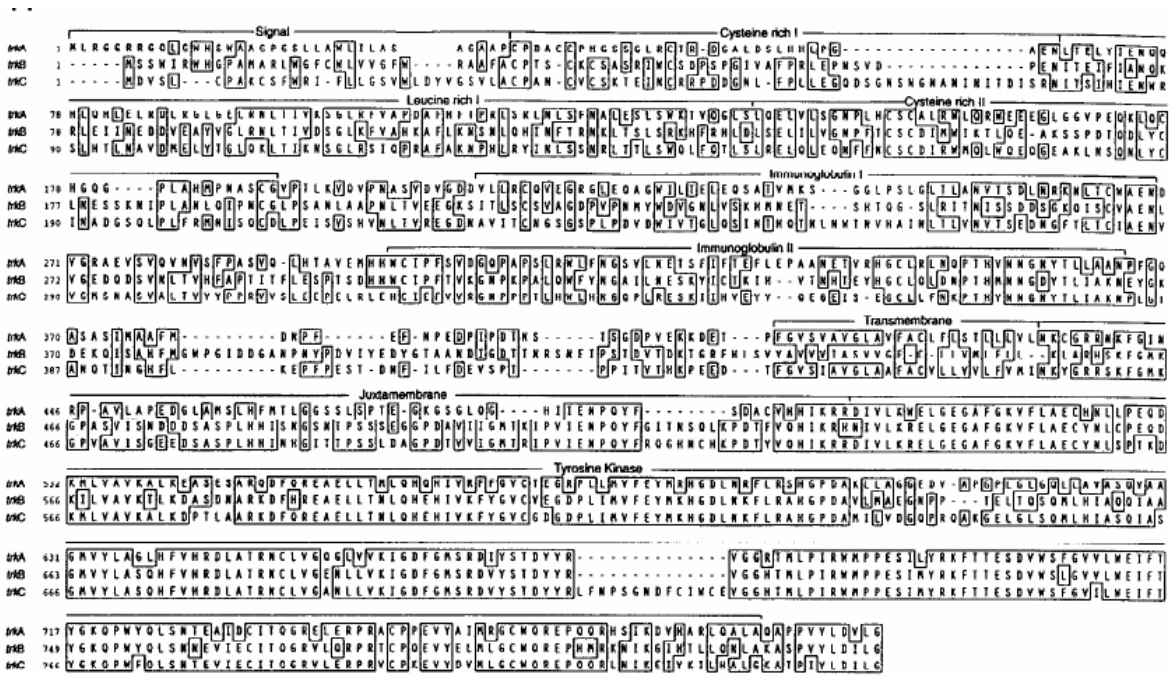


Figure 1.13 *Alignment of peptide sequences of three human trks (Shelton et al, 1995)*

Neurotrophins actions are mediated by transmembrane full length Trk receptors possessing tyrosine kinase activity.

Specifically NGF binds and activates primarily TrkA, BDNF and NT-4/5 TrkB and NT-3 TrkC (fig.1.14).

There are also truncated TrkB and TrkC receptors completely lacking the intracellular region and so the enzymatic domain, which is the signal transductor.

All the neurotrophins also bind with equal affinity to a common low-affinity (~10^{-9}M dissociation constant) receptor, called p75NTR (Squinto et al., 1991).

It was demonstrated that p75NTR (belonging to the tumour necrosis family receptor super family) has an important role in mediating Trk receptor signalling and, in the absence of neurotrophins, it may cause neuronal apoptosis.

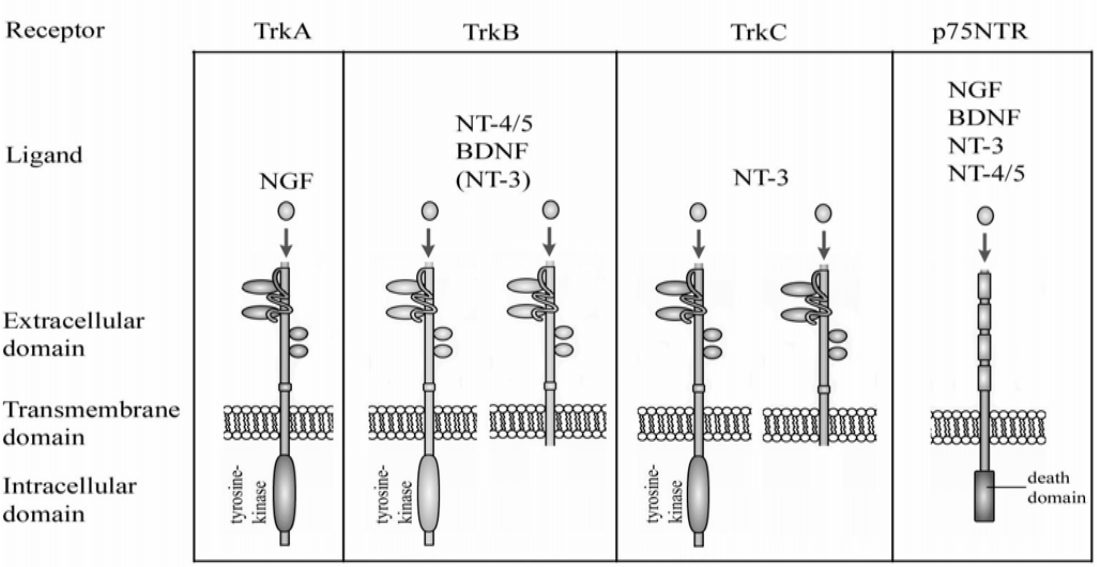


Figure 1.14 *The receptors for neurotrophins (taken from Rantamaki T., 2006)*

Figure 1.15 *TrkB protein (from DBB)*

In the central nervous system of the adults, TrkB and TrkC receptors can be found in the hippocampus and throughout the cortex, while TrkA is expressed mainly in the basal forebrain (Huang and Reichardt, 2001).

The full-length Trk receptors are predominantly of neuronal origin, truncated forms of these receptors are often expressed by non-neuronal cells like glia instead.

Fritzsch in 1999 could show the differences in the expression patterns of the full-length and truncated TrkB receptors during development.

Full-length TrkB in particular is expressed already perinatally while truncated TrkB starts to be expressed a few weeks later.

Binding of BDNF to TrkB initiates dimerization and trans-autophosphorylation of the tyrosine residues in the intracellular domain of the receptor (Reichardt, 2006).

These phosphotyrosine residues act as docking sites for effector proteins that will trigger downstream signaling cascades, leading to various responses as the activation of protein kinase cascades, Ca^{2+} mobilization, and gene expression.

BDNF activates TrkB receptors in the same or neighboring cells to promote their survival and may also enhance synaptic plasticity.

Although TrkB levels change during development and show cell-specific expression patterns, not much is known about the mechanisms that regulate TrkB expression.

At least four isoforms of trkB are produced by alternative splicing of the primary transcripts of the TrkB gene.

Of these, only the full-length isoform, which contains an intracellular tyrosine kinase domain, is known to be capable of mediating BDNF signaling.

The other three truncated isoforms (T1, T2, and T-shc), which lack the intracellular kinase domain but possess the same extracellular BDNF binding domain as full length receptors, can also be generated by alternative splicing.

T1 is prominently expressed in the brain and probably acts as a dominant negative inhibitor of BDNF signaling by forming heterodimers with full-length trkB.

Ten conserved phosphorylation-regulated tyrosine residues are present in Trk receptors intracellular region.

After binding the dimeric ligand, Trk receptors dimerize itself and become autophosphorylated *in trans* in the intracellular catalytic domain.

Moreover autophosphorylation at the Trk kinase domain may induce the phosphorylation of other tyrosine residues in the receptor.

The phosphorylated tyrosines serve as docking sites for many of the intracellular enzymes and adaptors containing SH2 (Src homology) or PTB (phosphotyrosine binding domain) region (Huang and Reichardt, 2003).

Following the phosphorylation of the tyrosine residue Y816 phospholipase-Cγ1 (PLCγ1) is activated (Middlemas et al, 1994).

Hydrolysing the phosphatidyl inositides (PI) of the membrane, activated PLCγ1 can generate inositol tris-phosphate (IP3) and diacylglycerol (DAG), two typical intracellular signalling messengers.

IP3 increases $[Ca^{2+}]i$ by stimulating the release of Ca^{2+} from intracellular compartments so activating the signalling of a the intracellular Ca^{2+}-dependent molecules.

DAG activates DAG-dependent protein kinase-C isoforms, which in turn can regulate the ERK (extracellular signal-regulated kinase) signalling cascade.

Phospho-Y515 is the docking site for Shc adaptor protein, which eventually activates Ras-ERK and PI3k-Akt cascades.

BDNF effects are exerted: Ras-ERK pathway regulates neuronal differentiation and PI3k-Akt following events are important for cell survival and maintenance (Reichardt, 2006).

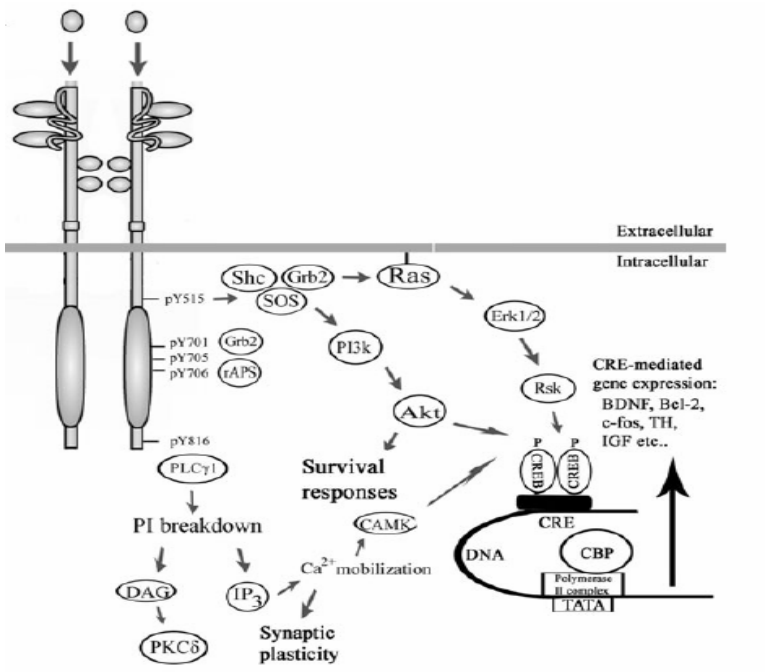


Figure 1.16 *Major intracellular signalling cascades regulated by neurotrophins. (taken from Rantamaki T, 2006)*

All Trk receptors are homologous in sequence, each comprising about 800 amino acids with the amino terminal ~400 residues forming the extracellular portion of the receptor where binding of the neurotrophins takes place.

They are multidomain transmembrane proteins that consist of an extracellular ligand binding domain containing two cysteine rich regions separated by a leucine rich domain (Haniu et al, 1995), a transmembrane region, and a intracellular part with the juxtamembrane domain that includes a Shc binding site, a tyrosine kinase domain and a tail region containing a PLC-γ binding site.

Although the precise three-dimensional structure of the entire extracellular fragment is not known, recognized sequence motifs have led to division of these receptors into five domains: one 3 tandem leucine-rich motif flanked by two cysteine cluster regions (domains 1-3,d1-d3) followed by two immunoglobulin domains (d4 and d5) of the C2 type belonging to the Immunoglobulin superfamily (Banfield et al, 2001).

Trk B is one of the most widely distributed NTRs in the brain, whose expression is high in such areas as the neocortex, hippocampus, striatum, and brainstem.

The second class of trk B receptors has the same extracellular and transmembrane domain as the other but contains a short cytoplasmic domain of 23 amino acids, lacking the entire kinase catalytic region.

Although, the Trk B locus is widely expressed in the central nervous system and peripheral nervous system, not all the trk B transcripts are distributed equally.

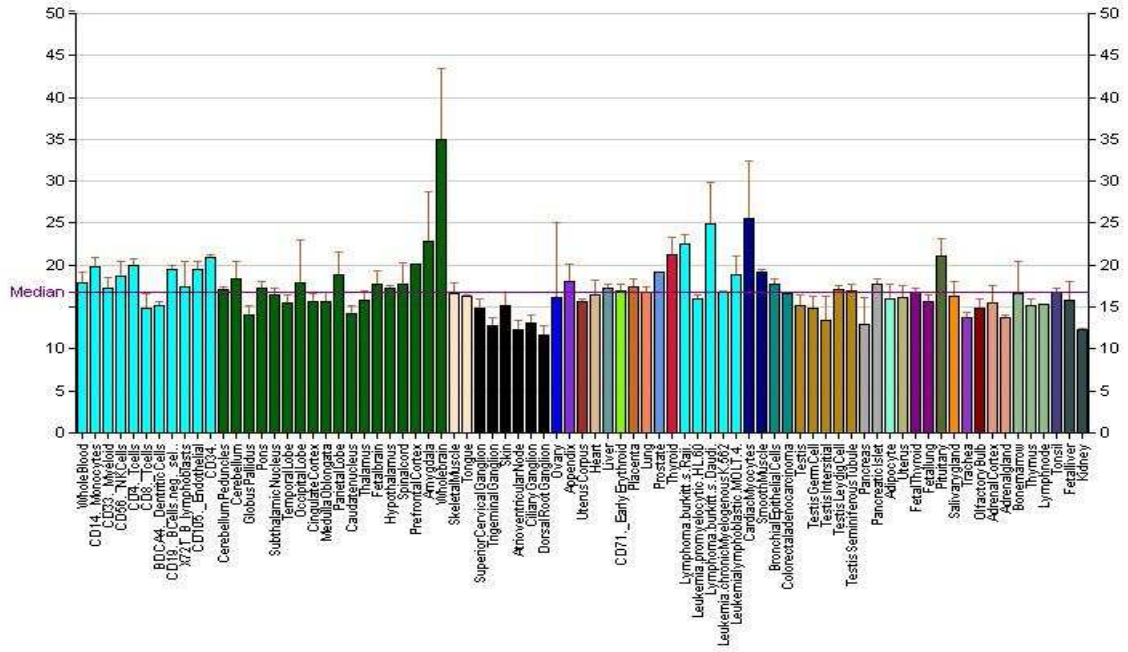


Figure 1.17 *TrkB expression (based on data from Su AI, Wiltshire T, Batalov S, et al (2004). "A gene atlas of the mouse and human protein-encoding transcriptomes". Proc. Natl. Acad. Sci. U.S.A. 101 (16): 6062–7.*

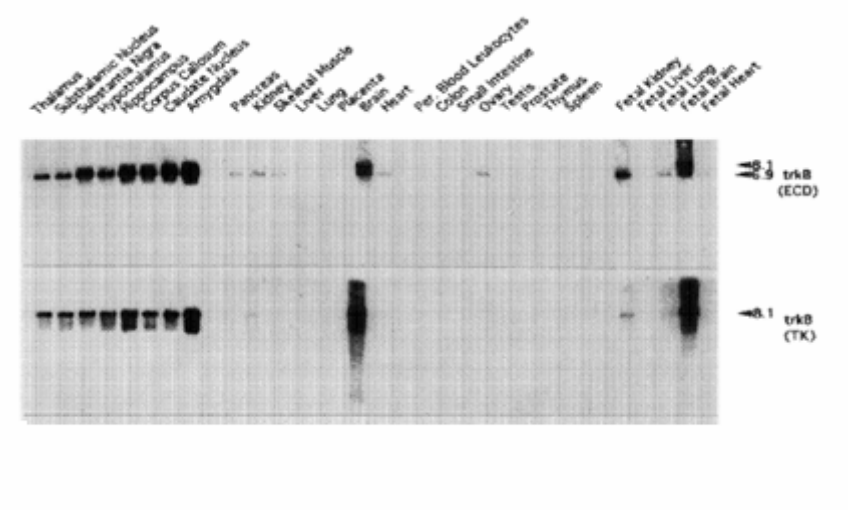


Figure 1.18 *TrkB expression (Shelton et al., 1995)*

2. MATERIALS AND METHODS

2.1 cDNA LIBRARY

To get the cDNA library we used the Creator SMART cDNA Library Construction Kit by Clontech Laboratories Inc (Takara bio).

This way we generated a full length cDNA Library from 1 μg of poly A+ RNA, as shown in fig. 2.1.

The library construction employed long-distance PCR (LD PCR; Barnes et al., 1994) for generating full-length cDNA.

In this protocol a modified oligo(dT) primer (CDS III/3' PCR Primer) primed the first-strand synthesis reaction, and the SMART IV Oligo served as a short, extended template at the 5' end of the mRNA.

When the RT reaches the 5' end, the enzyme's terminal transferase activity adds a few additional nucleotides, primarily deoxycytidine, to the 3' end of the cDNA.

The SMART IV Oligo, which has an oligo (G) sequence at its 3' end, base-pairs with the deoxycytidine stretch, creating an extended template.

RT then switches templates and continues replicating to the end of the oligonucleotide.

The resulting full length ss cDNA contains the complete 5' end of the mRNA, as well as the sequence complementary to the SMART IV Oligo, which then serves as a universal priming site (SMART anchor) in the subsequent amplification by LD PCR (Chenchik et al., 1998).

Only those ss cDNAs having a SMART anchor sequence at the 5' end can serve as a template and can be exponentially amplified.

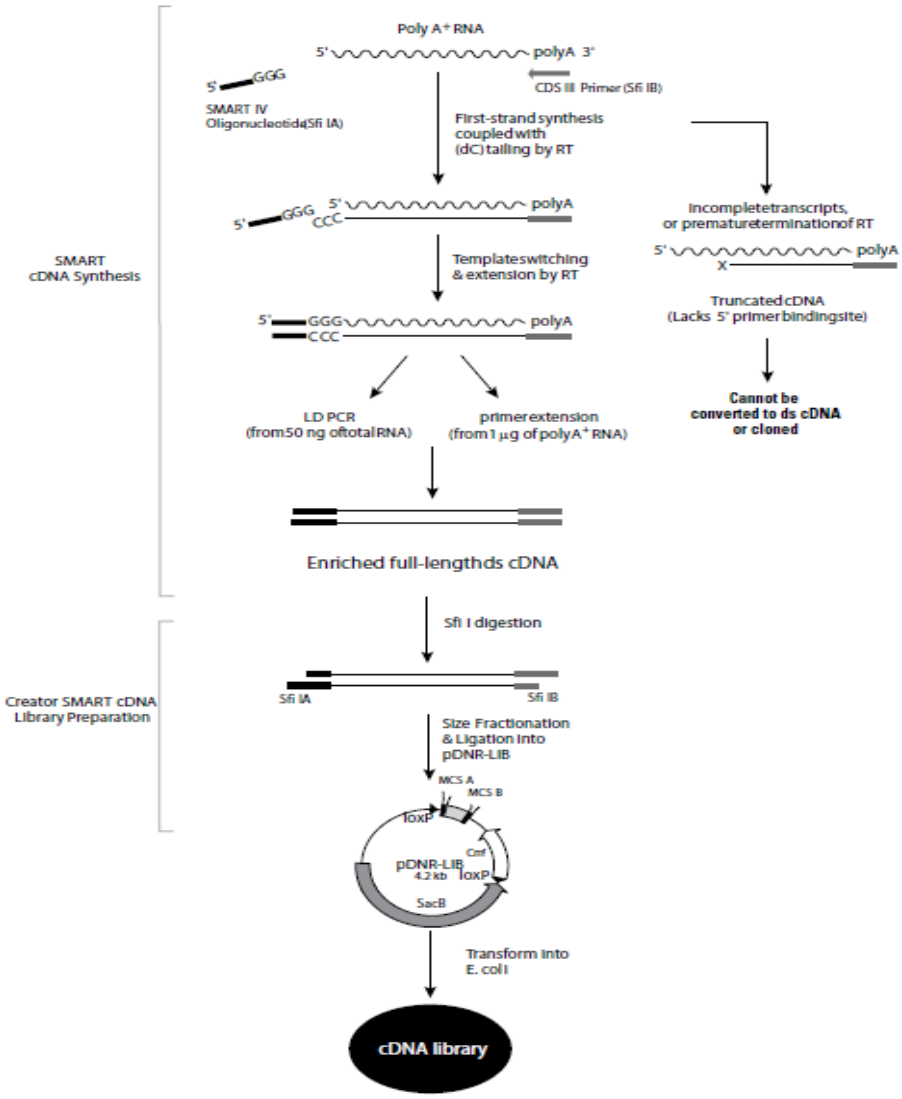


Figure 2.1 *Getting a cDNA library from RNApolyA+ (from Clontech kit)*

Incomplete cDNAs and cDNA transcribed from polyA+ RNA lack the SMART anchor so are not amplified. Thus, contamination by genomic DNA and poly A– RNA is eliminated.

This selective amplification allowed me to construct a cDNA library with a high percentage of full-length clones using a microgram of poly A+ RNA.

The next step in library construction was primer extension (Sambrook J. & Russell, D.W., 2001).

Similar to the first-strand cDNA synthesis described above, the CDS III/3' primer was used to prime the first-strand reaction, and the SMART IV Oligo served as a short, extended template at the 5' end of the mRNA. After first-strand cDNA synthesis, the primer-extension step generated full-length, ds cDNA.

2.1.1 FIRST-STRAND cDNA SYNTHESIS

The following reagents were combined in a sterile microcentrifuge tube:

- 1 µl RNA sample (1 µg poly A+RNA)
- 1 µl SMART IV Oligonucleotide (12 µM)

5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGG-3'

- 1 µl CDS III/3' PCR Primer (12 µM)

5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30N–1N-3'

(N = A, G, C, or T; N–1 = A, G, or C)

deionized H_2O was added to a total volume of 5 µl.

The tube was spun briefly in a microcentrifuge and left incubating at 72ºC for 2 min, then it was cooled down on ice for 2 min and spun again to collect the contents at the bottom.

To each reaction tube were added:

2.0 µl 5X First-Strand Buffer

- 250 mM Tris (pH 8.3)
- 30 mM MgCl2
- 375 mM KCl

1.0 µl DTT (dithiothreitol, 20 mM)

1.0 µl dNTP Mix (dATP, dCTP, dGTP, dTTP; 10 mM each)

1.0 µl Super Script Reverse Transcriptase 2U/µl

to a total volume of 10.0 µl.

After mixing and pipetting ten times, the tube was incubated at 42ºC for 1 hr and placed on ice to terminate first-strand synthesis.

Then 1 µl of Sodium Hydroxide (25mM) was added to the tube (to degrade RNA).

And it was left on ice after a incubation at 68ºC for 30 min.

2.1.2 DOUBLE STRANDED cDNA SYNTHESIS BY PRIMER EXTENSION

Preheated PCR thermal cycler has to be at 95ºC.

11 µl First-Strand cDNA

35 µl Deionized H2O

50 µl 2X Taq pfu mix

2 µl 5' PCR Primer (12 µM)

5'-AAGCAGTGGTATCAACGCAGAGT-3'

2 µl CDS III/3' PCR Primer (12 µM)

5'-ATTCTAGAGGCCGAGGCGGCCGACATG-d(T)30N–1N-3'

were mixed together to a total volume of 100 µl

the content was gently mixed and collected by centrifugation at the bottom of the tube.

PCR reaction :

95ºC preheated

72ºC 10 minutes

95ºC 1 minute

3 cycles 95ºC 15 seconds; 68ºC 8 minutes

A 5-µl sample of the product was analyzed on a 1.1% agarose/EtBr gel, alongside a 1-kb DNA size marker. The ds cDNA appeared as a 0.1–9 kb

smear on the gel, with some bright bands probably corresponding to the abundant mRNAs for the brain.

2.1.3 TRK B AMPLIFICATION

Specific primers were designed, corresponding to the ATG and the TAG codon which represent in turn the start and the end of the gene:

PCR reaction:

F 5' ATGTCGTCCTGGATAAGGTGGC 3'

R 5' CTAGCCTAGAATGTCCAGGTAG 3'

2 X Taq polymerase mix*	50 μl
Template (cDNA library)	1 μl
F primer (100mM)	1 μl
R primer (100 mM)	1 μl
dd water	47 μl
Total	100 μl

Taq+ mix 2X

1 ml 10x buffer

(100mM Tris-Cl pH 7.6 at 25ºC, 100mM $MgCl_2$, 75mM DTT)

200 μl Pfu 5U/μl

30 μl cresol red dye

250 μl dNTPs mix 40mM (10mM each)

10% glycerol

Water up to 10 ml

2X PCR MIX 10 ml

200µl Taq 5U/µl

2,5 ml sorbitol 2 M

0,5 ml dNTPS 40mM

0,6ml MgCl2 50mM

2 ml Buffer 10X

(670mM Tris-Cl; 150mM $(NH_4)_2SO_4$; 0,1% tween20 pH 8.8)

0,8 ml DMSO

70 µl cresol red

Water up to 10 ml

PCR reaction

95ºC 3 minutes

3 cycles

95ºC 1 minute

55ºC 1 minute

72ºC 2 minutes

25 cycles

95ºC 30 seconds

60ºC 30 seconds

72ºC 2 minutes

Check on agarose gel 1.3%

After proving that the gene amplified was actually TrkB full length (2469), the same PCR reaction was performed using a Taq polymerase mixed with

a Pfu polymerase which possesses 3' to 5' exonuclease proofreading activity in order to have nucleotide-misincorporation errors corrected.

Then the polymerase-generated PCR fragment had to be treated with just Taq polymerase before being cloned into pGEM-T-easy vector in order to get a polyA overhang.

So the purified PCR product was mixed with the 2X Taq mix and left

at 95ºC 1 minute (denaturation step)

and at 72ºC for 10 minutes (elongation step).

An electrophoresis was performed and the band was excised from the gel, transferred to a tube with the binding buffer of the gel DNA purification kit (from Yorkshire Bioscience Ltd) and melted at 50-60 ºC.

The fragment was purified with the specific spin columns and inserted into a cloning vector.

2.1.4 INSERTION IN pGEM-T-Easy

The fragment was ligated in pGEM-T-Easy from Promega, O/N 16ºC

$$\frac{\text{ng of vector x kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

	Control	ligation
insert	----	125ng
pGEM-T-easy	50ng	50ng
T4 ligase	3U	3U
2Xligase mix (Promega) and water Up to	30µl	Up to 30µl

PCR products generated using a nonproofreading DNA polymerase such as *Taq* DNA polymerase, which lacks 3´→5´ exonuclease activity, have a

single template-independent nucleotide at the 3´ end of the DNA strand (Newton and Graham, 1994).

This single-nucleotide overhang, which is most commonly an A residue, allows hybridization with and cloning into pGEM-T vectors, which have a complementary 3´ single T overhang.

We used the high-copy-number pGEM-T Easy Vector which contains T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the coding region for the α-peptide of β-galactosidase.

Inactivation by insertion of the α-peptide allows recombinant clones to be directly identified by colour screening on indicator plates containing X-Gal and IPTG.

After ligation the incubation period was extended to 16 hours at 4ºC in order to increase the number of colonies after transformation.

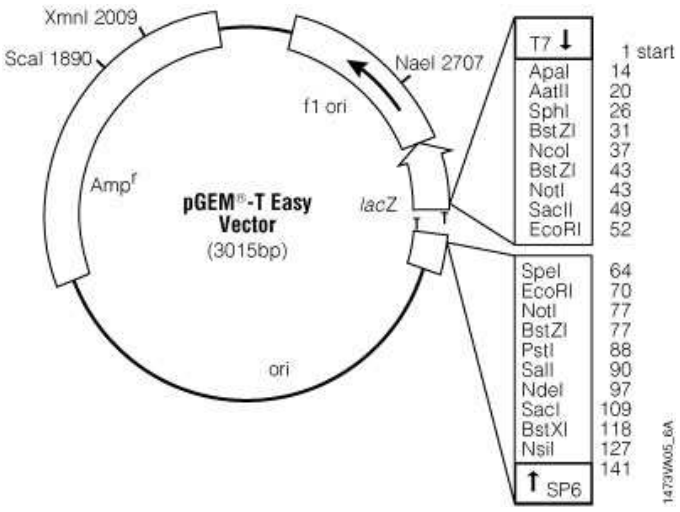


Figure 2.2 *pGEM-T-Easy Vector (Promega)*

2.1.5 DNA CONCENTRATION

DNA was precipitated adding 1 µl glycogen and 2,5 volumes of 96% Ethanol, left in a tube at -70ºC for an hours, centrifuged at 13.5 K rpm in a bench centrifuge.

The supernatant was discarded and the pellet was left to dry, then a small amount of ddH_2O was added.

2.1.6 ELECTROTRANSFORMATION

E.coli DH5α electrocompetent cells were thawed on ice and circa 50 µl were added to the DNA (in a pre-chilled centrifuge tube).

After mixing, cell suspension was transferred into a precooled cuvette which has two aluminium electrodes on its sides and put in the electroporator.

After the 0.5 seconds pulse the suspension was immediately transferred into a tube with prewarmed SOC medium which is made of

- 2% w/v bacto-tryptone
- 0,5% w/v bacto-yeast extract
- 10mM NaCl
- 2,5mM KCl
- ddH_2O to 1000 mL

Added fresh

- 10mM MgCl2
- 20mM glucose

and left in the orbital shaker at 37ºC for about an hour before plating about 100 µl onto LB agar Carbenicillin(Cb) plates 100µg/ml.

White colonies were chosen and an analysis of recombinant vectors by colony PCR and restriction enzyme digestion had to be performed because in pGEM-T vectors the insert can be cloned in either direction but just one is the correct one for transcription and translation of the gene.

Either a colony PCR with internal primers was done.

F in 5' GGGAACATCTCTCGGTCTATGC 3'
R in 5' GCCAAACTTGGAGTGTCTTGCC 3'

To mend the mutations found in Trk B it had to be cut from pGEM-T easy, made blunt and inserted in pBluescript KS +(pBSK) from Stratagene.
To get the proper amount of DNA we inoculated bacteria from a single colony obtained with a transformation of the original plasmid and grew it overnight in LB-Carbenicillin. Purification with the plasmid DNA purification kit from Yorkshire Bioscience followed.
VECTOR DIGESTION

TrkB in pGEM-T-easy	0,5 μg
Eco RI	2 units
Buffer Eco RI	10 μl
Dd water	Up to100 μl

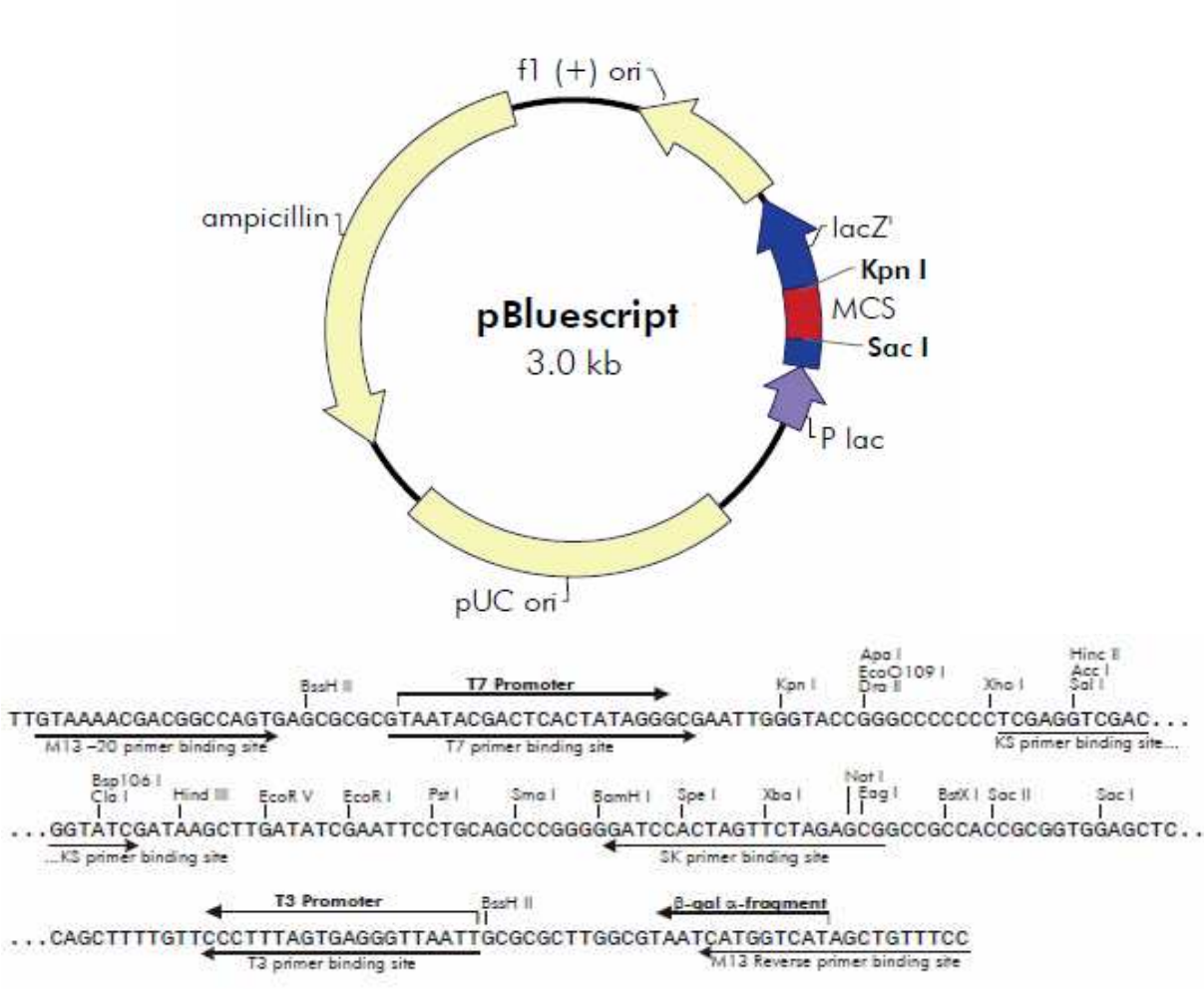


Figure 2.3 *pBluescript vector from Stratagene and the multiple cloning site (598-826 in the sequence)*

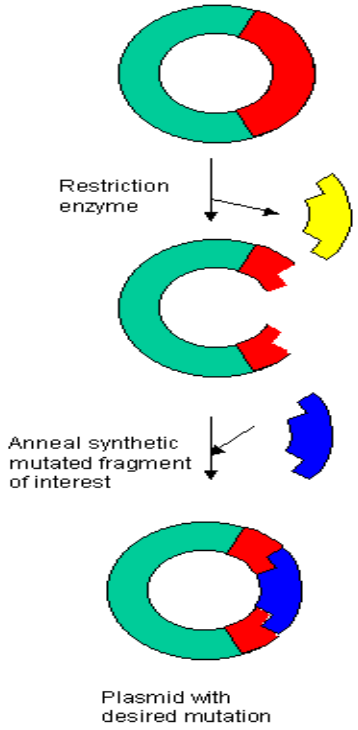
pBluescript(+) was cut with the restriction enzymes Hind III and Bam HI O/N 37ºC to eliminate Hind III site in its MCS (multiple cloning site) and made blunt, too.

Cutting Bam HI – Hind III fragment

For this purpose 0.5 μg of DNA clone were separately digested with:

2 units of BamHI (G▼GATCC; Amersham Pharmacia Biotech) in 20 mM Tris-HCl pH 8.5, 10 mM MgCl2, 1 mM dithiothreitiol, 100 mM KCl;

and 2 units of HindIII (A▼AGCTT; Amersham Pharmacia Biotech) in 10 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM dithiothreitiol, 50 mM NaCl; in 25 µl of reaction mix, incubated for 1 h at 37°C



- Getting both TrkB fragment and pBluescript (+) blunt
- 2X Taq mix was added to both DNA solution
- 95ºC 1 minute
- 72 C 10 minutes
- then 4ºC.

TrkB insertion into pBluescript (+) O/N 16ºC

TrkB cDNA was ligated into pBluescript (+) cloning vector, with 2u of T4 ligase (NEB) in 50 mM Tris-HCl, 10 mM MgCl2, 1 mM ATP, 10 mM Dithiothreitol pH 7.5, at 16ºC over night.

2.1.6 CHEMICAL TRANSFORMATION

Chemical competent cells from Novagen were thawed on ice, the ligation mix was in a pre-chilled tube on ice.

After mixing them together, the cell suspension was left 10 minutes on ice, then heat shocked at 42ºC for 30 seconds and immediately put on ice for 2 minutes before transferring it all in a tube containing 250 µl SOC.

Cells were left shaking (220 rpm) at 37ºC for about an hour and then 100 μl were plated on LB-Cb agar dishes.

Cutting TrkB pBSK(+) HindIII/NdeI

For this purpose 0.5 μg of DNA clone were separately digested with:

2 units of HindIII (A▼AGCTT; Amersham Pharmacia Biotech) in 10 mM Tris-HCl pH 7.5, 10 mM MgCl2, 1 mM dithiothreitiol, 50 mM NaCl;

and 2 units of NdeI (CA▼TATG; Amersham Pharmacia Biotech) in 20 mM Tris-acetate, 50 mM potassium acetate, 10 mM Magnesium Acetate, 1 mM Dithiothreitol pH 7.9 in 25 μl of reaction mix, incubated for 1 h at 37°C

Getting pBSK(+) blunt

pBSK(+)	0.5 μg
Blunting enzyme Fermentas	2 units
2X reaction Buffer	20 μl
Water	Up to 40 μl

After reinserting TrkB fragment into pBluescript with a ligation a site specific mutagenesis was performed cutting the 657 bp fragment in which both the mutations were found with two appropriate restriction enzymes: Hind III and Nde I and inserting it into a pGEM-T easy vector.

To mend mutations 6 primers were synthesized:

F pre Hind 5' GCATTTCCGATTGGAGCCTAAC 3'

R post Nde 5' CTACAAGATTTTCCGCCACACAAG 3'

and

Mut 1 f 5' CATTTCTGAAAAACAGCAACCTGC 3'

Mut 1 r 5' GCAGGTTGCTGTTTTTCAGAAATG 3'

Mut 2 f 5' GCAGATACCCAATTGTGGTTTGC 3'

Mut 2 r 5' GCAAACCACAATTGGGTATCTGC 3'

After several amplifications the mutations were eventually mended as the sequencing reaction with many internal primers confirmed.

trkB s1F 5’ GTGGAGGAAGGAAAGTCTATC 3’

trkB s1R 5’GGATCACCTGCCACACTAC 3’

trkB s2F 5’GGTCTATGCTGTGGTGGTG 3’

trkB s2R 5’CACCACCACAGCATAGACC 3’

trkB s3F 5’CGTCTGCGTGGAGGGCG 3’

trkB s3R 5’TGGAGGTTGGTCAGGAGC 3’

In the first step mutation number one was fixed using as a template the final products of F pre Hind – Mut1 R and Mut1F- R post Nde PCR reactions

After checking the sequencing results the second step gave the whole fragment aligning with the natural TrkB receptor performing a PCR reaction with F preHind-R postNde and using as a template the products of F pre Hind-Mut2 r and Mut2 F-R postNde.

2.1.7 REINSERTION OF THE TRKB FRAGMENT INTO FULL LENGHT TRKB

The fragment with the two fixed mutations was then cut from pBSK with Hind III and Nde I and reinserted into the pGEM-TrkB.

After several attempts the gene was complete and so we tried to insert it into a prokaryotic expression vector (pET29) in order to have it produced in a large amount by bacteria.

Results were not good as the whole gene was probably too large and in any case too difficult to produce via bacteria because of its chemical nature.

Being a transmembrane receptor means that the protein has either a hydrophobic and a polar nature, not mentioning that it needs many post-translational modifications to be active, and these are not feasible by bacteria.

2.2 INSERTING TRKB FL INTO PCDNA3.1

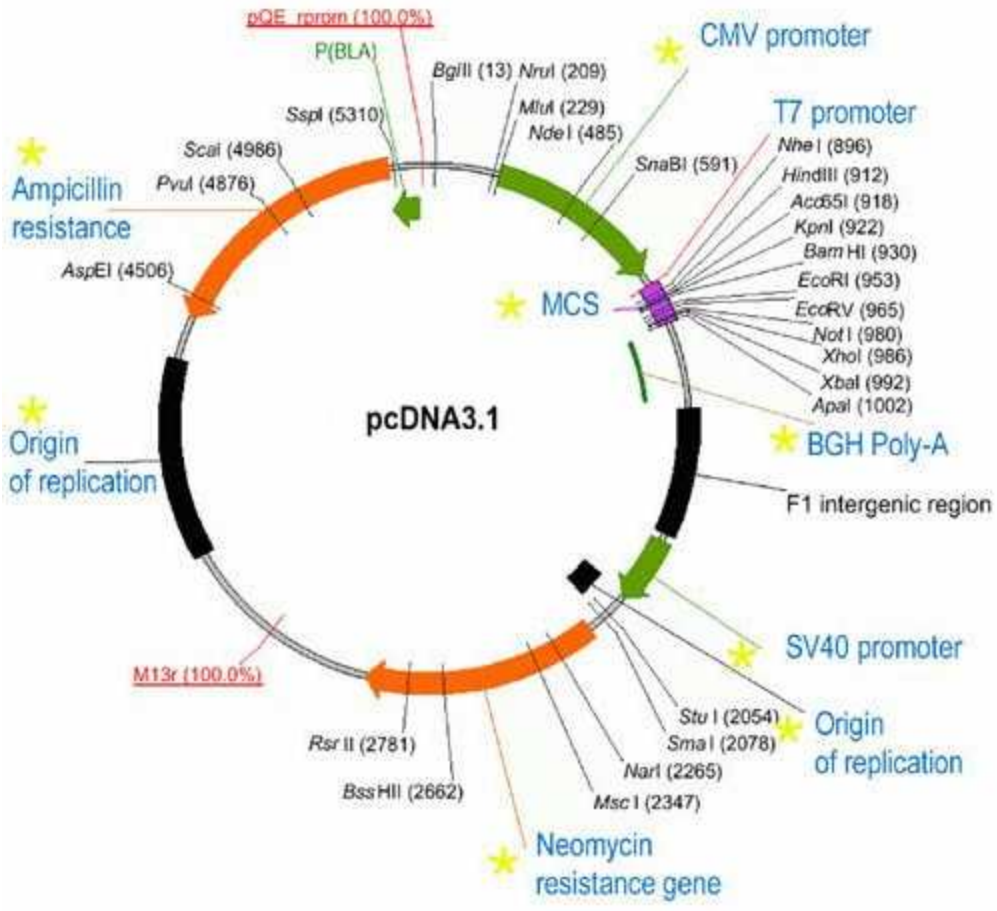


Figure 2.4 *pcDNA3.1 (adapted from stanxterm.aecom.yu.edu)*

pcDNA 3.1 was chosen as it could be transfected into eukaryotic cells.

Being TrkB gene human, it would be much better performing a phage display on eukaryotic cells as, in contrast with bacteria, they can provide the required post-translational modifications to the receptor.

After the transformation 3 clones resulted positive to the colony PCR and have been transfected, proving their efficiency.

To obtain the green fluorescent protein (GFP) thus making TrkB traceable in transfected cells, two suitable primers were designed:

F egfp Bam 5'CTTGGATCCGACATGGTGAGCAAG 3'
R egfp Bam 5'ACGGATCCCTTGTACAGCTCGTCC 3'

After obtaining GFP gene from an old construct, it was amplified and digestion with the restriction enzyme Bam HI for both the vector and insert (pcDNA3.1-TrkBfl) followed. An overnight ligation at 16ºC was performed.

Cells were actually transfected using Jet Pei from Polyplus.

A cell line derived from human cervical cancer (HeLa) was used.

2.2.1 CELL CULTURE

Cells were cultured in a 37ºC and 5% CO_2 incubator, in medium containing:

- DMEM (Dulbecco Modified Eagles's Medium):
- 10% FBS (Fetal Bovine Serum);
- 5% HS (Horse Serum);
- 1% PS (Penicillline-Streptomycine);
- L-Glutammine 2mM.

2.2.2 TRANSFECTION

Cells were grown in six wells plates containing glass coverslips till they reached a confluence around 50-70% and then transfected using JetPEI.

jetPEI™ compacts DNA into positively charged particles - called complexes - capable of interacting with anionic proteoglycans at the cell surface.

Upon binding to the cell membrane, the complexes are internalized via endocytosis. Once inside the endosomal compartment, the DNA is protected from degradation by jetPEI™. This is in part due to the unique property of this polymer to act as a "proton sponge", which in turn buffers the pH within the endosome.

This mechanism ultimately cause the breaking of the endosome and the release of the DNA and the complexes into the cytoplasm, thereby allowing nuclear transport for subsequent transcription.

Transfection procedure in a 6-well plate:

Per well, 3 μg of DNA were diluted in 150 mM NaCl to a final volume of 100 μl, mixed gently and spun down briefly.

The same was done with 6 μl of jetPEI™ per well. They were diluted in 150 mM NaCl to a final volume of 100 μl. Vortexed and spun down briefly.

The 100 μl jetPEI™ solution was added to the 100 μl DNA solution in the following order:

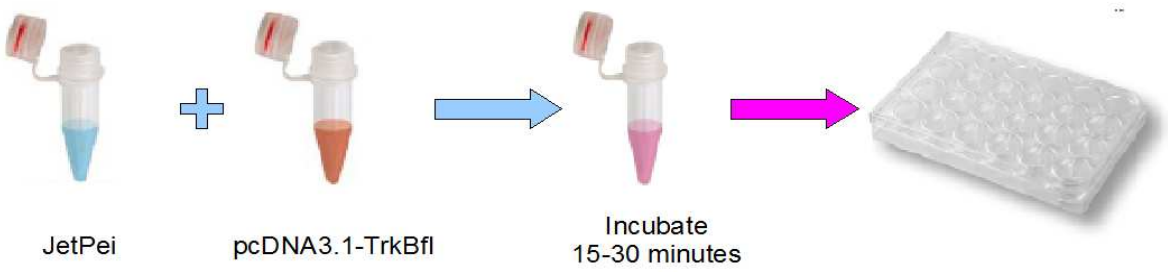


Figure 2.5 *Transfection with jetPEI*

The solution was vortexed immediately and spun down briefly.

A 15 to 30 minutes incubation at room temperature followed.

The 200 µl jetPEI™/DNA mix was added dropwise to the cells in 1 ml of serum-containing medium. The plate was then gently swirled to homogenize.

The next day, the medium was changed and immunofluorescence was performed within 24 to 48h following transfection.

2.2.3 IMMUNOCYTOLOGY ASSAY

Cells on the coverslips were washed in PBS and fixed in paraformaldehyde 4% in PBS 1X for half an hour at room temperature.

After washing with PBS cells were permeabilized with PBS-Tween20 0.1% for 15 minutes at room temperature.

A blocking buffer made with 5% goat serum in PBS was then added for 30 minutes at 37ºC in order to block aspecific sites.

Subsequently cells were left in a 1% solution of the primary antibody (anti TrkB rabbit polyclonal IgG, from Santa Cruz with the epitope

corresponding to amino acids 160-340 mapping within the extracellular domain of TrkB of human origin) in PBS overnight at 4ºC.

On the next day, following PBS washes cells were incubated in the dark at room temperature for 45 minutes with the secondary antibody diluted in PBS at different concentrations.

Secondary antibody from Santa Cruz biotechnology Inc., California was raised in goat and FITC conjugated.

Pictures were taken using a NIKON fluorescence microscope at a wavelength of 494 nm.

2.2.4 IMMUNOHISTOCHEMISTRY ASSAY

To check the effectiveness of the antibody some immunohistochemical assays on cochleas' sections were made.

The cochlea sections from rat, mouse and guinea pig were stained using Vectastain ABC kit from Vector Laboratories.

The paraffin embedded section slides were dipped into xylene and ethanol solutions at various concentrations and then washed in water and PBS.

After permeabilizing with PBS and Triton 0.1%, slides were covered with the blocking buffer for 40 minutes and then left overnight with the primary antibody at 4ºC in a humidified chamber.

The next day the primary antibody was washed away and an incubation of 30 minutes with the secondary antibody at room temperature in the dark followed.

The secondary antibody was detected with the avidin - biotin complex technique using a Vectastain Elite ABC kit (from Vector Laboratories,

CA) and developed with DAB (diaminobenzidine) from the same company.

Pictures were acquired with an eclipse 80i Nikon Microscope.

2.2.5 TRKB BINDING DOMAIN

PCR using the whole gene as a template was performed with these two primers

F2 5' CG**CATATG**GCACCAACTATCACATTTCTCGAATCTC 3'

R2 5' GC**GGATCC**CTATTAATGTTCCCGACCGGTTTTATC 3'

The PCR product was subcloned into pET15b to create the expression vector pET15b-TrkBIg2.

The amino acid residues from 286 to 430 comprise a second Ig-like domain of TrkB which has a beta sandwich structure.

In addition there are a further 21 residues at the NH_2 terminus, which constitute the histidine tag and associated thrombin cleavage sequence.

Chemical transformation was done using a special *E.coli* strain, BL21(DE3)pLysS, which is especially meant for the expression of proteins and produce themselves lisozyme.

Expression was carried out in accordance with the pET (Novagen) manual.

Cells were grown in LB (10 g of NaCl; 10 g of tryptone; 5 g of yeast extract; in 1L deionized H_2O) in two-litre flasks shaking at about 160 rpm in the orbital shaker at 37ºC till they reached an O.D. comprised between 0,4 and 1 (more or less 3 hours).

Protein expression was induced with addition of 1 mM IPTG, for about 3-4 hours.

Harvested cells were resuspended in 10% glycerol and frozen at -80ºC.

Expression of the protein was checked at different time with SDS PAGE analysis according to the Criterion manual from BioRad.

Pellets were frozen and thawed to let the lysozyme work and then sonicated for about 500 seconds, then washed with different solutions.

Solution 1

- 20 mM sodium phosphate buffers (pH 8-8.5)
- 0.1% Triton X-100
- 0.1 M NaCl

centrifuged at 4ºC for 30-60 minutes 4500 rpm

Solution 2 (3 washes)

- 20 mM sodium phosphate buffers (pH 8-8.5)
- 1% Triton X-100
- 0.1 M NaCl

centrifuged at 4ºC for 30-60 minutes 4500 rpm

Solution 3 (3 washes)

- 20 mM sodium phosphate buffers (pH 8-8.5)
- 1% Triton X-100
- 1 M NaCl

centrifuged at 4ºC for 30-60 minutes 4500 rpm

These washes removed all soluble matter, leaving inclusion bodies containing insoluble protein, while the centrifugation helped avoiding clogging of protein aggregates or cell debris on the Ni-NTA column.

2.2.6 PURIFICATION ON NI-NTA GEL

Purified inclusion bodies were solubilised in 8 M urea buffer (20 mM sodium phosphate, pH 8.5, 1 mM DTT) and incubated at room temperature for 2-24h with gentle shaking. TrkBIg$_2$ protein was purified on a His-Select Nickel Affinity Gel from Sigma.

This is an immobilized metal-ion affinity chromatography (IMAC) product consisting of a quadridentate chelate on beaded agarose charged with nickel that is designed to specifically bind histidine containing proteins.

HIS-Select Nickel Affinity matrix is 6% beaded agarose and is selective for recombinant proteins with histidine tags while it has a low non-specific binding of other proteins.

The affinity gel was poured into a clean chromatography column, washed with 1–2 volumes of deionized water to remove the ethanol storage solution and then equilibrated with 3–5 volumes of equilibration buffer.

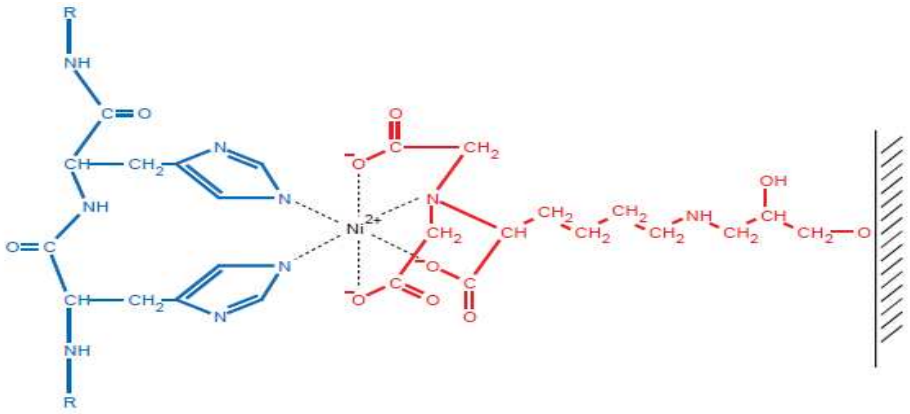


Figure 2.6 *The interaction between Ni-Nta beads and the Histidine tag (from Qiagen Qiaexpressionist)*

Purification was carried out under reducing conditions (20 mM sodium phosphate, pH 8.5, 8 M urea, 10 mM imidazole), and eluted using 300 mM imidazole after defining the best concentration for the elution.

2.2.7 SDS PAGE

After the elution on Ni-Nta column and the gel filtration other SDS PAGE assays were performed following the Criterion Manual instructions, so running buffer (1x 25 mM Tris; 192 mM glycine; 0.1% SDS) and loading buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 25% glycerol; 0.01% Bromophenol Blue; 350 mM DTT) were prepared.

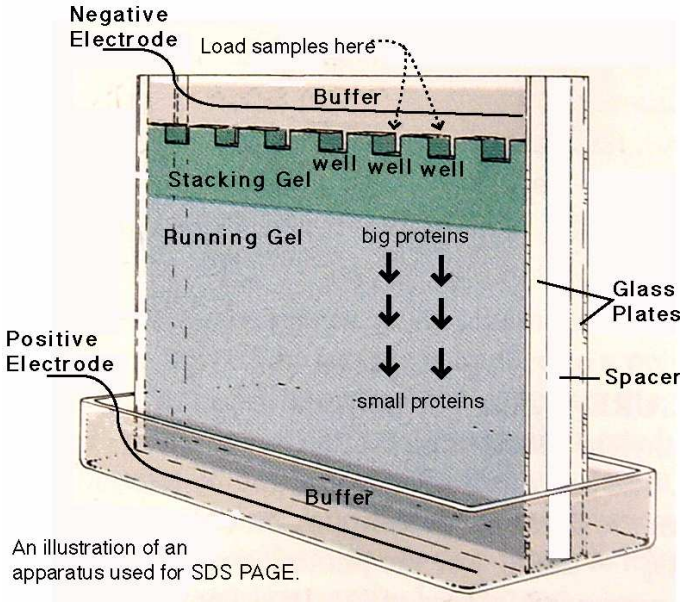


Figure 2.7 *SDS PAGE vertical gel (from http://web.chemistry.gatech)*

An unstained molecular weight marker was purchased from Fermentas with bands at 116; 66.2; 45; 35; 25; 18.4; 14.4 Kda. The molecular weight marker was heated for 5 minutes at 95ºC while samples – after being diluted in the loading buffer – were heated at 65ºC for 20 minutes as the protein could be damaged by such a high temperature.

The different samples were then loaded on a precast Criterion Tris-HCl gradient gel 10-20% from Bio-Rad which provides the best resolutions for proteins weighing 10-100KDa.

After the running step – generally performed overnight with a low constant voltage–gel was washed three times with deionized water for at least 15 minutes to remove SDS and dyes.

About 50 ml of the staining solution (Bio-Safe Coomassie from BioRad) were added and left incubating for 30-60 minutes shaking from time to time.

In the destaining step the gel was washed in water for an extended period (either overnight, or over a couple of days) to get a better resolution.

2.2.8 REFOLDING

Refolding took place under nonreducing conditions (20 mM sodium phosphate, pH 8.5, 100 mM NaCl) on a Superdex 200 gel-filtration column (Amersham bioscience).

Superdex prep grade is a preparative gel filtration medium with a composite matrix of dextran and agarose. This matrix (fig 2.8) combines the gel filtration properties of cross-linked dextran (Sephadex) with the physical and chemical stabilities of highly cross-linked agarose, providing high selectivity and resolution.

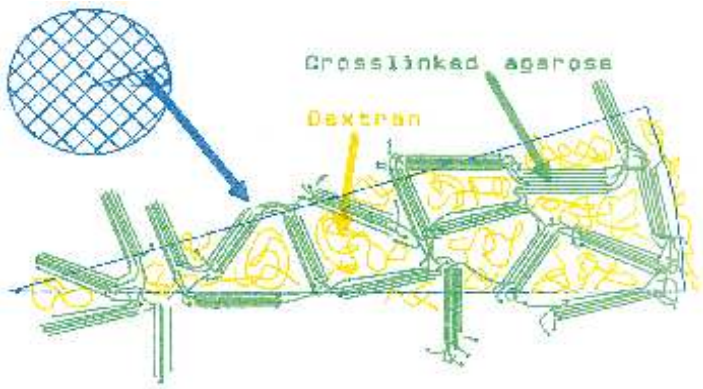


Figure 2.8 *Hypothetical view of a section through a bead from Superdex (Amersham Bioscience) whose mean particle size is 34µm.*

150 ml of the Superdex 200 slurry were poured into a XK 26/40 column avoiding bubbles. Void volume (61 ml circa) was calculated loading a saturated solution of bromophenol blue and blue dextran (see figure).

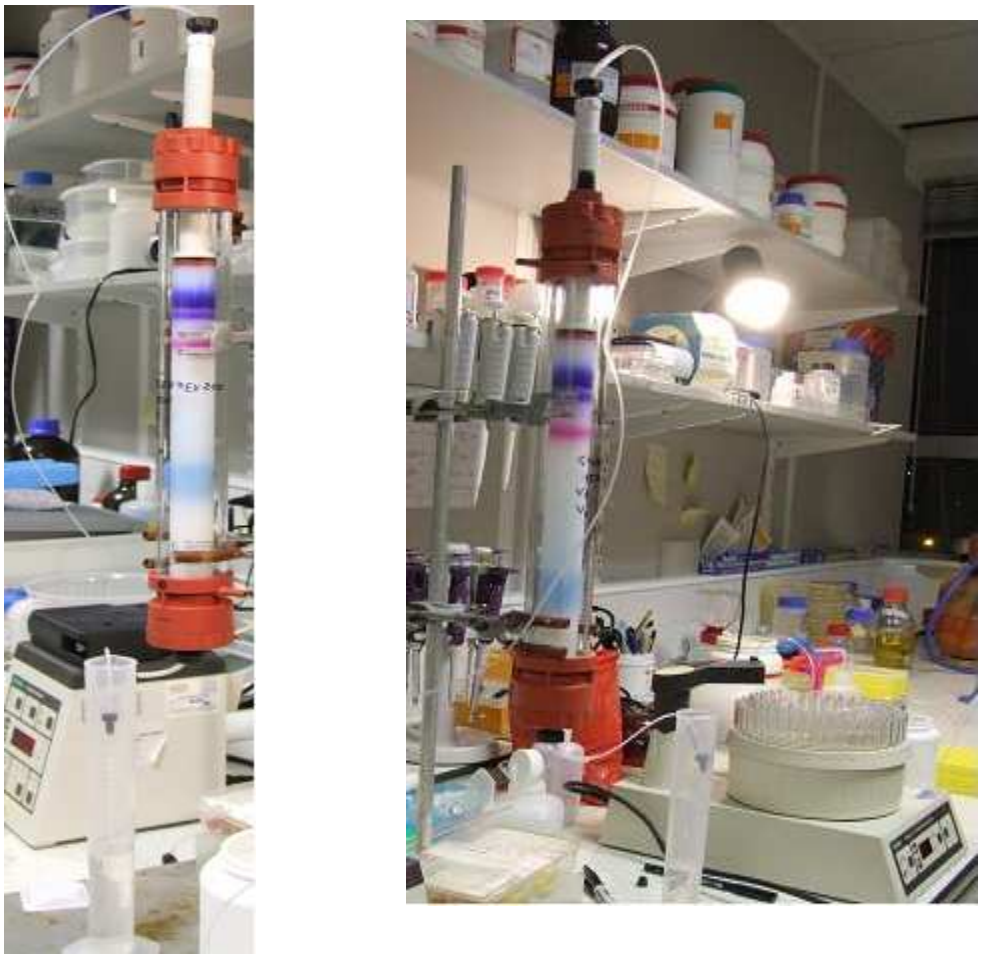
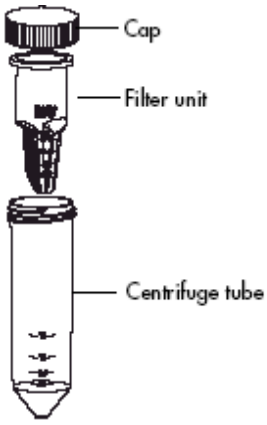


Figure 2.9 *Experimental measure of the void volume in the gel filtration column*

Fractions eluted from the NiNta column were concentrated with an Amicon Ultra centrifugal filter device, 5.000 NMWL .



The tubes were spun at 2000g for about 45-60 minutes and concentrated to the final volume of 2-3 ml maximum.

Flow rate was approximately 1.50 ml/minute.

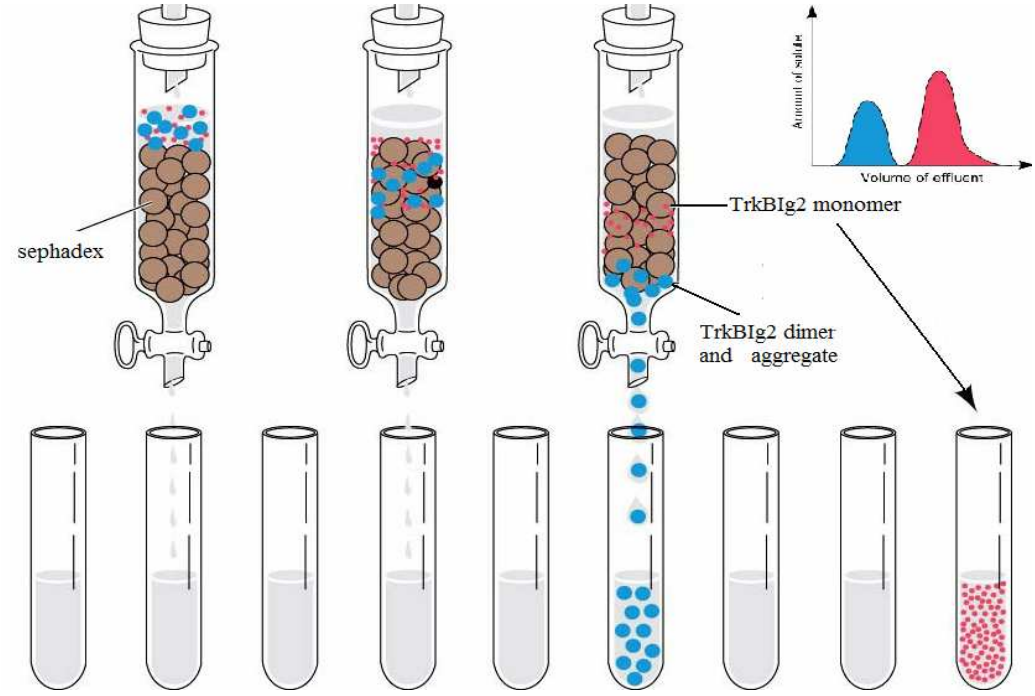


Figure 2.10 *Gel filtration technique*

The presence of the protein in the fractions was checked mixing some microliters of Biosafe staining solution with the eluted and comparing it with a known concentration of BSA and coomassie.

Figure 2.11 *Checking the presence of BSA protein in the fractions eluted from the superdex column*

$TrkBIg_2$ monomer was found in fractions collected 100-130 ml after loading the sample.

Separation from dimer and aggregate was finally achieved as it was demonstrated further.

An SDS PAGE and a native PAGE gel were ran to confirm the presence of TrkB binding domain, fractions from the peak corresponding to a molecular weight of approximately 18.5 kDa actually contained TrkBIg2 monomer.

For the native gel, proteins were prepared in a nonreducing, nondenaturing sample buffer that maintained the proteins' secondary structure and native charge density. All the conditions were the same as before apart from the fact that DTT and SDS were not used.

PHAGE DISPLAY

Phage display is a selection technique in which a library of bacteriophages, each displaying a different peptide sequence, is exposed to an immobilized target (Smith and Petrenko, 1997; Arap, 2005)

After the incubation the unbound phage is washed away while specifically bound phages stick to the target.

Elution of the phages from the receptor is done washing with an excess of its natural ligand .

Phages eluted from the target are used to infect bacteria and thus be amplified, this process has to be repeated at least 3 or 4 times.

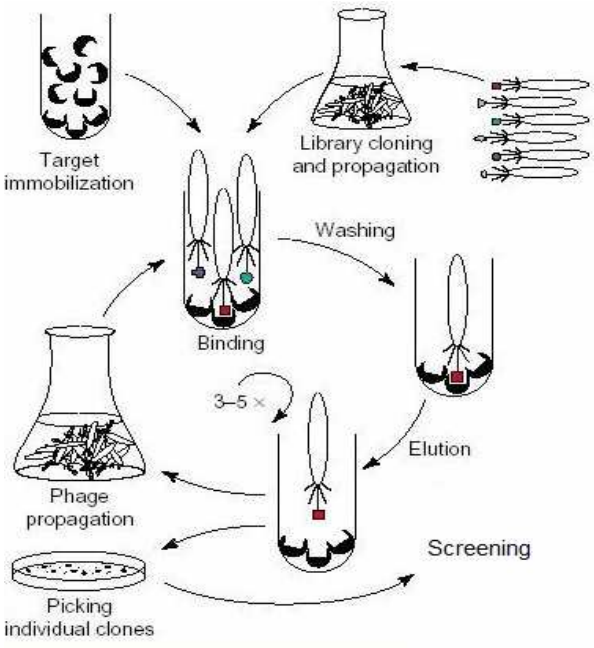


Figure 2.12 *The phage display technique in brief*

Single colonies are then chosen isolated and sequenced.

2.2.7 THROMBIN CLEAVAGE

Fractions eluted from the gel filtration column containing TrkBIg2 were incubated with Ni-Nta gel, phages and thrombin (from Sigma) in a solution containing 20 mM Tris-Cl, 150 mM NaCl, pH 8.4.

The protein is fused with a His tag and presents a cleavage site for thrombin before the actual binding domain.

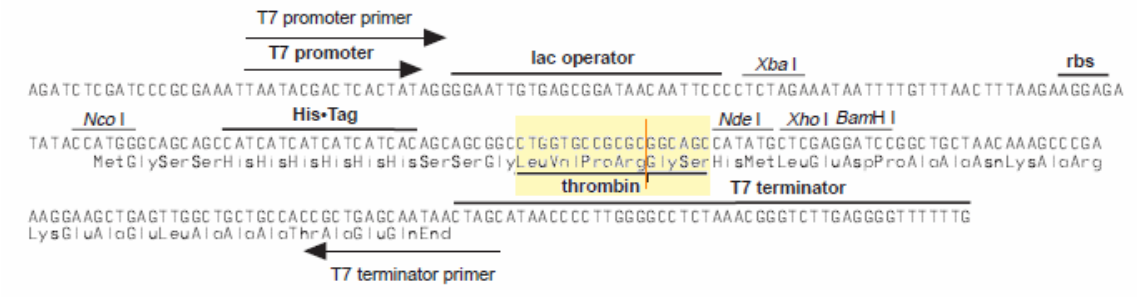


Figure 2.13 *Thrombin Cleavage site in pET-15b*

To this effect only the phages actually attached to the protein can be washed away and eluted after thrombin's cut, while the beads and the His tag will remain in the tube.

2.3 BDNF

Concerning brain derived neurothrophic factor we followed more or less the same scheme as the one used for TrkB.

We obtained the gene from the same human brain cDNA library using the specific primers:

F 5'ATGACCATCCTTTTCCTTAC 3'

R 5'CTATCTTCCCCTTTTAATGG 3'

With a PCR reaction with Taq polymerase

95ºC 3 minutes

3 cycles

95ºC 1 minute

55ºC 1 minute

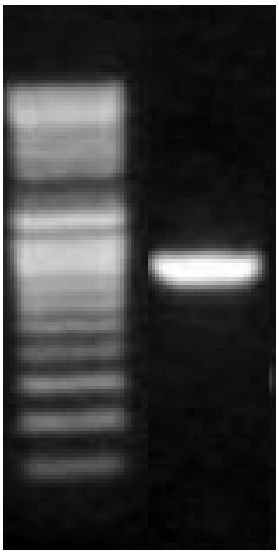
72ºC 1 minute

25 cycles

95ºC 30 seconds

60ºC 30 seconds

72ºC 40seconds

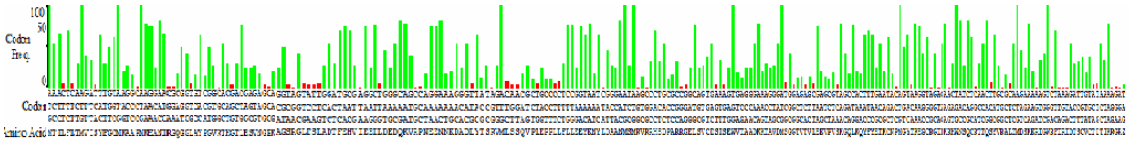


Checked on agarose gel 1.3% (as in figure, band at 744bp)

After confirming the size of the neurothrophin gene and amplifying it with Taq/Pfu polymerases mix we inserted it into pGEM-T-easy with the technique of T/A cloning.

We then inserted the gene into pet28 and transformed BL21(DE3)pLysS cells from Novagen, following PET manual to obtain the expression of the protein.

We checked translatability of BDNF codons in *E.coli* with a program based on codons usage in bacteria found on the website http://www.faculty.ucr.edu/~mmaduro/codonusage/usage.htmtranslated and the fraction of sense codons below threshold (=10.00):was 60/247 (24%), as in figure.



Colors: = less than 10% of codons for same amino acid; = at least 10%

Figure 2.14 *E.coli codon usage analysis*

A new BDNF gene was synthesized using five different primers each about 100bp long which included two suitable restriction sites, a histidine tag, a thrombin cleavage site and the ribosome binding site (RBS).

The 100-mers, HPLC purified were provided by Yorkshire Bioscience

BDNF – F1

5'TATACCATGGGCAGCAGCCATCATCATCATCACAGCAGCGGCCTGGTGCCGCGCGGCAGCCACTCCGACCCGGCTCGTCGTGGTGAACTGTCCGTTTG 3’

BDNF – F2

5’GGCTCGTCGTGGTGAACTGTCCGTTTGCGACTCCATCTCCGAATGGGTTACCGCTGCTGACAAAAAAACCGCTGTTGACATGTCCGGTGGTACCGTTACCTTC 3’

BDNF –F3

5’GTCCGGTGGTACCGTTACCGTTCTGGAAAAAGTTCCGGTTTCC

AAAGGTCAGCTGAAACAGTACTTCTACGAAACCAAATGCAACCC
GATGGGTTACACC 3’

BDNF –F4

5’GCAACCCGATGGGTTACACCAAAGAAGGTTGCCGTGGTATCG
ACAAACGTCACTGGAACTCCCAGTGCCGTACCACCCAGTCCTAC
GTTCGTGCTCTGACTATGG 3’

BDNF –F5

5’CGTTCGTGCTCTGACTATGGACTCCAAAAAACGTATCGGTTGG
CGTTTCATCCGTATCGACACCTCCTGCGTTTGCA
CCCTGACCATCAAACGTGGTCGTTAGCTCGAGAC 3’

The amplification of this brand new gene was performed in successive steps and the final product was inserted into a pGEM-T-easy vector.

Two mutations were detected therefore a mutagenesis had to be done with the undermentioned primers:

Fix first mut F: 5’CGAATGGGTTACCGCTGCTGACAAAAAAAC 3’
Fix first mut R: 5’GAGATGGAGTCGCAAACGGACAGTTCACC 3’
Fix second mut F: 5’TTTGCACCCTGACCATCAAACGTG 3’
Fix second mut r: 5’CGCAGGAGGTGTCGATACGGATG 3’

Afterwards BDNF was inserted into pET28a and expressed by *E.coli* cells.

As for TrkBIg2 purification inclusion bodies were collected after harvesting and sonicating the biomass.

Clarified inclusion bodies were poured into a Ni-nta column and the protein was finally eluted washing with an imidazole solution (200mM).

The most suitable refolding strategy is under evaluation.

2.4 PHAGE DISPLAY ON GUINEA PIGS

Guinea pigs were chosen as models for the in vivo phage display as they had bigger ears and cochleas thus resulting easier to handle.

Caviae at different ages were weighed (400-800g), anesthetized and shaved.

Behind the ear, an incision was cut allowing the surgeon to operate.

A cochleostomy was done with the subsequent injection of 10 µl phages into the perilymphatic space through the round window membrane.

The hole was closed with some tissue and dental cement.

After 15-45 minutes the animals were sacrificed and cochleas explanted.

They were washed in different solutions of water, TBS (50 mM Tris-HCl (pH 7.5), 150 mM NaCl) and TBS Tween and dried with a gauze.

After putting the cochleas in a tube they were smashed and crashed with a pestle and incubated with glycine chloride buffer for 5 minutes at room temperature.

The supernatant was taken after centrifuging on a bench centrifuge at 10krpm (rotation per minute) and a solution of Tris at pH 8.9 was added.

Titering followed.

About 100µl were used to infect 300ml bacteria cells in LB in their log phase.

Biomass was grown in an orbital shaker at 37 ºC for 3-4 hours and 0.5 ml chloroform were added.

Following centrifugation the supernatant was collected and mixed with 1/6 volumes of PEG-NaCl (20% (w/v) polyethylene glycol–8000, 2.5 M NaCl).

A succession of centrifugations and resuspensions in TBS and PEGNaCl was performed resulting in a final pellet containing the amplified phages.

These phages after being titered could be used to start a new round of the selection.

3 RESULTS AND DISCUSSION

The neurotrophic factors are released by neuronal target tissue in order to regulate differentiation of the neurons during development and assure their survival throughout adult life (Maness et al. 1994; Terenghi; 1999).
Furthermore it has been demonstrated that neurotrophins could promote nerve survival after an injury (Gillespie et al., 2002) which makes them potential therapeutic agents against neurodegeneration.
Brain derived neurotrophic factor is also of vital importance for neuron survival and especially in the inner ear, as its absence leads to an almost complete loss of innervation and consequently, functionality (Fritzsch et al 1997).
The binding of this neurotrophic factor to its natural receptor TrkB triggers signalling which is crucial for axonal outgrowth, neuronal survival, neurotransmitter release and synaptic plasticity.
It may be therefore of great help in people affected by sensorineural hearing loss and using cochlear implants, as it has already been demonstrated that it maintains neuronal cells alive and this proved to be necessary for the efficacy of the prosthesis.

I joined a European project which is working at the creation of multifunctional nanoparticles that could be used as drug carriers for a target delivery, in particular in spiral ganglion neural cells in the ear.

BDNF could direct nanoparticles to this target when binding to its specific neuronal transmembrane receptor TrkB.

The issue with its application is related to its big size that makes it difficult to deliver it.

Our aim was then to find some smaller peptides mimicking BDNF action and affinity for its receptor TrkB in order to attach them to these new generation nanoparticles and direct them to their cellular target.

As our goal was attaining BDNF mimetic peptides, we worked to get its full length receptor purified in order to perform the phage display on it.

Our starting material was a polyA+ RNA library from human adult brain from which we could finally obtain TrkB gene with the PCR having designed the proper primers.

After subcloning it into a cloning vector and fixing the mutations occurred during the DNA amplification, we finally inserted it in a prokaryotic expression vector.

Due to its transmembrane protein nature, though, expression in bacteria proved to be very complex and difficult.

The coexistence of the hydrophobic and hydrophilic domains makes the production of the protein almost impossible for prokaryotic cells.

This fact forced us to find a different solution.

We obtained a cDNA library from RNA polyA+ from the whole adult human brain using Creator SMART cDNA Library Construction Kit.

Our aim was getting the gene coding for the tyrosine kinase receptor TrkB as it was the target of the phage display.
Random peptides 12-mers expressed at the N-terminus of a minor coat protein of the M13 phage have to stick to the receptor binding domain (Smith, G.P. and Scott, J.K.; 1993).

This domain is exposed outside the cell thus in the extracellular domain of the protein.

In our study we also needed the intracellular kinase domain as we wanted to check the efficacy of the interaction after the binding.

Investigating in literature I found many different isoforms of TrkB gene (Klein et al 1989, 1990) according to the type of tissue they were expressed and I chose the one representing the full length receptor.

Despite the fact it was longer than the other variants we decided to go for it as we also wanted the protein structure to be the closest possible to the natural one expressed in the neurons of the spiral ganglion in the inner ear.

In literature the sequence 2469 bp (base pair) long was selected: it codes for the full length functional receptor for brain derived neurotrophic factor (Shelton et al., 1995).

All three domains are represented in this 822 aminoacids long protein: the extracellular domain, the single transmembrane segment and the intracellular one possessing a tyrosine-kinase fragment (Shelton et al., 1995)

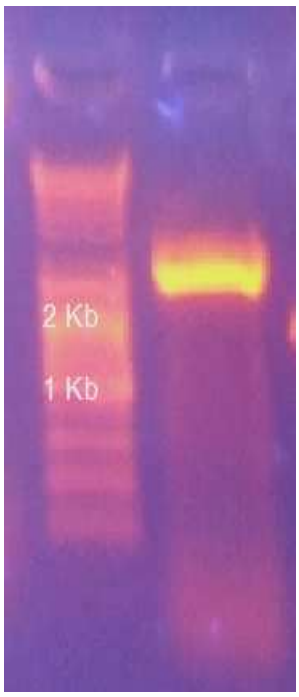


Figure 3.1 *TrkB on the gel, 2469 bp.*

Specific primers based on the coding sequence were designed, the forward one called F1 including the three bases corresponding to the start of the gene and the reverse one starting from the TAG codon which represent the end of the gene (R1).

Using 1µg of the cDNA library taken from human brain as a template a number of polymerase chain reactions were performed in order to amplify the gene sequence and so get a good amount of the DNA.

After getting the DNA and checking the size of it on gel (2469 bp as shown in figure 3.1) we subcloned it into a pGEM-T-Easy vector with the method of the T/A cloning.

PCR products generated using a nonproofreading DNA polymerase such as *Taq* DNA polymerase, which lacks 3´→5´ exonuclease activity, have a single template-independent nucleotide at the 3´ end of the DNA strand.

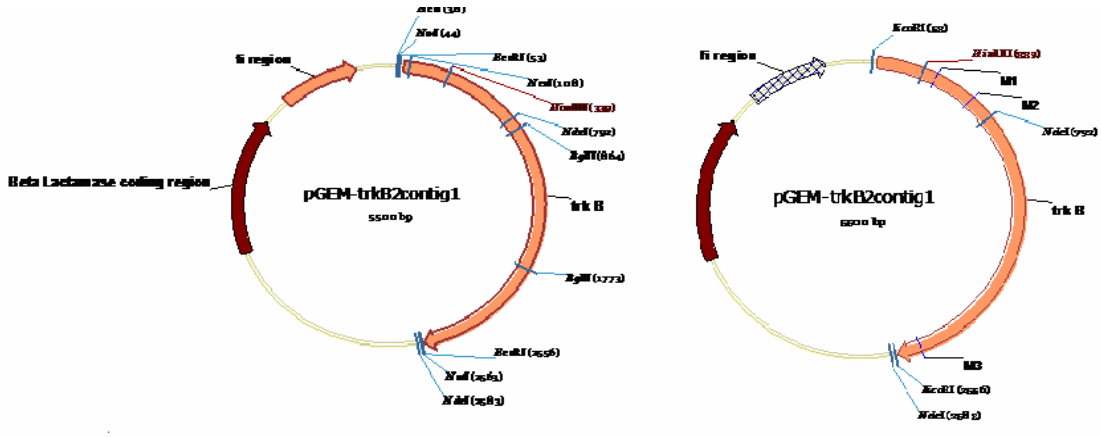


Figure 3.2 *TrkB full length in pGEM-T-easy*

The best of the clones was aligning with TrkB but presented three mutations at 343, 578, 2337 bases (see figure 3.2).

One of the mutations (at 2337) was not significative as it did not affect the final translation into protein whereas the other two had to be fixed.

With a site specific mutagenesis the mutations were eventually mended and the fragment (fig.3.3) cut with the appropriate restriction enzymes could be reinserted into the original pGEM-T easy vector containing the other part of the gene.

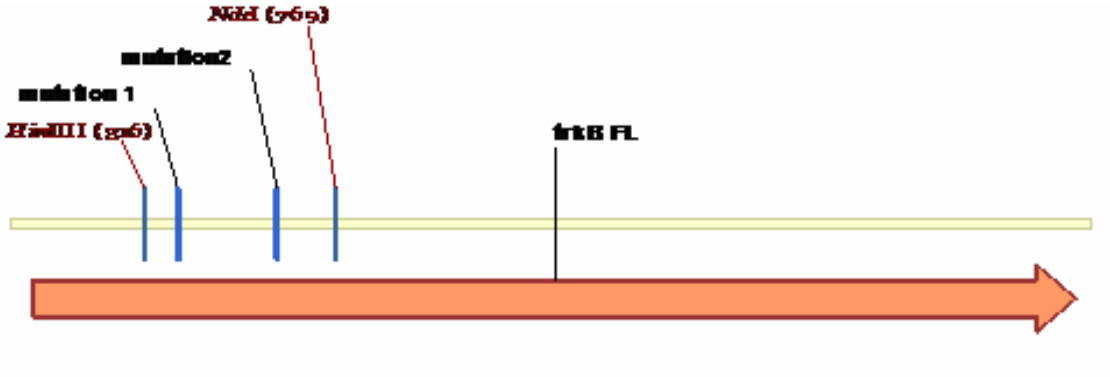


Figure 3.3 *Position of mutations and primers in TrkB full length*

After several attempts the gene was complete and so we tried to insert it into a prokaryotic expression vector (pET29) that allowed to get a large amount of the protein which is the target of the phage display.

Results were not good as the whole gene was probably too large and in any case too difficult to produce via bacteria because of its chemical nature.

Being a transmembrane receptor infact, means that the protein has either a hydrophobic and a polar nature, not mentioning that it needs many post-translational modifications to be active, and these are not feasible by bacteria.

In literature I could find numerous studies aiming at determining which part of the TrkB extracellular domain was specific for the neurotrophin binding.

The LRMs of TrkA and TrkB have been reported as essential for ligand binding, in particular the second LRM. Many studies demonstrated that

interaction with the Ig-like domains of TrkA is crucial for NGF binding (Ultsch, M H et al., 1999; Urfer et al, 1995).

In particular the second Ig-like domain, closest to the transmembrane-spanning region, provides the main contact for both NGF binding to TrkA and NT3 binding to TrkC (Urfer et al, 1998).

In various articles (Haniu et al, 1997; Naylor et al, 2002) it was demonstrated that the actual binding domain of the receptor is a small part of the protein spanning from nucleotides 953 to 1397 of the human TrkB gene, which corresponds to amino acid residues 286 to 430 of the mature protein

Naylor et al. (2002), fully proved the efficacy of the ligand binding with surface plasmon resonance assays so confirming that the second Ig-like domain of TrkB is the main docking region for BDNF. So we designed new primers to obtain only the binding domain from the whole gene. After subcloning the PCR product we created the expression vector pET15b-TrkBIg2.

The plasmid contained an N-terminal His-tag with an adjacent thrombin cleavage site.

This enabled an easier purification of the protein with a commonly used technique in which recombinant proteins tagged with 6-10 poly Histidines can be purified in one step by ion metal affinity chromatography (IMAC) as described in Porath et al., 1975.

This purification method takes advantage of the fact that several metal ions, and in particular Nickel and Cobalt exhibit affinity to Histidine residues (Schmitt, J., Hess, H., and Stunneberg, H.G.; 1993; Lindner et al, 1992) .

Transformation was done using a special *E.coli* strain, BL21(DE3)pLysS, especially meant for the expression of proteins: they in fact produce themselves lisozyme allowing their own degradation, in practice.

The presence of the protein and the best parameters for bacteria induction and washing were checked every time, step by step with SDS PAGE assays as shown in fig 3.4.confirming the data of a previous article by Naylor et al.; 2002 (see fig.3.4)

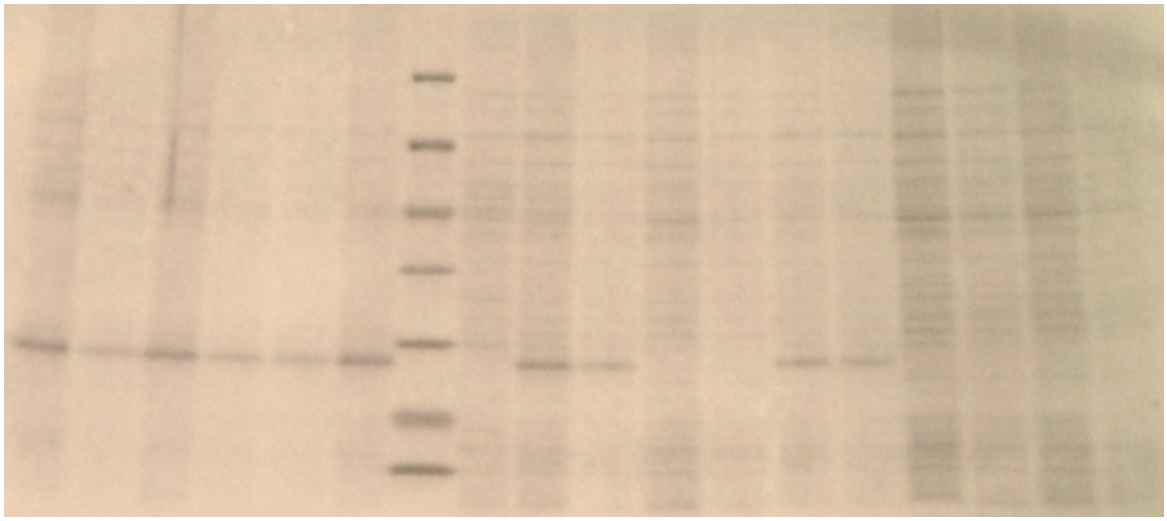


Figure 3.4 *$TrkBIg_2$ expression in different clones. Lanes 1 to 4, 7and 8 uninduced cells, time zero. Lanes 5, 6, 9, 10 expression after 2 hours induction with IPTG, 11 BL21(DE 3)pLysS not transformed with the plasmid pET15b- $TrkBIg_2$, 12 molecular weight marker, lanes 13 to 18 expression after 24 hours induction.*

Bacteria had expressed the protein TrkB in inclusion bodies (Bowden et al, 1991) which resulted to be around 20 KDa, as it was expected.

After harvested cells were sonicated we could start clarifying the inclusion bodies containing the protein following Palmer, I, Wingfield, PT 1995.

Purified inclusion bodies were solubilised in a urea containing buffer as in our reference article protein was found in the urea-soluble fractions.

A chromatographic Ni-nta column was used, under reducing conditions in order to obtain only the secondary structure, more favourable for the protein-bead interaction.

TrkBIg$_2$ was then eluted trying different concentrations of imidazole in order to find the one that most suited the purification.

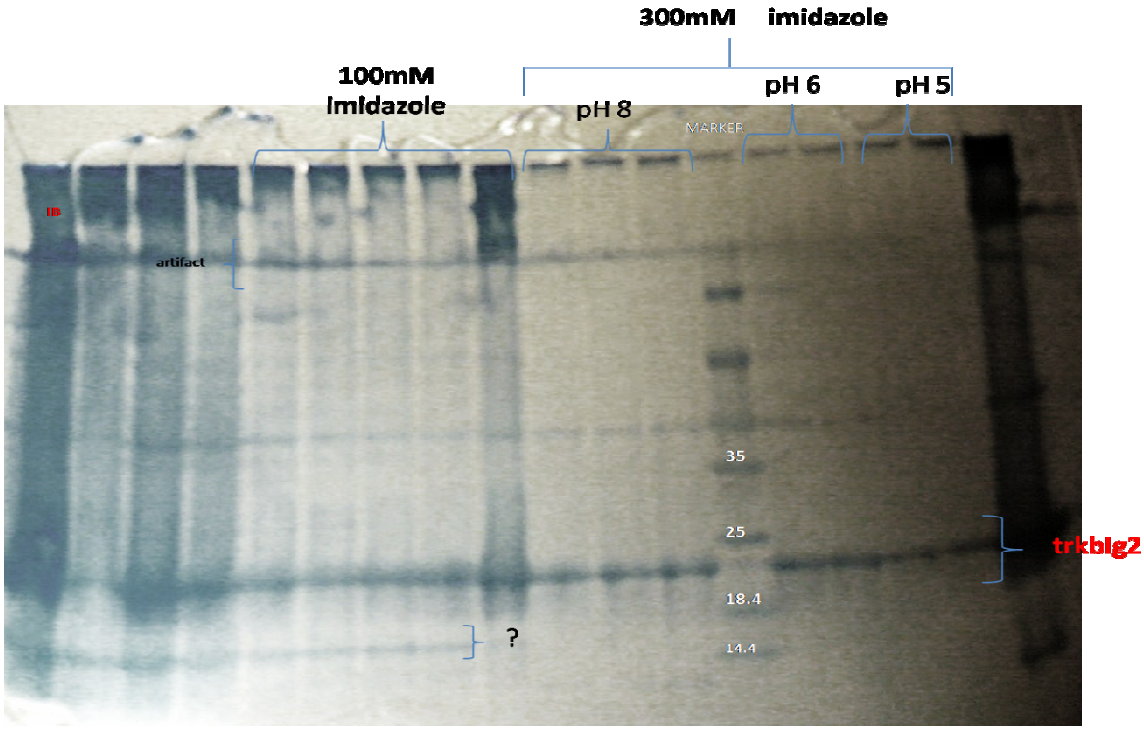


Figure 3.5 *The protein TrkBIg$_2$ eluted from the column at different concentration of Imidazole.*

In the end we found out the best concentration was 300 mM Imidazole as shown in figure 3.5.

The fractions containing the protein eluted from the Immobilized metal ion affinity chromatography, were concentrated and poured into a Superdex 200 gel-filtration column. Nonreducing conditions were maintained in order to get the protein refolded as close as possible to its natural 3D structure (Jaenicke and Rudolph, 1990).

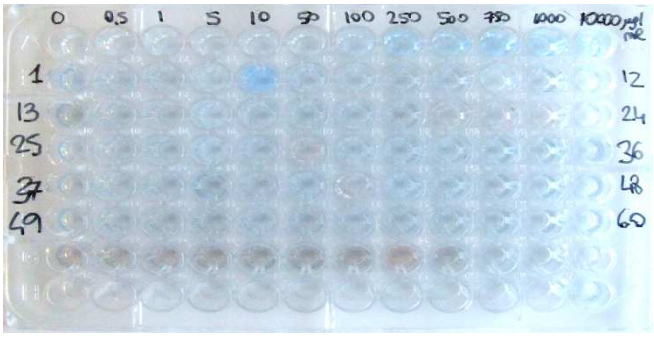


Figure 3.6 *Checking the presence of the protein in the eluted fractions*

In the first row there are various concentrations of BSA and the staining solution, from the first well of the second row on there are all the fractions eluted from the gel filtration column. In well number 5 is visible a big amount of protein due to the presence of aggregates and the dimer and from well 31 on the second peak of TrkBIg2 monomer is detectable.

We aimed to get the protein refolded in its natural conformation (figure 3.7), thus able to interact with its natural ligand or - as TrkB had to be used the target for the phage display - a 12mer peptide.

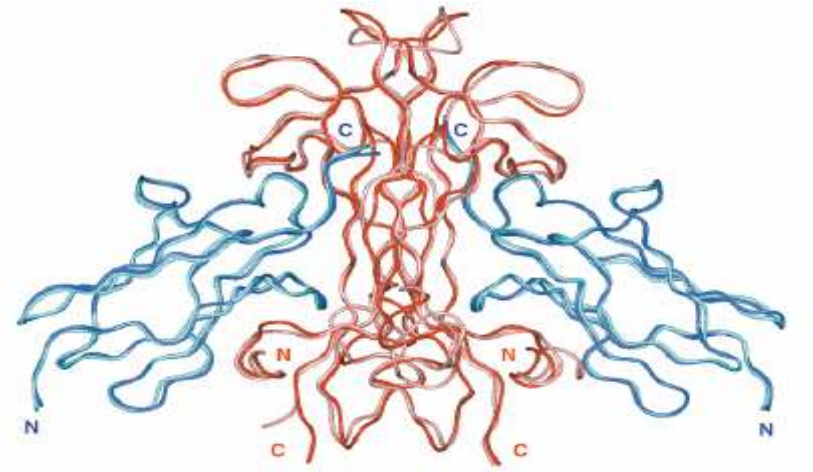


Figure 3.7 *The model BDNF-TrkBIg2 complex (dark red–dark blue)*
Taken from Naylor's article, 2002

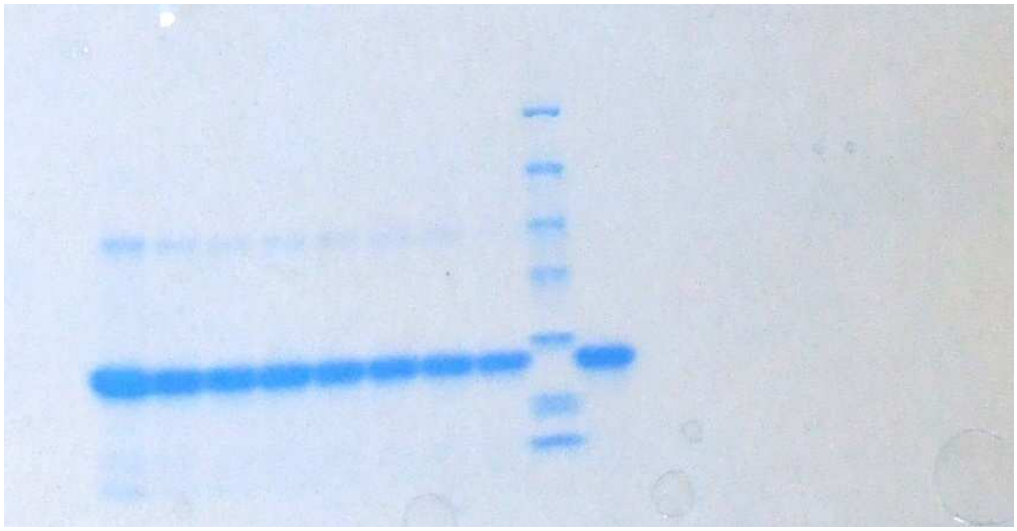


Figure 3.8 *Fractions eluted from the gel filtration column*

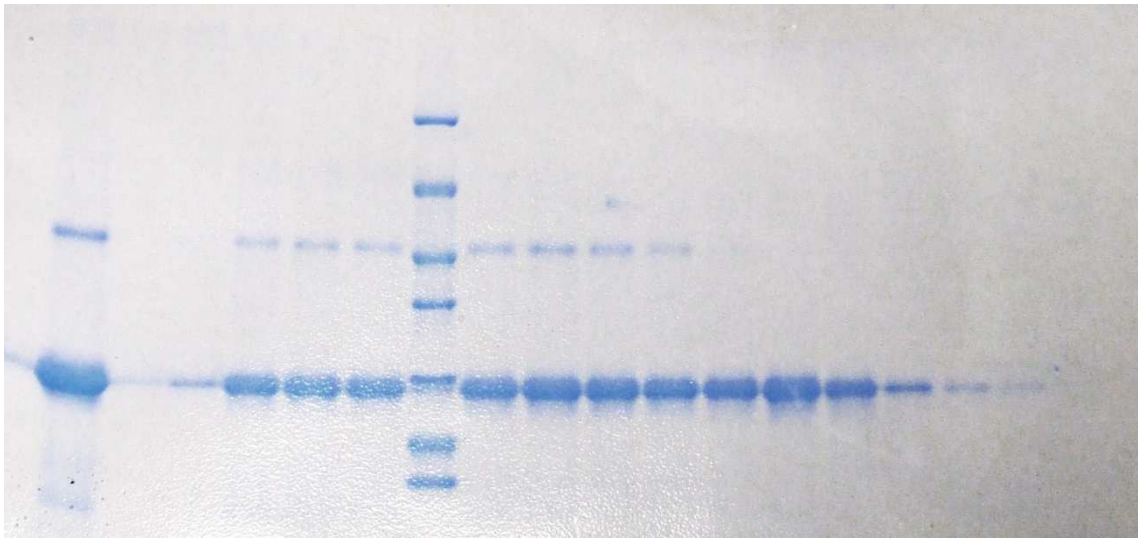


Figure 3.9 *Fractions eluted from the gel filtration column*

In the first well fractions eluted from the Ni-nta gel before loading them on the superdex column, from well 2 on fractions collected progressively from the gel filtration column (superdex)

Comparing TrkB purification progress with others performed with known molecular weight proteins like BSA and Lysozyme, we estimated that the protein had to be eluted approximately after a certain volume of buffer had flowed through.

The binding domain was found in the pool of eluted fraction but unluckily the presence of another protein weighting about 45kDa, probably naturally produced by the *E.coli*, was as well detected, as shown in figures 3.8, 3.9.

We then performed a native protein electrophoresis in order to investigate on the presence of the contaminant or aggregate in the various fractions and to know if the protein could be somehow detached from its dimer.

This technique does not use any denaturant agent thus leaving the proteins intact, in their native form, as they have to be used in the phage selection.

This allowed separation of TrkBIg2 monomer from dimer and aggregate. And we could eventually see that fractions from the peak corresponding to a molecular weight of approximately 18.5 kDa contained $TrkBIg_2$ monomer.

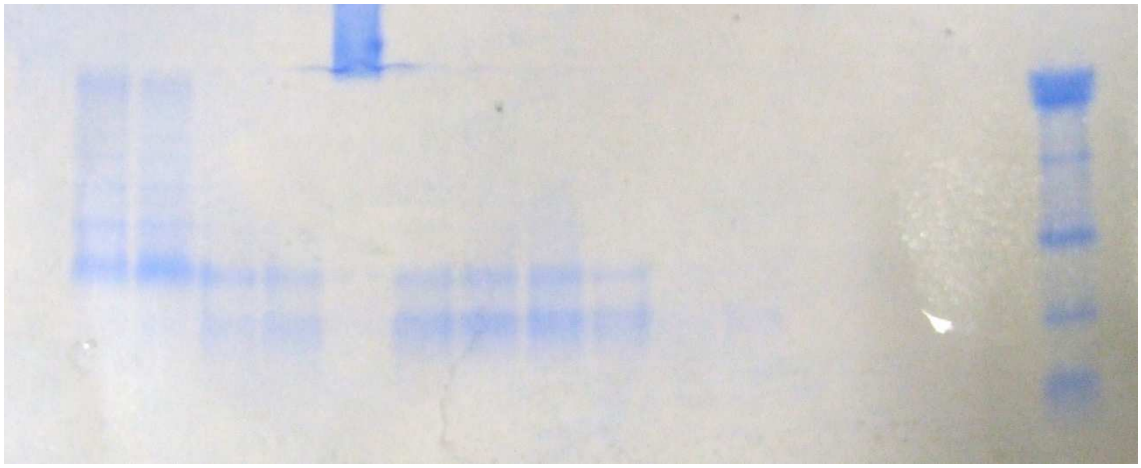


Figure 3.10 *In the native gel, the monomer is separated from the dimer*

We then pooled all the fractions and added the Ni-Nta gel in order to immobilize the His tagged binding domain to the beads. This represented the first step of the phage display.

Our idea was letting the phages stick to the Nickel-bound protein, washing away the aspecific phages and subsequently cutting the protein from the beads taking advantage of the presence of a thrombin cleavage site between the His tag and the protein.

We found that a similar experiment had been performed on Nickel-beads-attached protein with a different enzyme (Clackson T, Lowman H, 2004).

This way we could perform the phage display technique in just one go.

When using phage display in fact, it is always necessary to “subtract” from the selected phages the ones that did not actually bind the target but something else like, in this case, the beads could be.

Cutting the protein with thrombin instead, the beads with the His tag (which is obviously not a part of the binding domain) and the phages attached would be left, while only the specific phages would be collected.

The parameters to set the thrombin cleavage in beads were set.

BDNF

As during phage display a wash with a competitor is needed to have the phages detached from the target, we worked to get TrkB natural ligand, BDNF.

The gene was scratched from the same cDNA library from adult human brain obtained while we were studying TrkB.

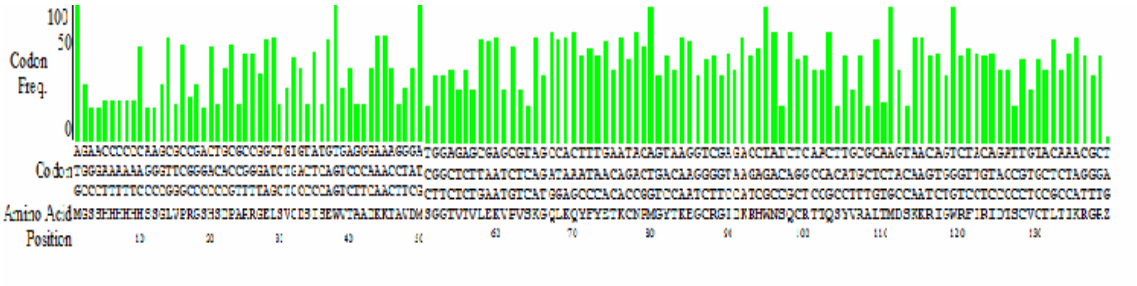
In literature many isoforms of the neurotrophin could be found but as they have mainly the same coding sequence we chose the most frequent which is a DNA fragment 744 bp long (Lewin and Barde, 1996)

The gene, amplified by specific primers, was inserted into a prokaryotic expression vector. After transforming the proper cells and inducing them we realized in an SDS page assay that the protein was not expressed by *E.Coli.*

This could be probably due to the fact that *E.Coli* and bacteria in general have a different codon usage from the eukaryotic organisms (Phillips et al , 1987)

So we “translated” all the nucleotide sequence of BDNF in something that could be more easily “read” by bacteria (figure 3.11).

Moreover we found out that we could try to get just the mature form of the NT synthesized which was about 300bp shorter.



Colors: ■= less than 10% of codons for same amino acid; ■= at least 10%

Fraction of sense codons below threshold (=10.00): **0/140 (0%)**

Figure 3.11 *E. coli Codon Usage Analysis for the new BDNF*

So we designed a completely new “easily translatable” BDNF and we finally obtained the DNA, performing many amplification cycles with 10 overlapping primers.

When the BDNF gene was successively inserted into a cloning vector and sequenced some mutations were found, due to proofreading mistakes by the polymerase and they were fixed with a site specific mutagenesis.

After sequencing BDNF gene it was inserted into a pET28 expression vector and the protein expression in *E.coli* was achieved as shown in figure 3.12.

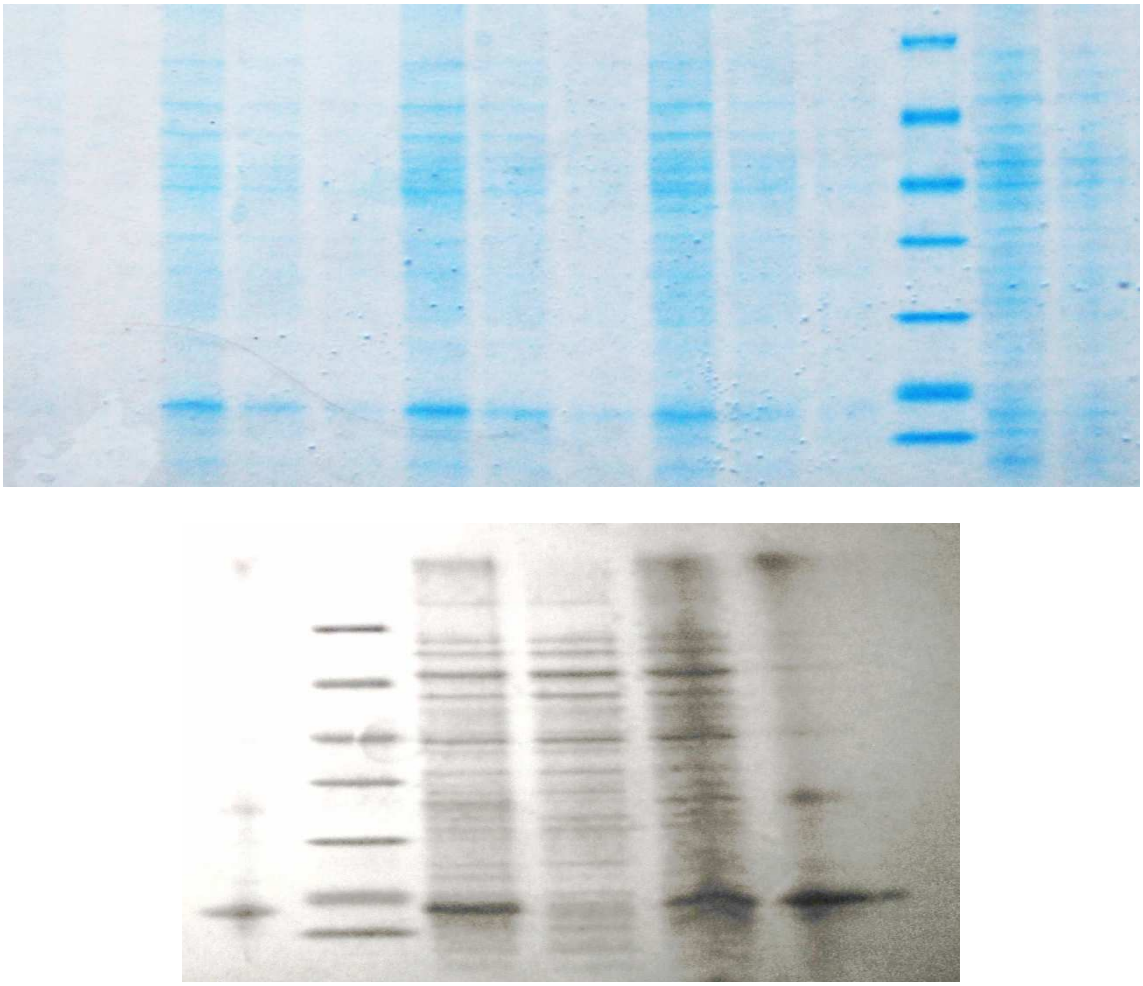


Figure 3.12 *E. coli BL21cells expressing BDNF, selecting the best clone and setting the time of induction*

TRK B FULL LENGTH

We also took into consideration the possibility of performing a phage display in vitro on the real protein, expressed by eukaryotic cells, thus more reliable as a model.

Trk receptors are heavily *N*-glycosylated in their extracellular portions.

As Watson et al. in 1999 demonstrated, glycosylation serves two distinct functions: regulation of Trk surface expression and prevention of ligand-independent Trk activity.

Trk core protein is continuously active *in vitro*.

Consequently, factors that alter Trk receptor *N*-glycosylation can profoundly modulate the activation capacity of the receptor.

This could represent a problem in production of TrkB in *E.coli* as the receptor will certainly not be N-glycosilated.

N-glycosylation is instead a normal post-translational process in eukaryotic cells.

This was the reason why eukaryotic cells were chosen to produce the best targets for the selection of peptides despite the much longer time the process requires.

We prepared a plasmid containing TrkB full length gene inserted into an eukaryotic vector, pcDNA3.1 (the structure is shown in figure 3.13).

The starting material for the transfection was a cell line derived from human cervical cancer cells (HeLa).

About 40 hours after the transfection, we could prove that the receptor was actually expressed by the cells as the immunocytology assay confirmed.

Cells' fluorescence was in fact detectable following the immunoreaction, (figures 3.14), thus demonstrating first of all that the sequence coded for the protein and subsequently that Hela cells could be used for both transient and stable transfection.

The presence of the BDNF receptor was also confirmed in cochleas' sections from mouse, rat and guinea pig with an immunohistochemical assay (fig 3.15).

TrkB was especially detected in cavia's inner ear. This fact made us take into consideration that the phage display on guinea pig could be as well performed, giving comparable results to the one obtained on the human model.

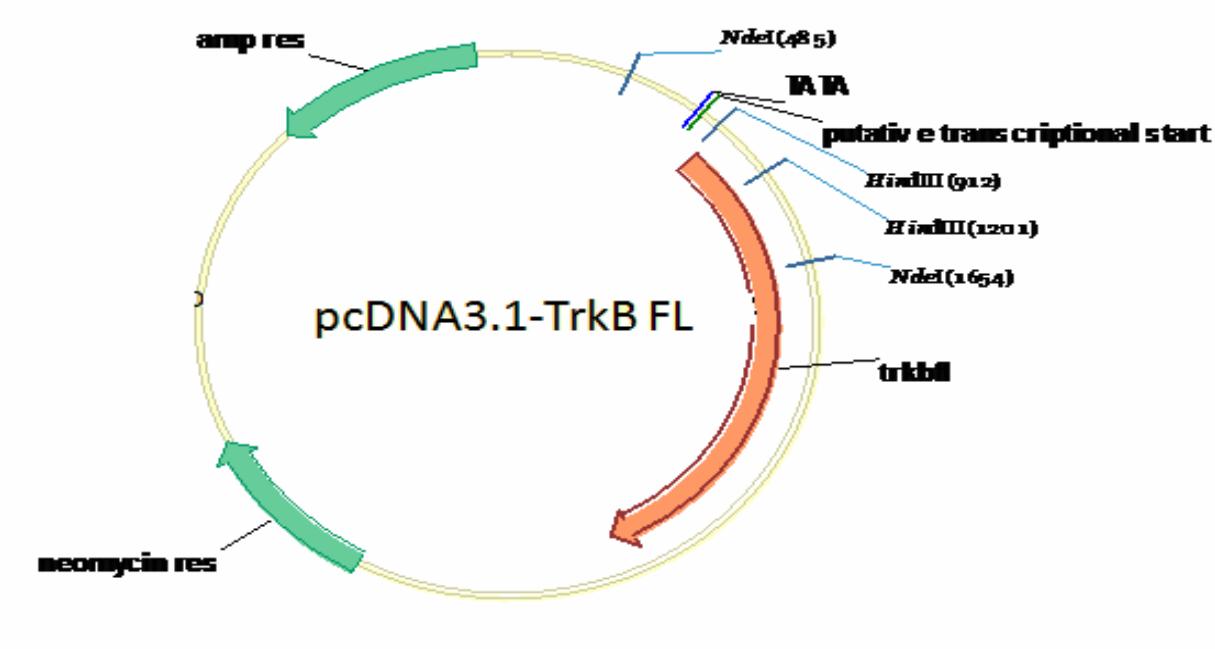


Figure 3.13 *TrkB full length in pcDNA3.1*

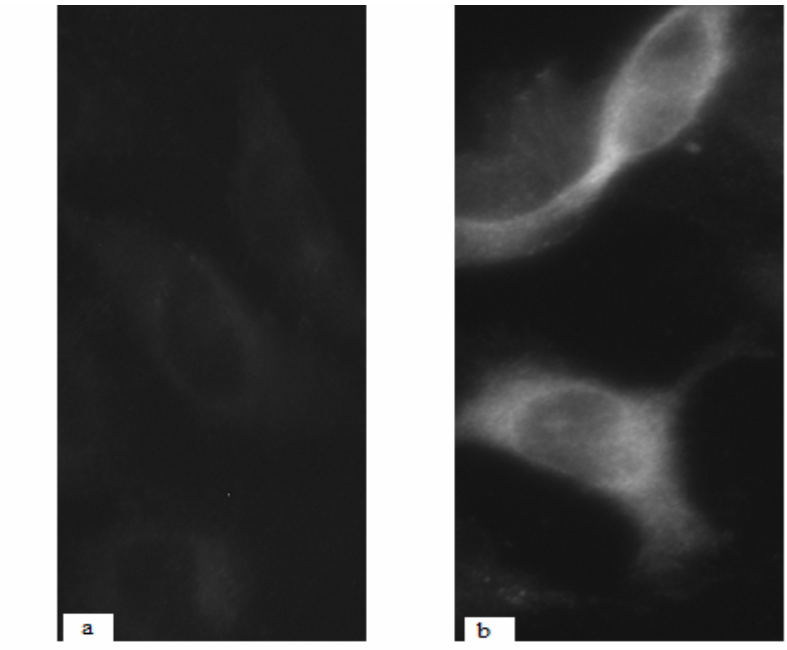


Figure 3.14 *Immunocytofluorescence of Hela cells. a) negative control, b) transfected cells*

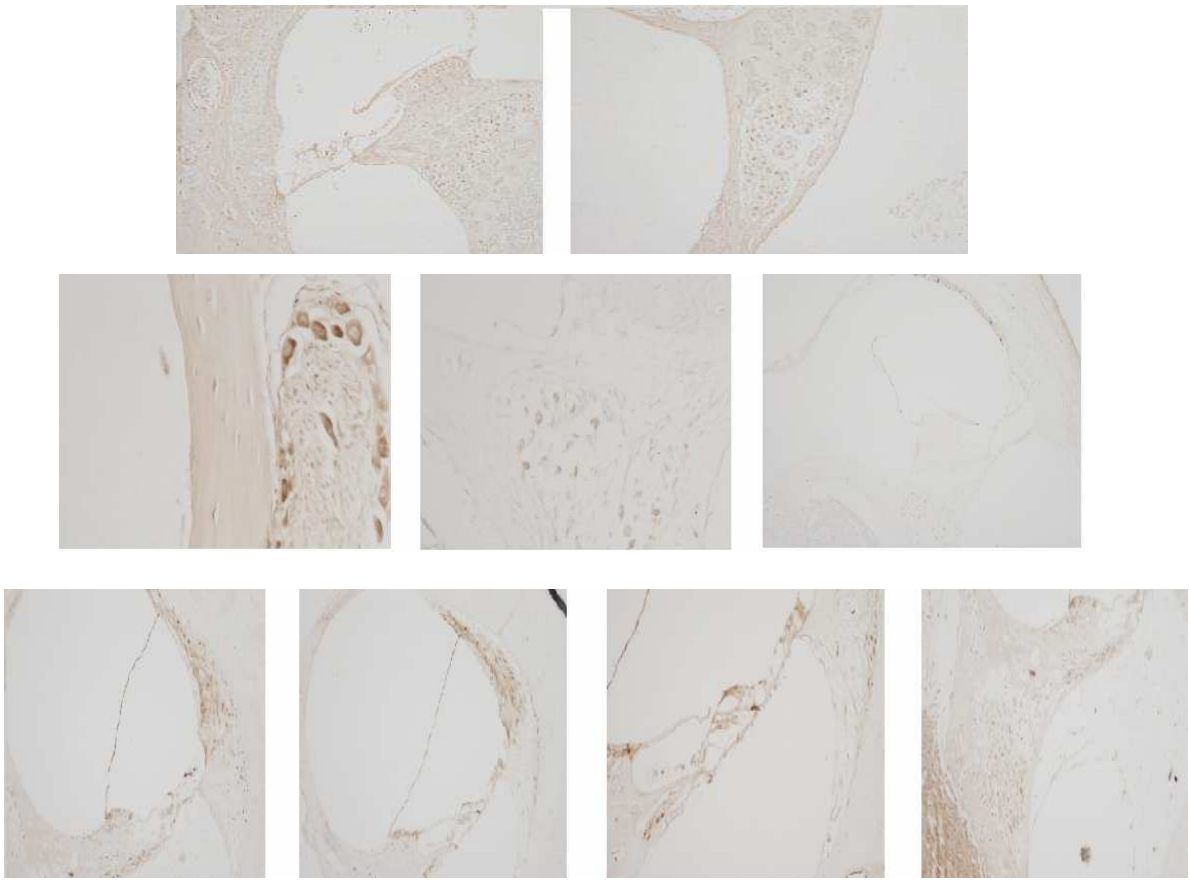


Figure 3.15 *TrkB detected in cochleas from rat (first lane), mouse (second lane) and guinea pig (third lane).*

A simpler and quicker method to detect the ectopic TrkB is having it fused with a fluorescent protein like GFP.

Having the receptor traceable in transfected cells could help us in the creation of a stable cell line containing TrkB.

Green fluorescent protein is one of the most useful reporter genes for marking transfected cells *in vivo*, it is expressed in the outer dermal layer of the Pacific Northwest jellyfish Aequorea victoria.

GFP autofluorescence provides a green bioluminescent light to the jellyfish in response to a calcium-dependent energy transfer step involving a GFP-associated protein called aequorin.

Exposure to ultraviolet light causes GFP to autofluoresce a bright green color within living cells, even in the absence of aequorin, calcium, or any other cofactor or substrate.

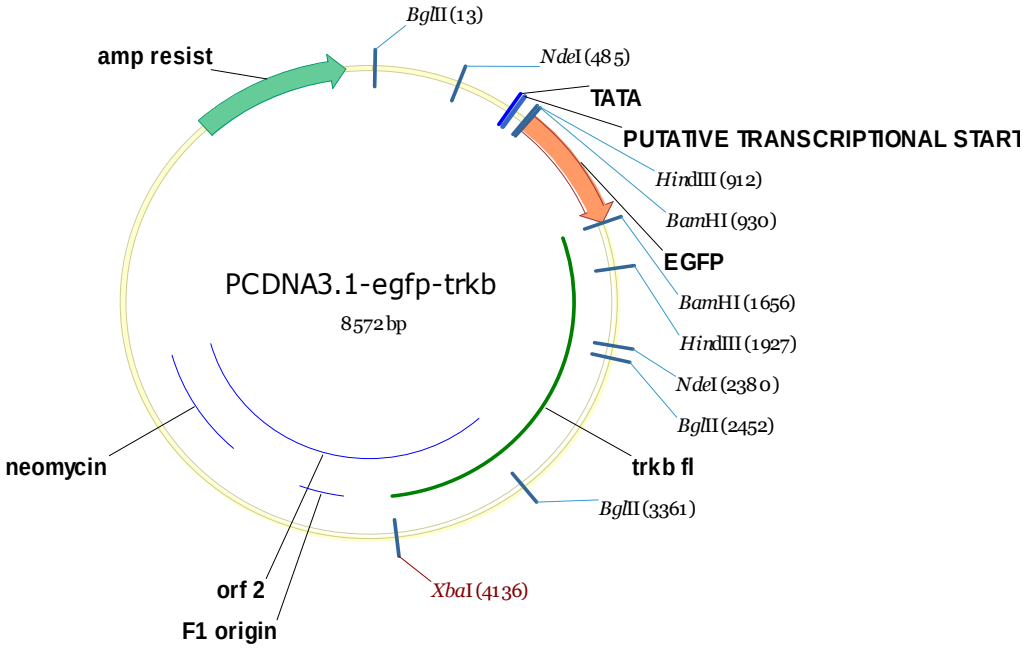


Figure 3.16 *pcDNA3.1 construct with TrkB and the gene coding for the green fluorescent protein GFP.*

We excised GFP sequence from the backbone of an EGFP vector and inserted that in the pcDNA3.1-TrkB vector we already had.

We successfully transfected cells as shown in figure 3.16.

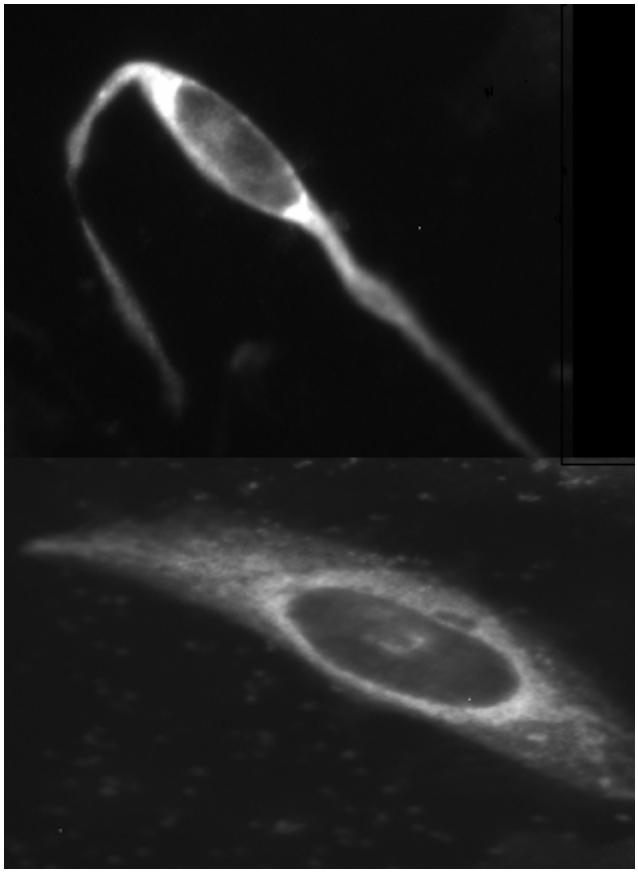


Figure 3.17 *Hela cells transfected with GFP-TrkB full length*

As the huge therapeutic potential of neurotrophins is known many attempts were made to obtain smaller and handier peptides, not all of them were successful and many times the mimetic molecules resulted in being antagonists instead (O'Leary and Hughes, 2003; Zhongcai et al, 2003).

A good way to narrow the different options is comparing peptides sequences obtained from different sources.

Considering the great homology of neurotrophic factors within species (Shelton et al, 1995) we took into consideration the possibility of performing the peptide selection in vivo, too as it had been done already (Rajotte et al 1998 Sergeeva et al, 2006).

We infact performed the same technique on guinea pigs cochleas.

After cochleostomy and incubation of the phages, caviae were sacrificed and cochleas collected.

Harvested phages were used to infect bacteria, and the pool of peptides could still be enriched as three rounds were performed.

4. CONCLUSIONS

Communication disability in profoundly deaf individuals can be alleviated by cochlear implants, which are electromagnetic devices that directly stimulate the sensory neurons.

In most cases in fact, auditory impairment results from the death of sensory hair cells in the Organ of Corti, while the auditory nerve remains functional.

This fact has great significance as cochlear implants work only if suitable numbers of sensory neurons are preserved.

The auditory sensory neurons usually undergo a slow, nearly complete degeneration in the absence of hair cells and this degenerative process accelerates when the entire Organ of Corti degenerates.

Prevention of death of auditory sensory neurons is thus of great therapeutic importance, even if hair cells cannot be rescued or regenerated.

A target delivery of therapeutic agents to the neurons of the spiral ganglion in the cochlea could be a huge enhancement in this sense.

The Brain Derived Neurotrophic Factor has been taken into consideration to direct nanoparticles with a payload of drugs towards TrkB receptors expressed in neurons' membrane.

Smaller peptides mimicking its effect have to be used as BDNF can't be administered.

One of the aims of the Nanoear European project we are involved in is attaching these peptides to nanoparticles that could then be injected into the inner ear.

In collaboration with Yorkshire Bioscience Ltd I worked to set all the parameters to perform the phage display technique.

This technique allows the selection of specific peptides using the natural receptor as a target.

My task was then to provide the target and the best conditions for the experiment to be carried on.

A study in literature was taken on to find the most representative target which is an isoform of BDNF receptor expressed in particular in the inner ear.

The gene coding for this receptor, TrkB, was obtained and transfected in mammalian cells in order to get the protein with its natural structure and all the post-translational modifications.

The more specific binding domain of the protein was also expressed in large quantities in bacteria and a protocol to purify it was optimized.

Based on the great homology among species, an in vivo phage display was also performed using guinea pigs' cochleas, which is still in progress.

This study lays the foundations for the screening of peptides mimicking BDNF and hopefully ameliorating cochear implant design and performances.

Even if the road to walk is still long, the results obtained here are the first promising steps in the process of finding what it is very likely to become a new kind of drug.

The peptides that are about to be screened, included in nanoparticles easier to administer in the inner ear in fact have a great potential either for the target delivery and, as drugs themselves, for the protection and the maintenance of the auditory system in cochlear prosthesis users.

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