4 RESULTS

4.1 PART I. Development of a large-scale *in situ* hybridization to screen for putative BDNF regulated transcripts expressed in a subpopulation of adult DRG neurons

The aim of this experiment was to develop a high through put *in situ* hybridization method for adult DRG neurons to find transcripts with a restricted expression pattern in the DRGs. We had an enriched DRG cDNA library for the transcripts influenced by BDNF (Shin, et al., Unpublished results). This was an enriched library obtained by a subtraction procedure that enriches the downregulated transcripts in BDNF mutant DRGs with respect to control DRG. This library was used to establish the technique. BDNF regulates the functional properties of SA neurons (Carroll, et al., 1998). It was observed that there was no loss of sensory neurons in BDNF-/- animals and adult BDNF mutants compared to control (Carroll, et al., 1998). In addition, administration of BDNF in the heterozygous mouse could restore the deficits in mechanosensitivity of slowly adapting mechanoreceptos (SAMs) back to and beyond that found in control. This clearly indicated that down stream targets of BDNF might be sufficient for a phenotypic expression. In DRGs, 20-40% neurons expressing BDNF receptor TrkB are SAMs (McMahon, S.B., 1994). We expected the transcripts influenced by BDNF to be expressed in this subpopulation of neurons.

In this first part of my Ph.D work, a high through put whole mount adult DRG *in situ* hybridization protocol was developed. Many transcripts with a subpopulation specific expression pattern were obtained by screening the enriched library. After the identification of clones, regulation of some interesting genes by BDNF was tested by quantitative-PCR (qPCR) for their expression level in BDNF mutant and control DRGs. It was also tested if the expression level of the regulated transcripts in DRGs could be regulated in the presence and absence of BDNF in DRG neuron primary culture by qPCR. Overview of experiments done is summarized in Fig.13.

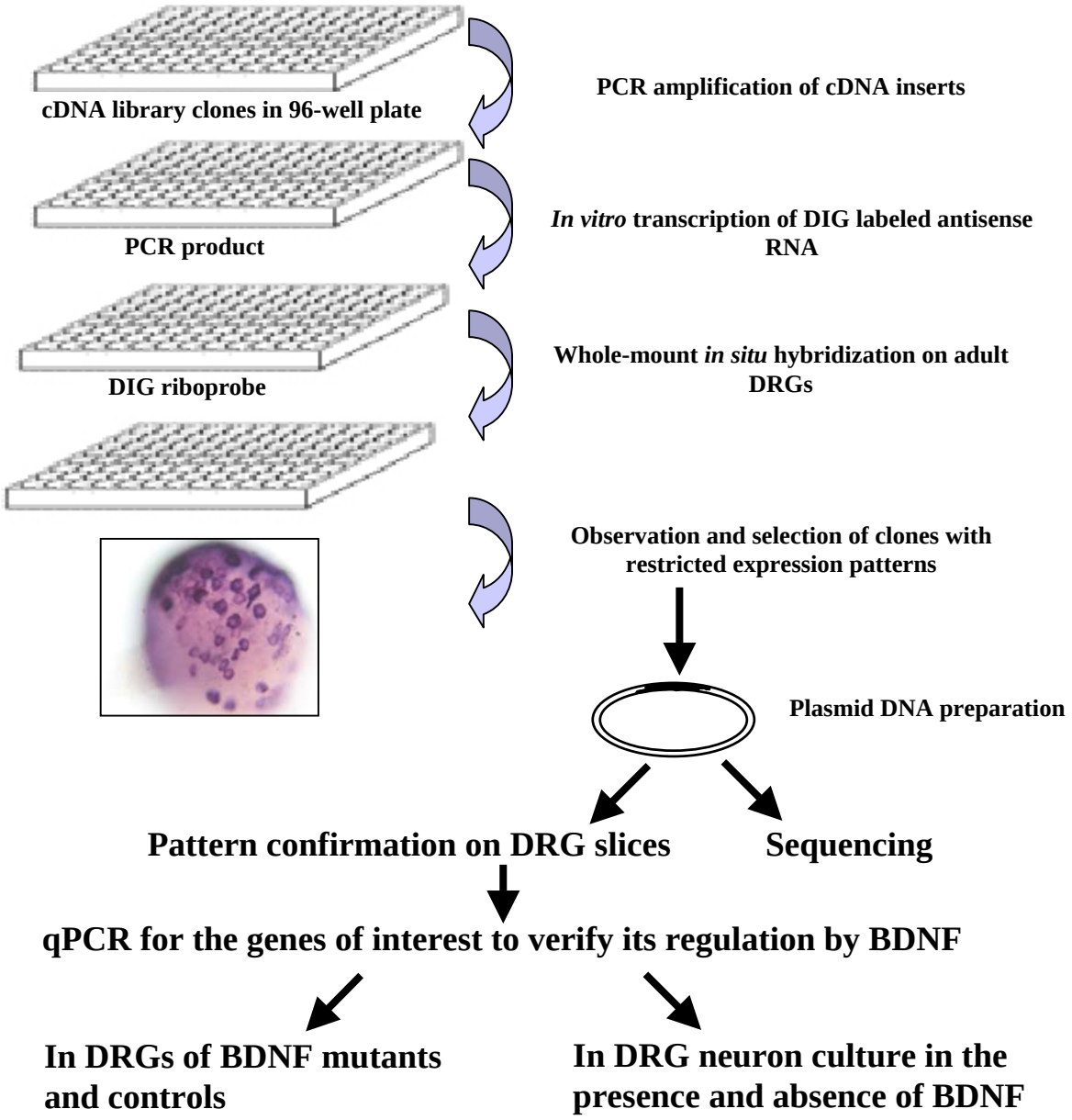


Fig.13 Overview of high-throughput whole-mount *in situ* hybridization and verification of candidate genes for the influence of BDNF in its expression

4.1.1 Identification of genes with a subpopulation specific expression pattern.

After the enrichment of the transcripts, by a PCR based enrichment, they were ligated and were used to transform the bacteria. After transformation, the transformants were sent to be picked and plated commercially (RZPD, Berlin, Germany). In total 2688 clones were picked and plated in a 384 well microtitre plates. To do the screening they were replated into 96 well plate format. A PCR based enrichment step was used in making the library. We screened 384 clones initially to see whether it would be possible for us to identify clones for cDNA that are selectively expressed in a subpopulation of DRG neurons. The majority of the tested clones (93.23%) did not have any expression or were expressed in a ubiquitous manner. Those clones with a restricted expression pattern were identified after sequencing (Fig.14). It was also observed that many clones were identified more than once (Table 03). Some wells contained multiple clones. These were not studied further. Clones with a restricted expression pattern were identified to be coding for mitochondrial proteins, proteins involved in cell adhesion, receptor proteins, proteins involved in calcium transport, protein folding, electron transport, microtubule based processes, different kinases and hypothetical proteins whose function is not yet known (Table 03).

We also wanted to verify the restricted expression pattern observed in whole mount *in situs* on DRG sections of control and on BDNF mutants. Similar expression pattern was observed for the clones obtained from whole mount *in situs* on *in situs* with DRG sections. (Fig.15). *In situ* hybridizations on sections were performed by our collaborators in Montpellier, France (courtesy Steeve Bourane, Stephanie Venteo and Patrick Carroll).

4.1.2 Many of the genes we found with a regulated expression pattern were also regulated by BDNF

Once we could find genes with a restricted expression pattern, we were interested to know whether these genes were down regulated in BDNF mutant DRG compared to control DRGs. We did qPCR experiments to target the expression level of the identified transcripts in control compared to the BDNF mutant DRGs. The results (Fig.16) indicated that some genes like Spock1, Paqr5, Tubb5, Aurkaip1 and Fstl1 were down regulated but some like Hsp90ab1, Prkar1a and Zwint were up regulated. For some genes, which includes Sncg, ATP synthase 6 and Hspa8 there was no statistically significant difference in the expression level. For other transcripts (Riken gene A230083H22, Tmem50a, Vbp1) the increase and in Anxa6 the decrease was not significant.

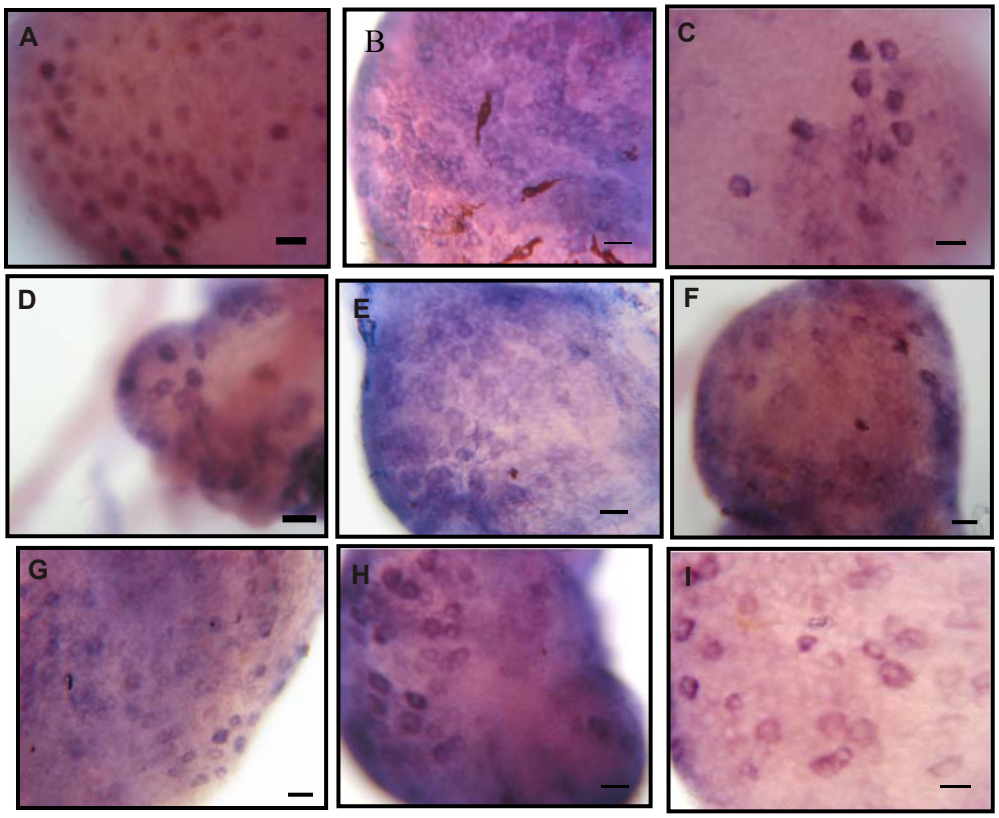


Fig.14. Examples of transcripts with a restricted expression in WT DRGs found after the library screening. Results of qPCR experiments comparing to the WT, showed Aurkaip1 (A), Tubb5 (B), Prkar1a (C) and Hspa8 (D) down regulated. On the other hand, Paqr5 (E) and Fstl1 (F) were upregulated. A Riken cDNA (A230083H22 gene) (G) whose function not yet known, seems to be upregulated. Transcripts which were not regulated by BDNF, but with a subpopulation specific expression pattern like ATP synthase 6 (H) and Persyn (I) were also found in the screen. Scale bar is 50µm.

4.1.3 Expression of transcripts in the presence and absence of BDNF using DRG primary neuronal cultures

As we could see the effect of BDNF in the expression level of different transcripts we tried to test whether the identified genes could be directly regulated in DRG cells by TrkB activation. We hypothesized that presence of BDNF could alter the transcription level of different transcripts and in that case we would observe a change in expression of the gene of interest. qPCR was done on primary DRG neuronal cell culture in the absence and presence of two different BDNF concentrations (100ng/ml and 500ng/ml). We were not able to observe any difference in the level of transcripts comparing in the presence and absence of BDNF on DRG neurons cultured for 18 hrs (Fig.17). We were looking for the expression level of transcripts that were observed to be regulated in DRG neurons *in vivo*. We observed no change in the transcription level for the tested transcripts in the presence and absence of BDNF.

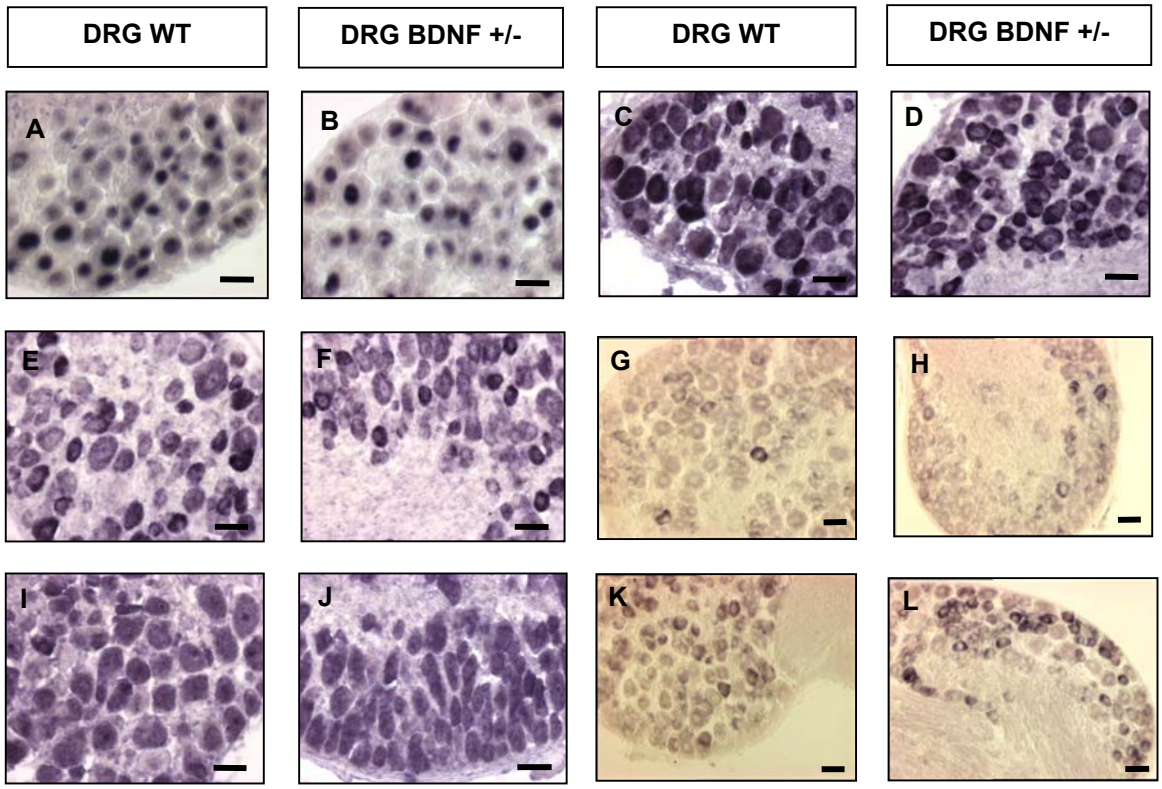


Fig. 15. *In situ* hybridization on WT and BDNF+/- DRG sections. Expression of different transcript showing subpopulation specific prominent expression pattern. Aurkaip1(A, B), Tubb5 (C,D), Fstl1 (E,F), Prkar1a (G,H), Riken cDNA A230083H22 gene (I,J), and Sncg (K,L). Scale bar is 50µm.
(Coutesy: Steeve Bourane, Stephanie Venteo and Patrick Carroll)

Clone No.	Gene	No. of Clones Repeated	Ref Seq ID	qPCR	Common name and Function
1	Riken gene.	01	NM_027532 3200002M19Rik	NO	Unknown
2	Sncg	06	NM_011430	YES	Persyn or γ.synuclein. Unknown
3	Spock 1	01	NM_009262	YES	Testican, Cell adhesion, motility, proliferation, nervous system development
4	Pdxk	01	NM_172134	N O	pyridoxal kinase, Pyridoxin metabolic process
5	Paqr5	01	NM_028748	YES	Progestin and adipoQ receptor family member V, Receptor family protein
6	Anxa6	01	NM_013472	YES	Annexin A6, Calcium ion transport, regulation of muscle contraction.
7	mKIAA0528	01	AK172966	YES	Unknown
8	Vbp1	01	NM_011692	YES	von Hippel-Lindau binding protein 1, Protein folding
9	Qrsl1	01	AK038801	NO	glutaminyl-tRNA synthase (glutamine-hydrolyzing)-like 1, t-RNA synthase
10	mt-Co3	01	AK131579	YES	cytochrome c oxidase III, mitochondrial, Electron transport
11	Tubb5	01	NM_011655	YES	Tubulin, beta 5 ,Microtubule based process, mitotic spindle assembly
12	RIKEN cDNA A230083H22 gene	01	BC094224	YES	Unknown
13	Zwint	01	NM_025635	YES	ZW10 interactor (ZW10-interacting protein 1), Exocytosis
14	Prkar1a	01	NM_021880	YES	protein kinase, cAMP-dependent, regulatory, type I, alpha , Cell proliferation, mesoderm formation, organ morphogenesis, protein aminoacid phosphorylation, signal transduction
15	Aurkaip1	01	NM_025338	YES	Aurora kinase A interacting protein 1, Mitosis, Positive regulation of proteolysis, Protein ubiquitination
16	Hsp90ab1	01	NM_008302	YES	Heat shock protein HSP 90-beta, Positive regulation of nitric oxide biosynthetic process, response to unfolded protein
17	ATP synthase 6	01	AK167037	YES	ATP synthesis
18	Fstl1	02	NM_008047.2	YES	follistatin-like 1, Unknown
19	Hspa8	01	NM_031165	YES	Heat shock cognate 71 kDa protein (Heat shock 70 kDa protein 8), Chaperon cofactor-dependent protein folding, protein folding, regulation of progression through cell cycle.
20	Tmem50a	01	NM_027935	YES	Transmembrane protein 50 A, Unknown

Table: 03: Interesting transcripts with a restricted expression pattern obtained after screening the library.

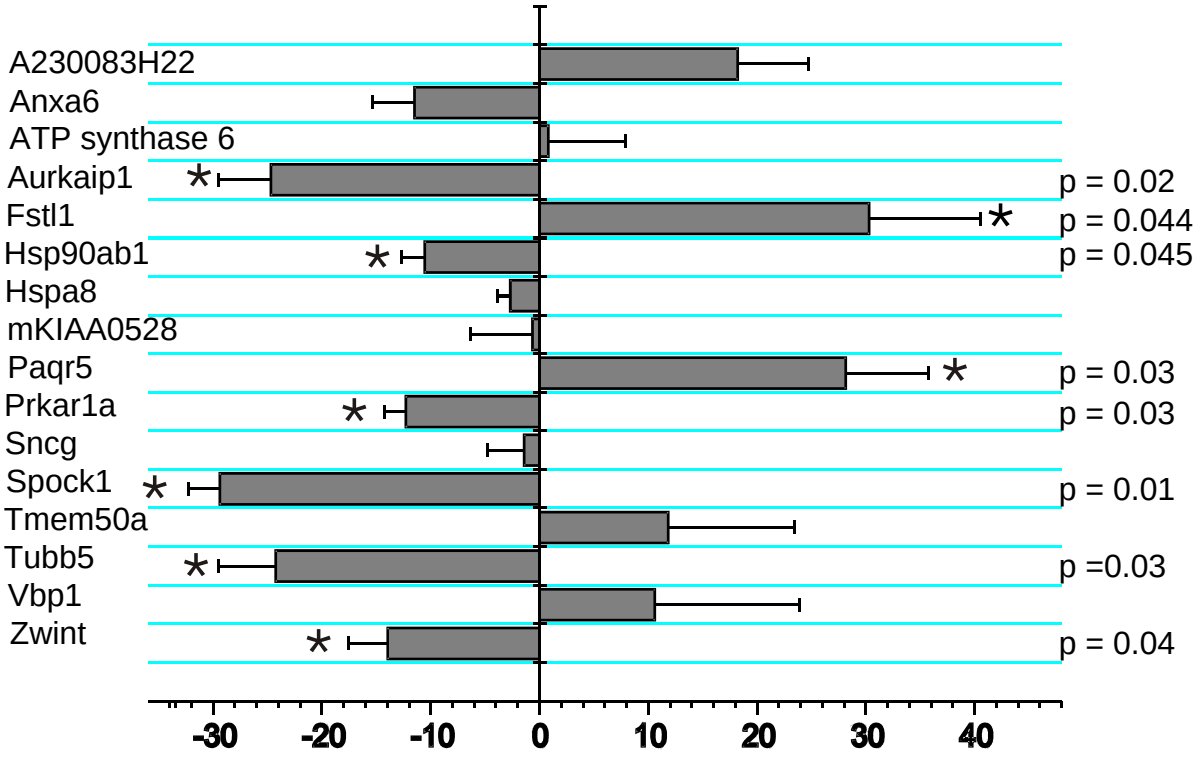


Fig.16. Percentage change of expression level of different transcripts obtained from the screen in heterozygote with respect to control. Different genes with a restricted pattern of expression observed in the high throughput whole mount *in situ* hybridization were selected for TaqMan PCR. Most of the genes were down regulated in the heterozygote.

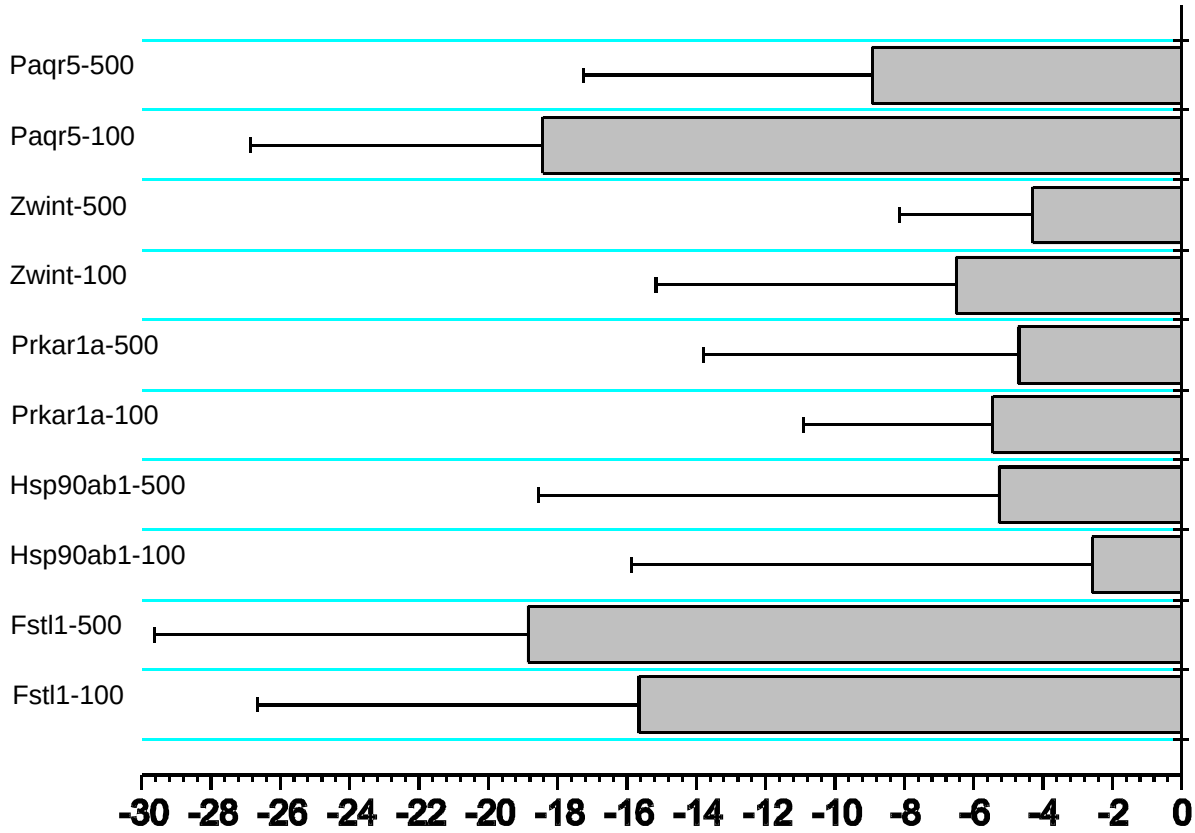


Fig.17. Percentage change of the expression of different transcripts with respect to no BDNF treatment. RNA from DRG neuron primary culture in the absence and in the presence of BDNF (100ng/µl and 500ng/µl) was used for qPCR of some of the genes, which were found to be significantly regulated in BDNF heterozygous DRGs.

4.2 PART II: Electrophysiological, anatomical, molecular biological and behavioral characterization of NT3+/-//NT4-/- double mutants (NT34 mutants)

In the second part of my work, I characterized the NT34 mutant to find molecule(s) involved expressed by NT3 and NT4 dependent sensory neurons. NT3 is shown to be important for the survival of proprioceptive neurons (Enfors P et al., 1994), and survival of D-hairs and Slowly adapting mechanoreceptors (Airaksinen MS et al., 1996). NT4 is shown to be important for the survival of D-hair mechanoreceptors later in the adult animal (Stucky CL et al., 2002). In the present study, we are looking at the defects of NT3+/-, NT4-/- double mutant DRG neuron function by doing electrophysiologicl recordings in an *in vitro* skin nerve recording, anatomically by electronmicroscopy and the varied expression level of different genes at the molecular level using gene chips. An over view of the experiments done is outlined in Fig:18.

4.2.1 Electrophysiology results

To characterize different sensory neuron types and their physiological properties age matched NT34 mutant and control mice were analyzed electrophysiologically using an *in vitro* skin nerve preparation. All the afferents were tested for stimulus response and Aß fibers were tested for velocity sensitivity. Classification of the units recorded is shown in Table 4.

Receptor type	**Control**			**NT34 mutant**		
	% Total	CV **m/s**	**vFT** (mN)	**% Total**	CV **m/s**	**vFT** (mN)
Aβ-Fibers						
RA	31.25 (5/16)	11.97±0.71	0.92 (0.4-1.4)	37.5 (9/24)	14.08±1.32	3.52 (0.4-1)
SA	68.75 (11/16)	16.81±1.78	1.55 (0.4-6.3)	62.5 (15/24)	13.4±0.77	1.43 (0.4-6.3)
Aδ-Fibers						
AM	57.14 (4/7)	3.23±1.6	4.175 (1.4-10.0)	82.61 (19/23)	5.01±0.55	2.31 (0.4-6.3)
D-hair	42.86 (3/7)	6.26±0.84	0.4	17.39 (4/23)	7.83±1.29	0.4

Table 4. Numbers and properties of of sensory afferents recorded

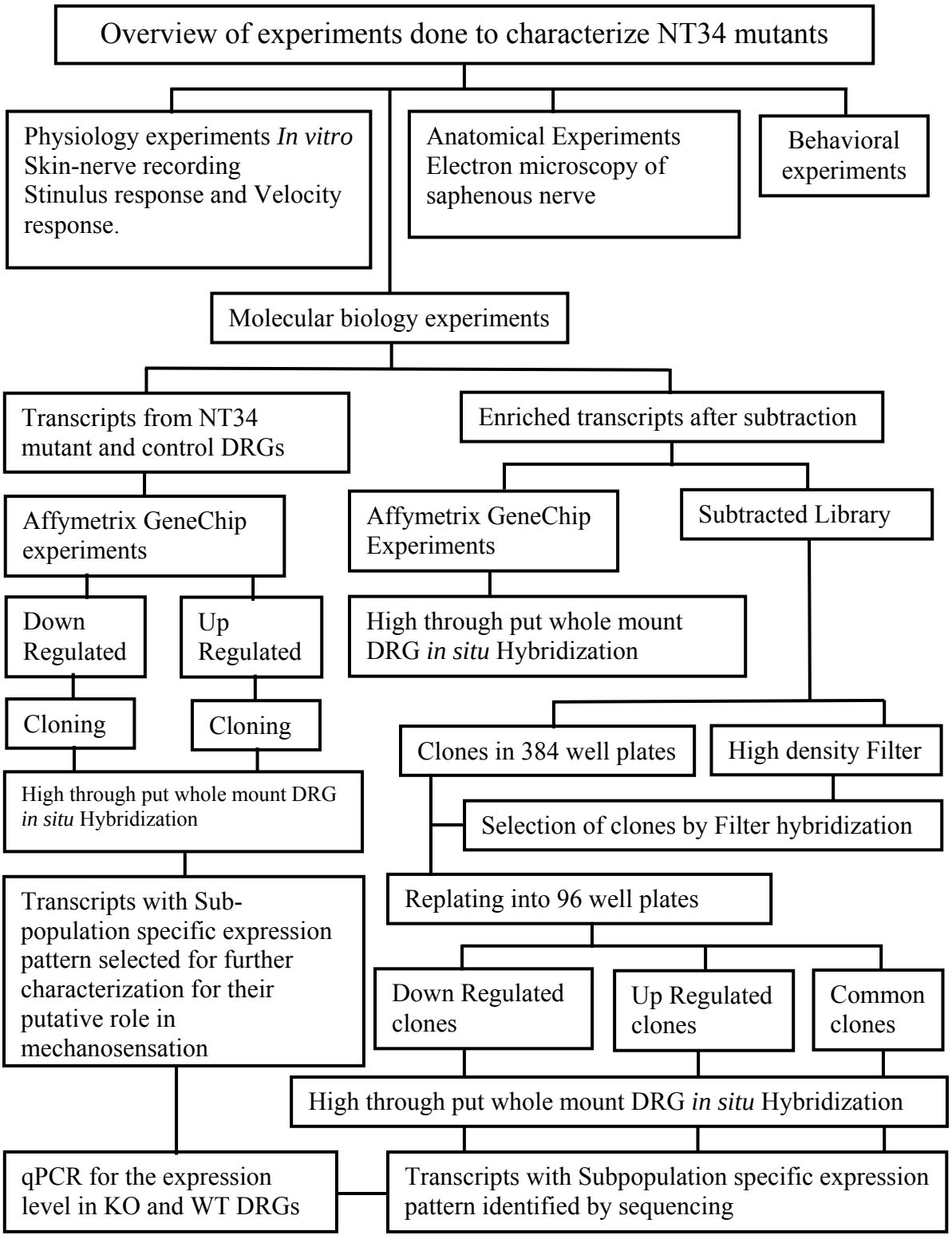


Fig. 18. Overview of experiments performed with NT34 mutants

4.2.2 No change was observed in the physiological properties of sensory afferents

There were previous reports indicating the loss of D-hair mechanoreceptors and slowly adapting mechanoreceptors in NT3+/- mice (Airaksinen, et al., 1996). NT4-/- mice showed loss of D-hair mechanoreceptors after 5 weeks of age. We used adult mice aged over 12 weeks (Stucky, et al., 1998, 2002). Using an *in vitro* skin nerve preparation we characterized different sensory neuron types and their physiological properties. All the afferents were tested for stimulus response and Aß fibers were tested for velocity sensitivity. The stimulus response data obtained from the control animals in these experiments was decreased in comparison to other control data (Christiane Wetzel). For a better assessment, control data obtained by CW is also plotted with my data. It was observed that the control data does not vary in different strains (Milenkovic N., et al., Unpublished data).

No change was observed in the physiological properties of all the different types of neurons assayed. Stimulus response of slowly adapting mechanoreceptors (SAMs) is shown in Fig. 19. As SAMs are sensitive to the movement of the probe sensitivity was measured during the movement of the probe (phasic response) and there was no change observed (Fig. 20).

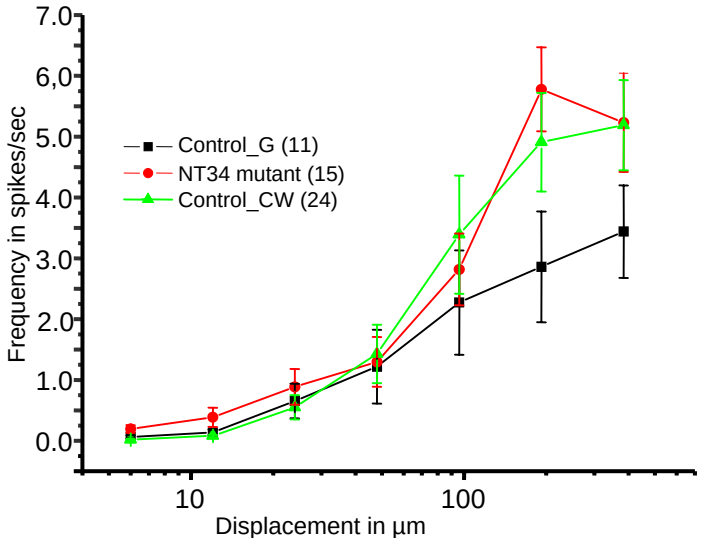


Fig. 19. Stimulus response of slowly adapting (SA) neurons of NT34 mutnat in comparison to controls. Control_CW is the reference data available in the lab.

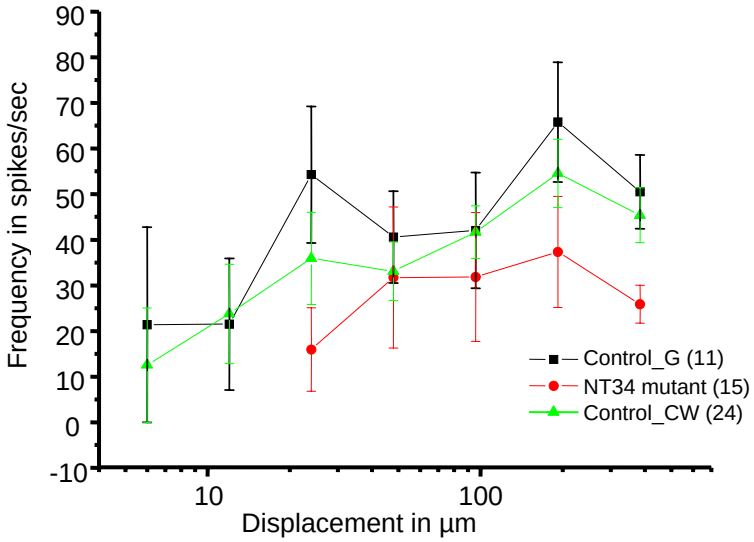


Fig. 20. Stimulus response of NT34 mutant slowly adapting (SAM) neurons in comparison to control datas. Sensitivity of the afferent during the movement of probe is analyzed. Control_CW is the reference data available in the lab.

Rapidly adapting mechanoreceptors (RAM) and D-hairs respond to movement of the probe. The sensitivity of these afferents was measured during the movement of the probe and no change was observed (Fig.21, Fig.22).

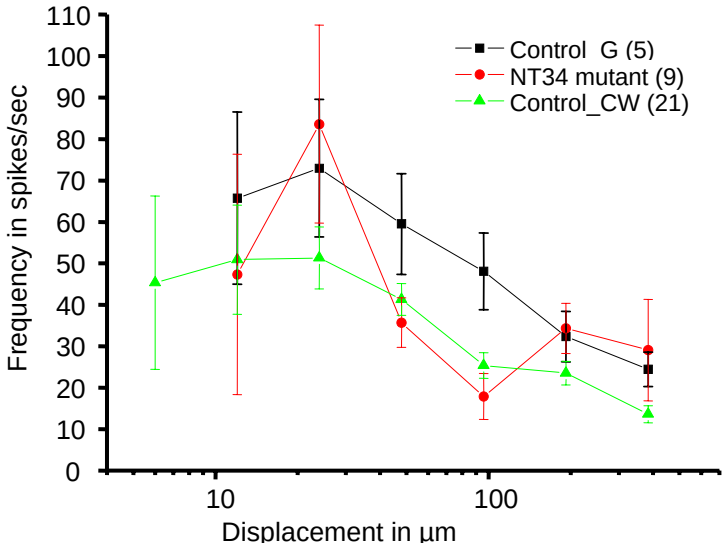


Fig. 21. Sensitivity of rapidly adapting mechanoreceptors of NT34 mutant and controls during the movement of the probe (phasic response). Control_CW is the reference data available in the lab.

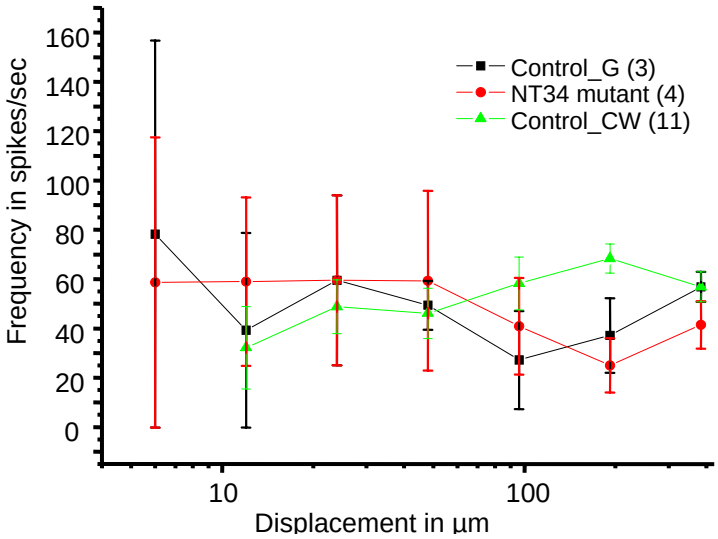


Fig. 22. Sensitivity of D-hairs of NT34 mutant and controls during the movement of the probe (phasic response). Control_CW is the reference data available in the lab.

AM fibers were tested for its sensitivity for different displacements of the probe. There was no change observed between the NT34 mutants and the control animals (Fig. 23).

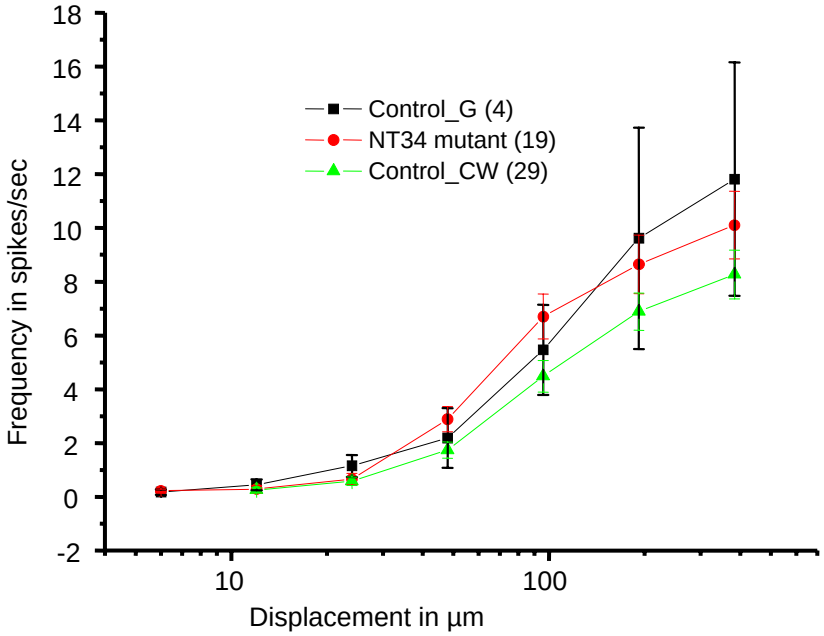


Fig.23. Sensitivity of AM fibers of NT34 mutant and controls during different displacement of the probe (Stimulus response). Control_CW is the reference data available in the lab.

4.2.3 Electron microscopy results:

The aim of this experiment was to assess quantitatively the loss of neuritis. The saphenous nerve was chosen, as it is purely cutaneous sensory nerve and well characterized anatomically and physiologically. It is reported that there is a loss of neurons in NT3 -/- as well as in NT3+/- mice (Ernfors, et al., 1994, Airaksinen, et al., 1996). NT4-/- is also shown to loose neurons, both during developmental (Conover, et al, 1994, Ernfors, et al., 1994) as well as in the adult stage (Stucky, et al., 2002). While dissecting the saphenous nerve it was noted that the nerve was extremely thin. We used NT34 mutants, NT3+/- and control age matched adult animals to compare electron micrographs of the saphenous nerve. We observed about 62.57% reduction in the myelinated fibers of NT34 mutants (227.75±29.04) where as it was about 28% for NT3+/- animals (438.33±33.51) compared to the controls (608.5±42.5). For unmyelinated fibers the percentage of reduction was 68% for NT34 mutants (832.10±77.98) and 47% for NT3+/- animals (1367.20±13.17) compared to controls (2594.87±585.75) (Fig.24).

4.2.4 Results from Molecular Biology Experiments:

We showed that there is a very marked decrease in both myelinated and unmyelinated axons in the saphenous nerve of NT34 mutants. We screened for genes whose expression is enriched or depleted in the DRGs of NT34mutants compared to controls. Such transcripts may be selectively expressed in NT3/4 dependent neurons (decreased) or NT3/4 independent neurons (increased). To find out these transcripts, we used a two-pronged strategy. One using an enriched pool of transcripts for down regulated genes in NT34 mutant obtained by suppression subtractive hybridization (SSH), by which a library was made and screened for transcripts with a restricted expression pattern in DRG neurons and one round of affymetrix gene chip experiments. In the second strategy I carried out gene chip experiments to compare the expression level of transcripts in control and NT34 mutant adult DRGs. Both upregulated and down regulated transcripts were cloned and the expression of regulated genes examined using whole mount DRG *in situ* hybridization procedure described previously. q-PCR was done to confirm the change of expression for a few of the interesting genes with a restricted expression pattern in adult control DRGs.

4.2.4.1 Experiments with enriched transcripts by SSH:

In the forward SSH protocol, we tried to enrich the transcripts down regulated in the NT34 mutant. In brief, adaptors are ligated to transcripts from control DRGs and transcripts from NT34 mutant DRGs were used to suppress the amplification of common transcripts during the pcr step. Because of the presence of adaptors, only the unsuppressed transcripts from Control DRGs are amplified. The library obtained is termed as "forward subtracted library". To enrich the upregulated transcripts in the NT34 mutant same procedure is repeated reversing the role of transcripts from NT34 mutant and control DRGs. The library thus obtained is termed as "reverse subtracted library". To identify the transcripts expressed in a subpopulation of DRG neurons, forward subtracted transcripts were used to make a library.

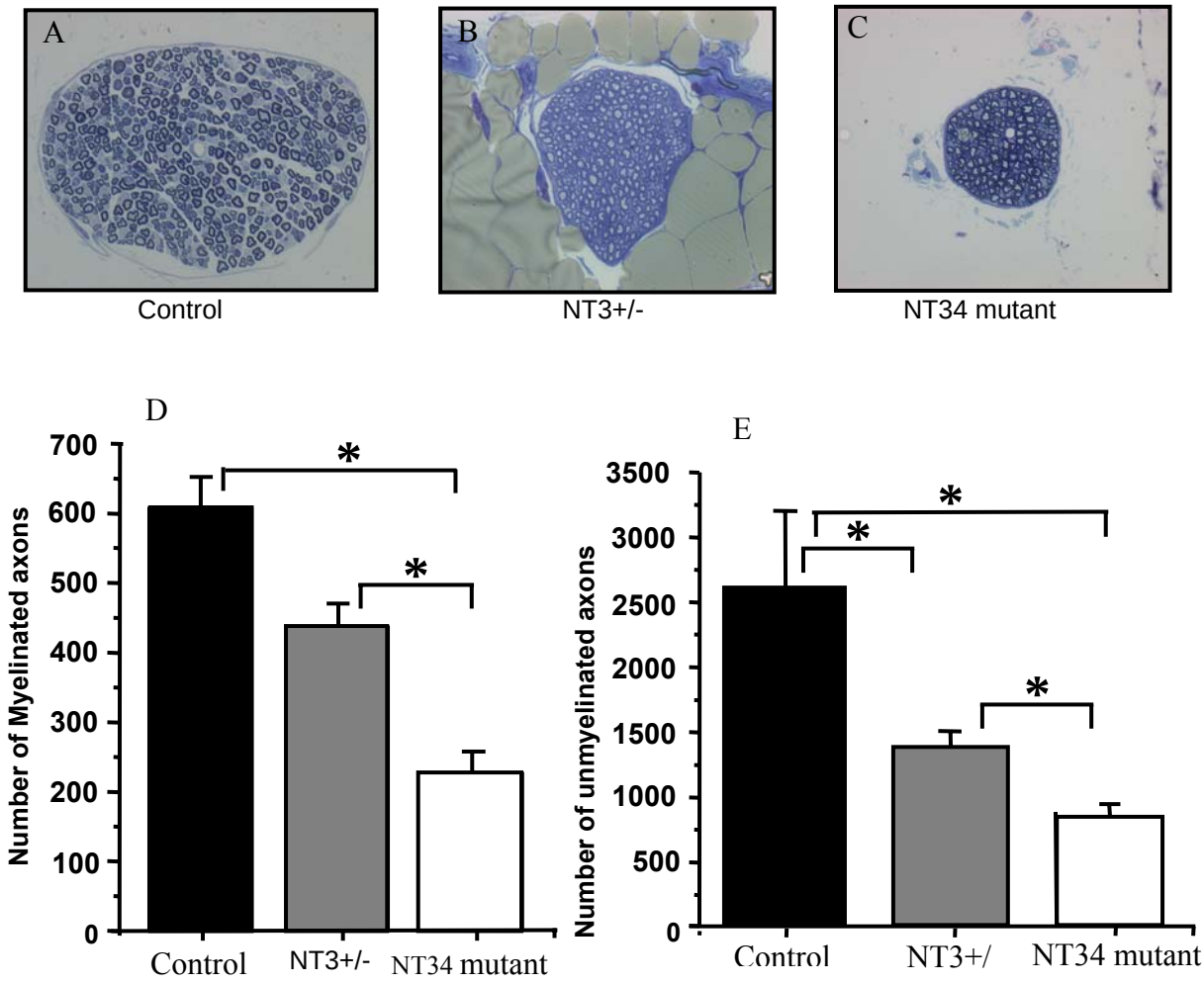


Fig.24. Electron microscopy analysis shows both myelinated and unmyelinated neurons are decreased in NT34 mutants in comparison to control and NT3+/- animals. A, B and C are semithin section of saphenous nerves from control, NT3+/- and NT34 mutants respectively. D) Comparison of myelinated axonal counts of Control, NT3+/- and NT34 mutants. E) Comparison of unmyelinated axonal counts of control, NT3+/- and NT34 mutants.

4.2.4.1.1 Library construction:

Enriched library for the transcripts that were regulated in the NT34 mutant DRGs was made. From the previous experiments with the subtracted BDNF library, it was observed that upregulated genes were present in the library enriched for down regulated transcripts. By doing the subtraction in both directions, down regulated and up regulated transcripts were separately enriched. The idea was to screen down regulated library which was spotted on to high density filters, with both the enriched pool of transcripts (forward subtracted for down regulated and reverse subtracted for up regulated transcripts) to identify the clones that were down-regulated and up-regulated. A total of 2688 clones of forward subtracted library were picked and spotted on to filters in duplicates and the corresponding clones cultured in 384 well plates. Picking up the colonies, spotting on to the filters and culturing them in 384 well plates were done commercially (RZPD, Berlin). This is termed as subtracted NT34 library for further reference.

4.2.4.1.2 Identification of clones with enriched transcripts in the subtracted NT34 library:

Enrichment was done in a pcr based protocol and hence a very high back ground of clones are expected with transcripts that are not enriched. So to avoid processing the clones with nonenriched transcripts, hybridization experiments were carried out. Clones with enriched transcripts were selected with their higher signal intensity compared to the unsubtracted control. By this procedure we could reduce the amount of clones to about 22% of the original library (Fig. 25). Clones with higher signal intensities were manually selected. Because of the difficult to exactly assign the clone position and in order to avoid missing clones of interest; selection was done in a lenient way. Out of 2688 clones, we selected 585, which were grouped as down regulated (398), up regulated (79) and 108 clones as common clones. Down regulated clones were from the hybridization with forward subtracted probes, upregulated clones were from the hybridization with reverse subtracted probes and the common clones were the ones which were present in both the lists. These were replated in to 96 well plate format. As internal controls, 216 clones, which showed no detectable signal or no enrichment, were also selected and replated.

4.2.4.1.3 Identification of clones with a subpopulation specific expression pattern:

Out of 585 selected clones, 344 clones (58.8%) showed a restricted expression pattern in DRG neurons. Among these 301 clones (87.5%) were found repeatedly in the selected clones (repeated by >1 clone). Examples of some of the clones with a restricted expression pattern are shown in Fig. 26 and the general list is described in Table 5.

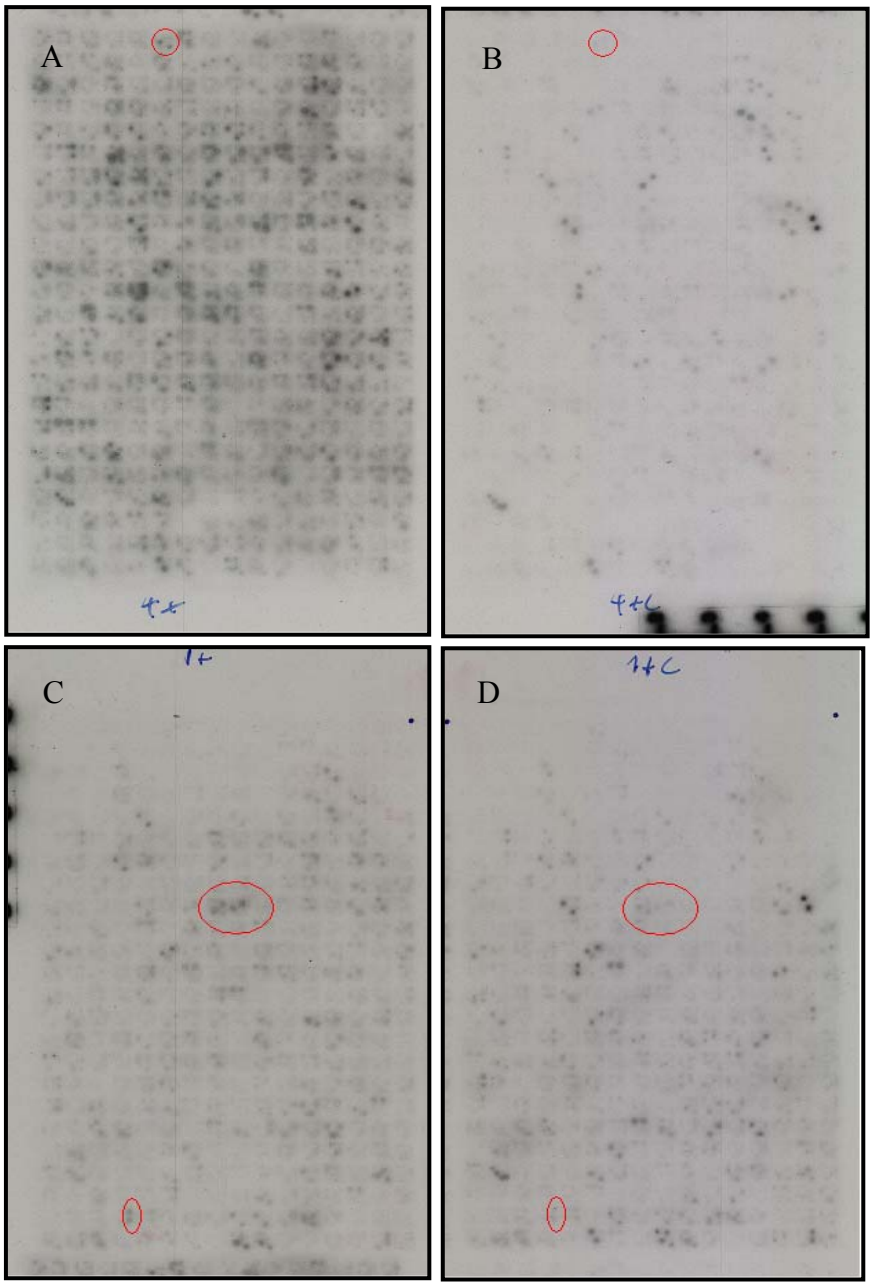


Fig. 25: Selection of clones for enriched regulated transcripts for whole mount DRG hight-thoughput insitu hybridization. A,B are High density filters hybridized with probes from forward subtracted PCR products and unsubtracted control respectively for the identification of enriched down regulated transcripts. C,D are hybridized with probes from reverse subtracted PCR products and unsubtracted control respectively for the identification of enriched up regulated transcripts present in the library. Clones with more signal intensity compared to the unsubtracted controls (like with red circles) were selected and replated.

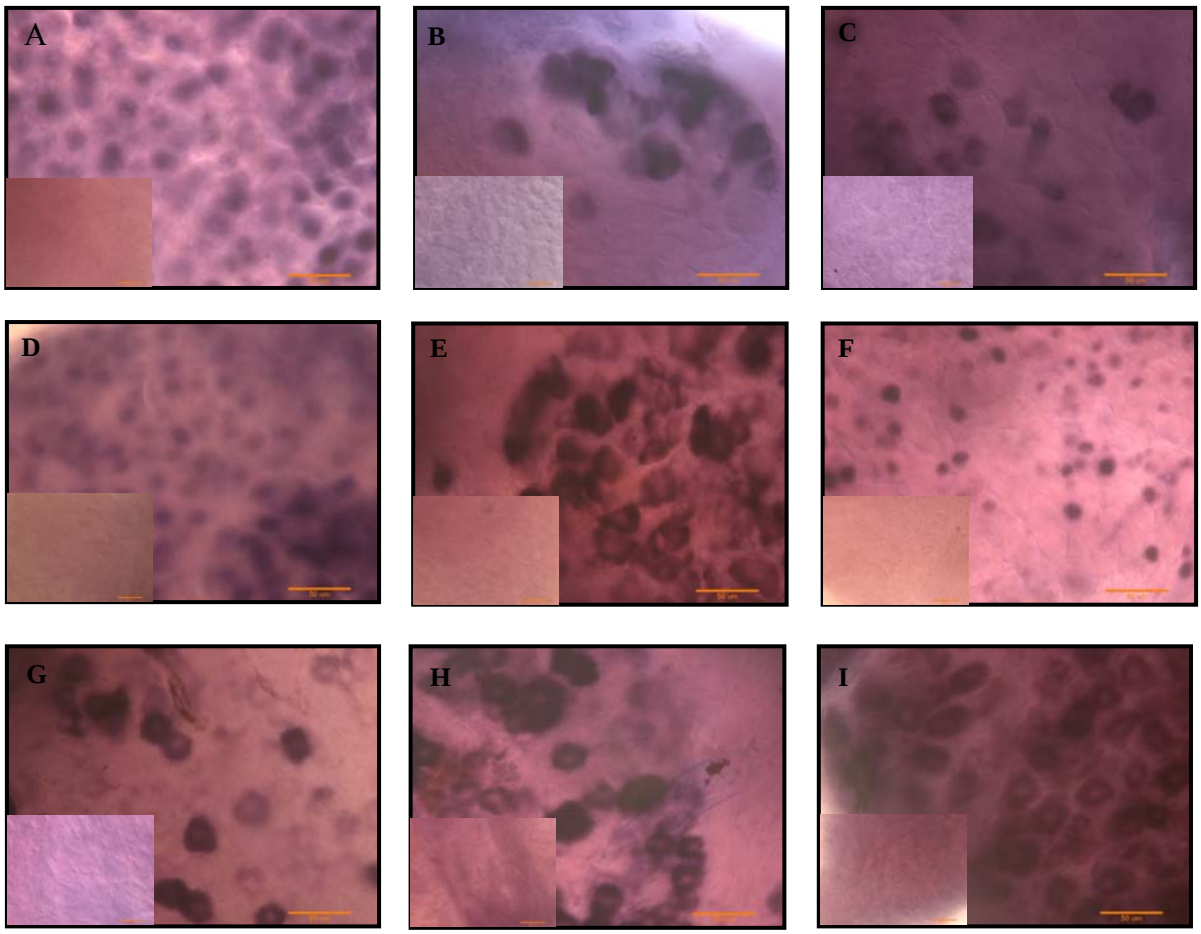


Fig.26: Some of the clones isolated from the enriched library, with a restricted expression pattern. A) Thymosin beta, B) Tubulin alpha 1, C) Hsp90ab1, D) Clone with no annotation, E) Sncg, F) Ramp2, G) Ppia, H) CD9, I) Gpsn2. Scale bar is 50 µm.

Sl no	No clones	D*	U#	C†	Name of the gene (Blastx)
1	39	35	1	3	Cytochrome oxidase B
2	37	21	0	16	Mitochondrial genome
3	26	25	0	1	NADH dehydrogenase 1
4	28	21	0	7	Cytochrome C oxidase
5	22	1	13	8	Partial similarity to thymosin beta
6	20	12	0	8	Tubulin alpha 1
7	19	18	0	1	Calmodulin (Calm2)
8	15	6	0	9	Hsp90ab1
9	8	1	6	1	No annotation
10	8	6	0	2	ATP8_Mouse
11	8	7	0	1	Cytochrome B
12	7	7	0	0	Thymosin, beta 4
13	6	5	0	1	Thymosin.
14	6	3	1	2	Persyn (Sncg)
15	5	0	0	5	Solute carrier family 20, member 2 (Slc20a2) intron
16	5	4	0	1	Receptor (calcitonin) activity modifying protein 2
17	5	4	0	1	Receptor (calcitonin) activity modifying protein 2
18	4	0	1	3	Partial similarity to Persyn
19	4	1	0	3	*Mus musculus* peptidylprolyl isomerase A (Ppia)
20	3	3	0	0	*Mus musculus* aldolase 1, A isoform (Aldoa)
21	3	3	0	0	ATPase inhibitory factor 1
22	3	3	0	0	NADH dehydrogenase subunit 1
23	2	0	1	1	*Mus musculus* tubulin, beta 3 (Tubb3)
24	2	2	0	0	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, ENSMUSG00000068487
25	2	2	0	0	NADH dehydrogenase 4, mitochondrial
26	2	2	0	0	*Mus musculus* ribosomal protein L41 (Rpl41)
27	2	2	0	0	*Mus musculus* myosin light chain, regulatory B (Mylc2b)
28	2	2	0	0	*Mus musculus* RIKEN cDNA 2010107E04 gene (2010107E04Rik)
29	2	1	0	1	CD9 antigen
30	2	0	0	2	*Mus musculus* strain VM mitochondrion,
31	2	1	0	1	*Mus musculus* thioredoxin 1 (Txn1)
32	2	2	0	0	*Mus musculus* glycoprotein, synaptic 2 (Gpsn2)

*Clones selected as Down regulated, # Clones selected as Up regulated, † Clones selected as Common clones.

Table 5: Annotation of repeated clones with a restricted expression pattern.

4.2.4.2 Oligo nucleotide array experiments:

Affymetrix Mouse Genome U74v2 set was used for the array experiments. Three independent experiments were performed to compare the gene expression in the adult age matched NT34 mutant DRGs and control DRGs. All animals used were more than 3 months old. In addition one experiment with subtracted and unsubtracted pool of transcripts was also performed. All the internal quality control criteria were satisfied in all stages of the experiment.

4.2.4.2.1 Comparison of the expression profile of adult DRGs from NT34 mutant and control mice:

Data from the experiments were analyzed to obtain two different populations of genes. First the genes those were down regulated in the NT34 mutants and second the genes that were up regulated. The criteria used for the selection were

- a) should be called present or marginally present
- b) it should be changed at least 1.5 times (fold change)
- c) the regulation should be observed in at least two out of three experiments.

86 genes matched these criteria that were down regulated in NT34 mutant where as 50 genes were found to be up regulated.

4.2.4.2.2 Search for genes with a subpopulation specific expression pattern

The candidate genes were cloned and *in situs* performed on whole mount DRGs to identify transcripts with a restricted expression pattern. Probes in the chips are designed to target a specific region in a transcript, which is called as a target sequence. It varies irrespective of the size of gene. This was taken for designing the primers to clone the probes intended for *in situ* hybridization. Probes ranging from 100 to 400 bp were cloned depending upon the size of the target sequence. If the target sequence was very small or if there could be no primers designed, then the consensus sequence was taken for designing the primer in such a way that the primer binding sites were at the target sequence or flanked the target sequence. In other words, the amplified product contained the full target sequence or a part of it.

4.2.4.2.3 Some of the transcripts with an altered expression level in NT34 mutants are expressed only in a subpopulation of adult DRG neurons:

Out of 87 down regulated candidate genes, 71 were cloned and whole *in situs* performed on whole mount DRGs. 20 clones (28%) showed a subpopulation specific expression pattern (Table.6, Fig. 27).

Groups	Gene names	No of genes
Extracellular matrix/cell adhesion molecules	**Cd44, Spp1, Vsnl1,**	3
Ion channels, channel modulators and membrane transporters	Scn4b, Clcn3, **Atp2b2,** Kcnd1, **Atp1a3, Ensa,** Atp5e, **Atp6v1h,** Atp1a1, Slc17a7, Atp6v0a1,	11
Receptors	Gpr64**, Gabbr1,** Gfra2, Dp1, Sorl1,	5
Other membrane proteins	Disp2, Tmem2, Tm4sf3**,** Vamp2,	4
Microtubule and transport associated	**Kif5b,** Desmuslin, **Mtap1a,** Mapre3, Phactr1, Dncli2, 6720463E02Rik,	7
Non annotated transcripts	138408_at, **2010315L10Rik, 1810020D17Rik,** AA415817, **D13Bwg1146e, 114131_at, 2900075B16Rik,** B130005I07Rik, 135232_at, 3830431G21Rik, 1110054A01Rik, C030027L06Rik, 5730496F10Rik, **166856_f_at,** E130013N09Rik, 1500004A13Rik, BC026657, 110291_at, 139147_at, 2810436B12Rik, E230022H04Rik, 2900073H19Rik,	22
Synapse associated proteins	**Lynx1**,	1
Transcription factors and associated proteins	Pou4f2, Runx2, Ap1gbp1, Mga, Runx1,	5
Calcium binding proteins	Syp, Pcp4,	2
Lipid binding proteins	Stard4, Cpne3, Fabp3,	3
Nucleotide binding proteins	Rit2, Sept4, Sept3,	3
Proteins binding to other molecules	Cadps2,	1
Kinases	**Dapk1, Ak1,** Pik3r1, **Dgkh**, Pik3r1	5
Enzymes other than kinases	Metap1, **Chd5**, Lipin1**,**	3
Electron transport	Ndufa5, BC034664, Cox6c,	3
Stress induced	Stip1,	1
Immune response related	Ccl27,	1
Signaling associated	Rassf3, Arhgef12, Rapgef5, Apc,	4
Heat shock proteins/protein folding proteins	Pfdn2,	1
Oncogene family	Rab6,	1
Other proteins not in the above categories	**Sncg,4**	1

Table 6: List of genes that are found to be down regulated in NT34 mutant DRG in Affymetrix experiment. Name of genes written in red showed a subpopulation specific expression pattern in whole mount DRG *in situ* hybridizations.

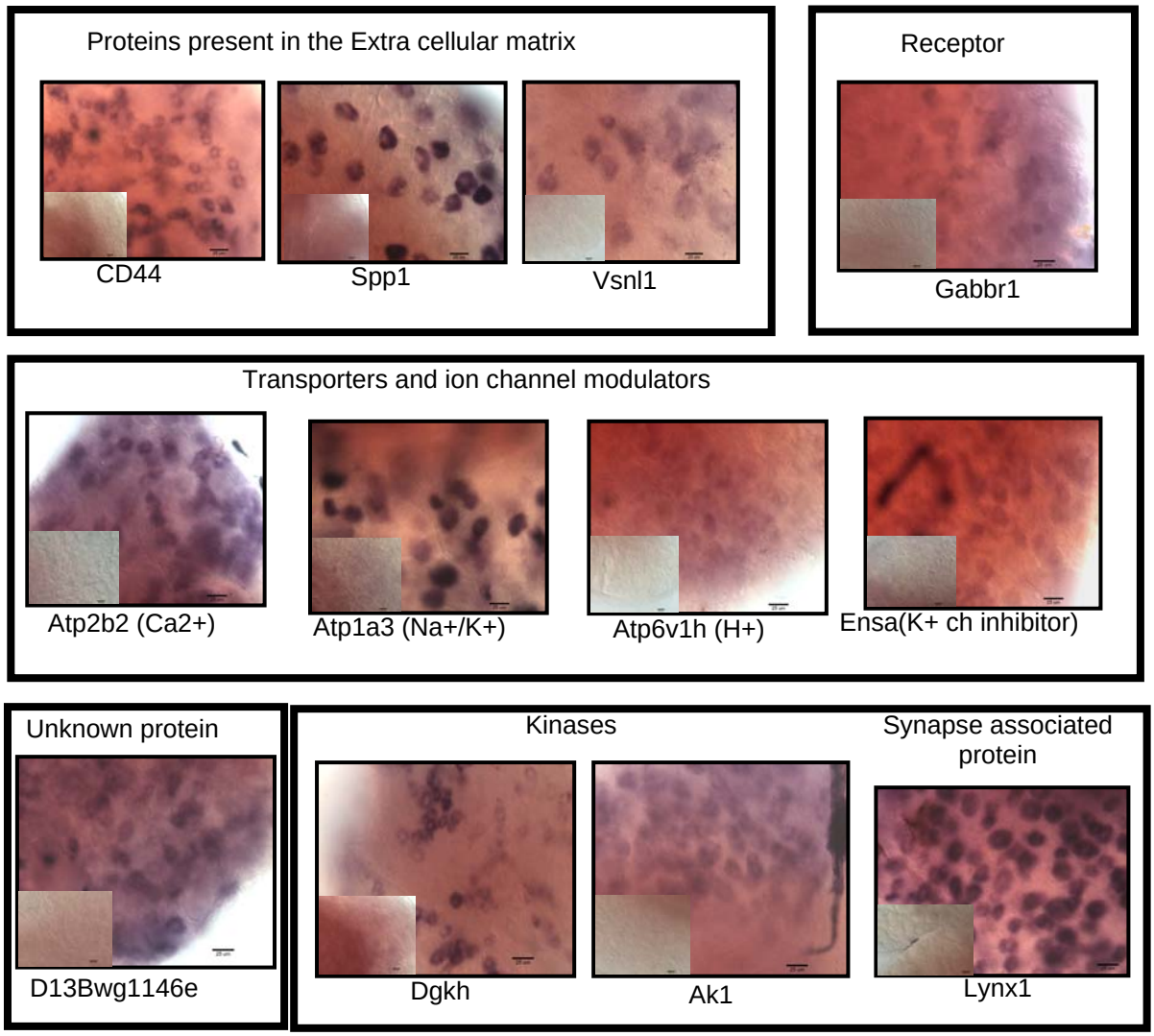


Fig.27: Examples of down regulated genes with subpopulation specific expression pattern observed in adult whole DRGs. scale bar is 25µm.

Out of the 50 genes that satisfied our criteria, 41 genes were cloned. It was observed that 38 genes (92%) showed a subpopulation specific expression pattern (Table. 7, Fig. 28).

Groups	Gene names	No of genes
Extracellular matrix/cell adhesion molecules	**Fbln2, Itgb1bp1, Itgb1bp1, Col5a2, Pcolce2, Col16a1, Crtac1**	7
Ion channels, channel modulators and membrane transporters	**Slc35b2, Kcnv1**	2
Gap junction channel	**Gja1**	1
Other membrane proteins	**Pcnx**	1
Microtubule and transport associated	**Rhou, Spnb2**	2
Non annotated transcripts	**AA407659, 9430098E02Rik, 4631408O11Rik, 162249_f_at, AA415817, 5730494N06Rik, LOC232532, MGI:1351330, Gm1052, 2900074C18Rik, 164941_f_at, Rai16, 139508_at, 4930455C21Rik**	14
Transcription factors and associated proteins	**MGI:1916782**	1
Kinases/Regulators	**Gadd45a, Pkib, Csnk1e, Camk4**	4
Enzymes other than kinases	**Dpp3, Pnp, Txndc7, Fkrp, Blvrb, Ddah1**	6
Immune response related	**Lst1, H2-D1**	2
Growth factors / Hormones / Cytokines / associated receptors	**Gal, Bdnf, Igfbp6, S100a11**	4
Mitochondrial proteins	**Gm137, Nudt9**	2
Development associated proteins	**Nnat, Tagln2**	2
Histone	**Hist3h2ba**	1
Apoptosis related	**Card10**	1

Table 7: Up-regulated genes in gene chip experiments. 50 genes were found to be up regulated in the DRGs of NT34 mutant mice compared to the control animal DRGs. 41 genes were cloned and whole mount *in situs* done. Written in red are the genes with a subpopulation specific expression pattern.

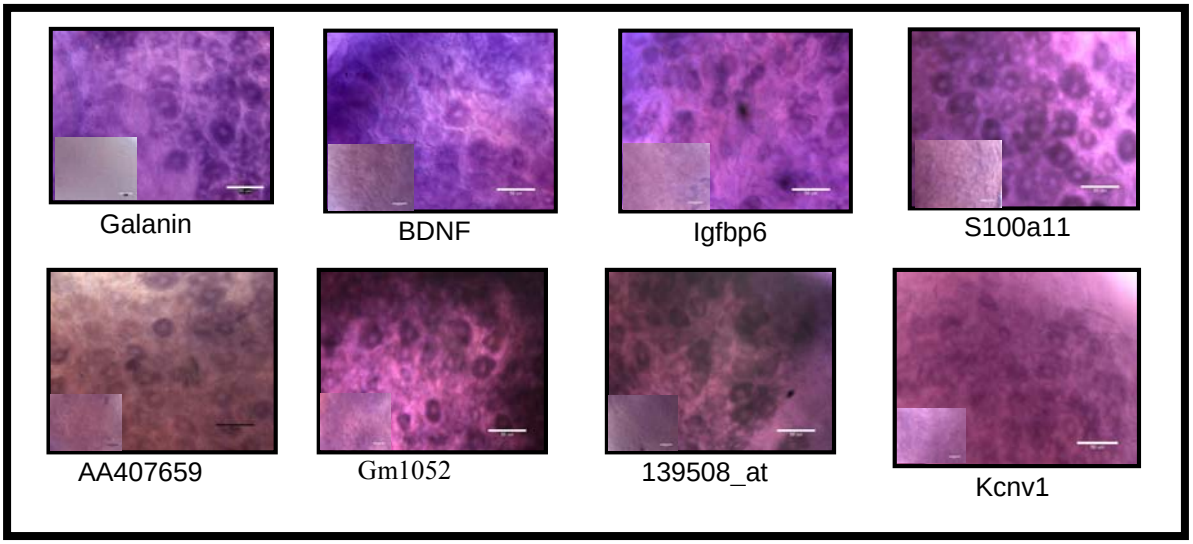


Fig. 28: Examples of up regulated genes with subpopulation specific expression pattern. Scale bar is 50µm.

4.2.4.3 Gene chip experiments with enriched pool of transcripts:

One round of experiments were performed with subtracted and unsubtracted cDNA pools. cRNA was transcribed from the pcr products and were used for hybridization on to Affymetrix set. As it was an enriched pool of transcripts, I was keeping the cut off filter at 2.5. In other words, only the transcripts enriched by 2.5 times were taken for further analysis. Out of 118 genes fitted to this criterion, 45 were cloned and high whole DRG *in situs* performed. We found that S100b (S100 protein), Nefh (neurofilament, heavy polypeptide), Map1lc3b (microtubule-associated protein 1 light chain 3 beta), Ankrd46 (ankyrin repeat domain 46), Atp6ap1 (ATPase, H+ transporting, lysosomal accessory protein 1), Ctsb (cathepsin B), Calm1 (calmodulin 1), Gpm6b (glycoprotein m6b), Cplx1(complexin 1), 6330417K15Rik, Npal3 (NIPA-like domain containing 3), Ppp1r11 (protein phosphatase 1, regulatory (inhibitor) subunit 11), Lrp11 (low density lipoprotein receptor-related protein 11), SPP1 (Secreted phosphoprotein 1), showed a subpopulation specific expression pattern.

4.2.4.4 Determining the expression level of some of the genes obtained in the screen with a restricted expression pattern

Seven genes were selected to compare the expression level in NT34 mutant and control DRG. Three independent qPCR experiments were conducted. Ramp 2 expression is increased in the NT34 mutant DRG in comparison to control (Fig. 29). Out of 9 genes that referred to as down

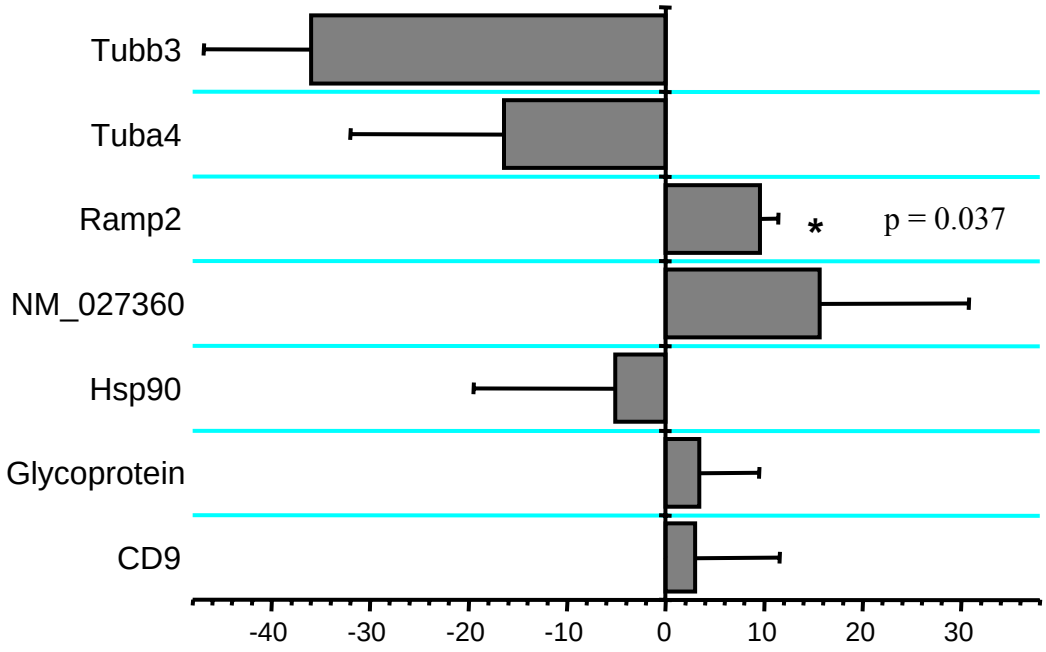


Fig.29 Percentage change of expression level of different transcripts obtained from the screen in NT34 mutant with respect to control. Different genes with a restricted pattern of expression observed in the high throughput whole mount *in situ* hybridization were selected for TaqMan PCR.

regulated in the NT34 mutant DRGs, in the affymetrix experiments, one transcript for which there was no annotation, was observed to be upregulated (Fig. 30).

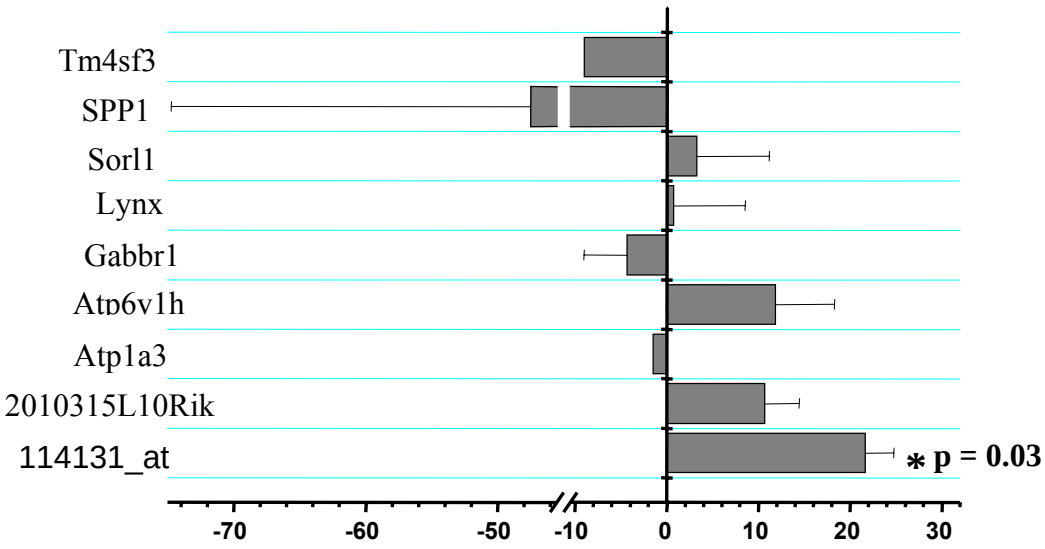


Fig.30. Percentage change of expression level of different transcripts observed in gene chip experiment to be down regulated in NT34 mutant compared to control.

4.2.5 Results of Behavioral test

4.2.5.1 Sand paper based tactile acuity test:

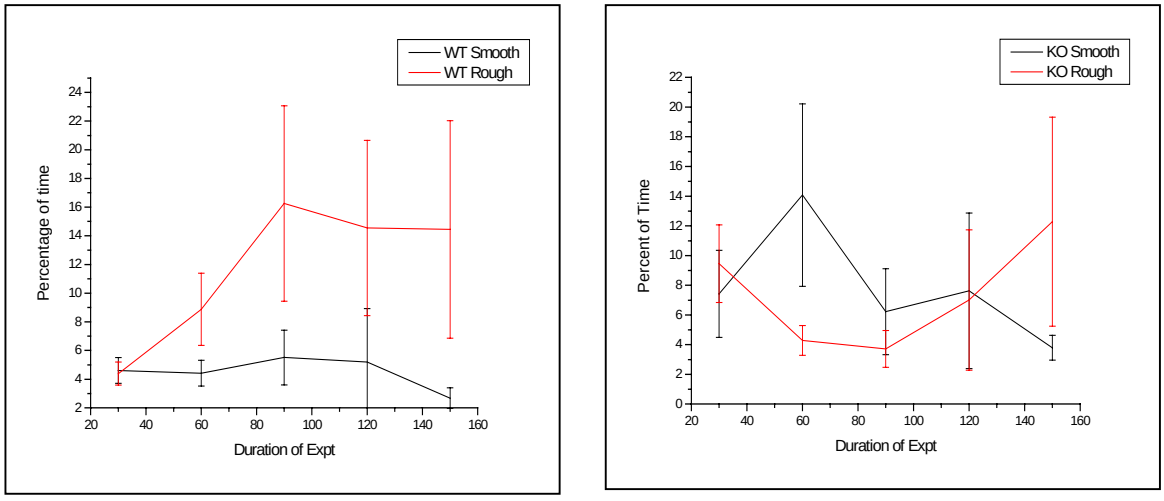


Fig. 31. Left pane is the time spent by control animal in each quadrant where the rough and the smooth surfaces were introduced. Right panel is the shows the NT34 mutant behavior.

Because of the profound loss of neurons, we decided to check whether these were able to discriminate between two different surfaces. Percentage of time spent in the quadrant where the rough surface was introduced, seemed to be high for the control animals where as for the NT34 mutant, no change was observed. These animals were not as active as the control. So the results only hinted that these animals could be tactile insensitive. When grid based tactile acuity was performed we could not conclude the data for the same reason.

4.3 PART III. Functional analysis of a candidate gene "Secreted phosphoprotein 1 (SPP1)" obtained from the screen for its role in mechano-transduction

Out of many genes with a restricted expression pattern, we decided to examine if SPP1 (secreted phosphoprotein 1) plays a role in mechano-transduction. This gene was selected because it was down regulated in the mutant compared to the control in 2 out of three independent affymetrix gene chip experiments. In addition it was also increased in the subtracted cDNA population enriched for the down regulated transcripts, compared with the unsubtracted cDNA population in one round of affymetrix gene chip experiment.

Secreted phophoprotein 1 (SPP1) commonly known as osteopontin (OPN) has other names like BSPI, BNSP, Eta, ETA-1, OP, minopontin, Apl-1, Opn, Ric, Opnl, Bsp. It was described first as a marker for transformed cells (Senger, et al., 1979) and was cloned later from rat osteosarcoma cell line (Oldberg, et al., 1989). This protein with its RGD (Arginine-Glycine-Aspartate) domain like fibronectin, vitronectin, collagen, laminin binds to integrins. SPP1 binds to different integrins $\alpha_4\beta_1$, $\alpha_8\beta_1$, $\alpha_v\beta_1$, $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_9\beta_1$ in a RGD dependent and independent manner thereby modulating different functions performed by these integrins like cell attachment, migration, chemotaxis, intracellular signaling and modulation of neuronal regeneration following injury (Reviewed in Rittling and Chambers, 2004). SPP1 also binds to splice variants of CD44- a hyaluronan receptor- initiating and modulating chemotaxis in a dose-dependent and RGD independent manner (Desai, et al., 2007). Recent evidence from chemotaxis of peritoneal macrophages provides evidence for the existence of an intracellular form of SPP1 which could act through G protein coupled receptors and CD44 (Zhu, et al., 2004). It was also observed that SPP1 increases the CD44 anchoring of MMP-9 metalloproteinase (Desai, et al., 2007). Association of CD44 to MMP-9 is shown to be a mechanism for tumor invasion (Yu and Stamenkovic, 1999). A role for SPP1 in the inhibition of cytochrome *c* oxidase (Gao, et al., 2003) and its association with elastin in dermis and aorta (Pasquali-Ronchetti, and Baccarani-contri, 1997) was also reported. It is expressed in a variety of tissues including bone, dentin, cementum, hypertrophic cartilage, brain, DRGs, gall bladder, breast kidney, cochlea and others (Reviewed in Sodek, et al., 2000). In many of these tissues only specific cell types express SPP1 (Lopez, et al., 1995). Increased expression level of SPP1 is also observed after mechanical stimulation in cells *in vitro* and in *in vivo* (Kubota, et al., 1993, Klein-NulenId, et al., 1997, Carvalho, et al, 1998, Miles, et al., 1998). Recently it

was reported the observation of increased mechanical withdrawal threshold in SPP1 mutants, hinting a possible role of SPP1 in mechanotransduction (Marsh, et al., 2007).

In the present study we asked whether SPP1 plays any role in mechanotransduction using electrophysiology and behavioral experiments. An overview of the experiments is shown in Fig. 32.

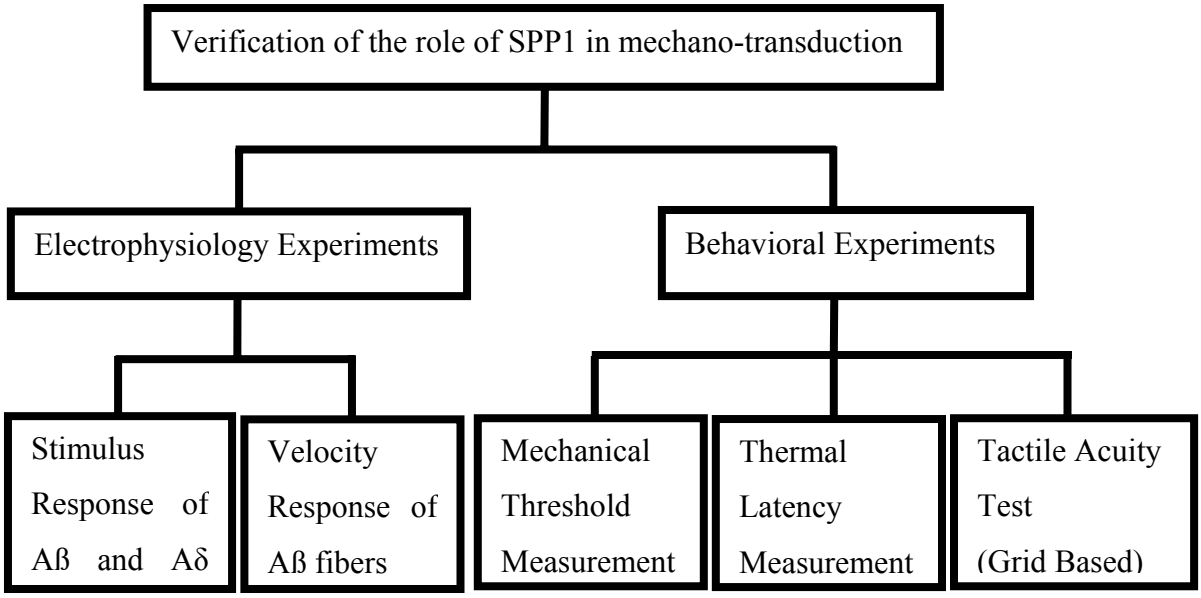


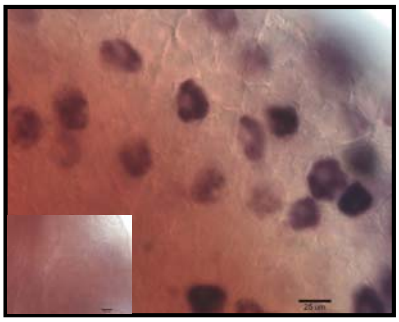
Fig. 32. An overview of experiments performed using SPP1 mutants.

4.3.1 SPP1 expression is observed mainly in medium sized cells

Cells in the DRGs can be classified grossly as large, medium and small. Even though there is an overlap between the properties of neurons irrespective of the size and other properties like conduction velocities and mechanical threshold of activation, (Djouhri and Lawson, 2004), in general, small neurons could be considered as nociceptors, medium neurons as Aδ fibers and large ones as Aß fibers. The cell diameters of neurons expressing SPP1 were calculated from the micrographs of DRGs, after whole mount *insitu* hybridization. It is observed that the majority of the cells expressing SPP1 were medium sized (Fig.33):

4.3.2 Results from electrophysiology experiments

It has been reported that in rat, SSP1 is expressed in many large or medium sized neurons in Trigeminal ganglia (TG), dorsal root ganglia (DRG), in Mes5 neurons and nerve fibers at the base of vibrissal follicles (Ichikawa, et al., 2000, 2001). Recently SPP1 expression was shown in a subpopulation of neurons in the mouse DRG and was observed to be co-localized with NF200, which is a marker for myelinated neurons (Marsh, et al., 2007). They also observed



SPP1

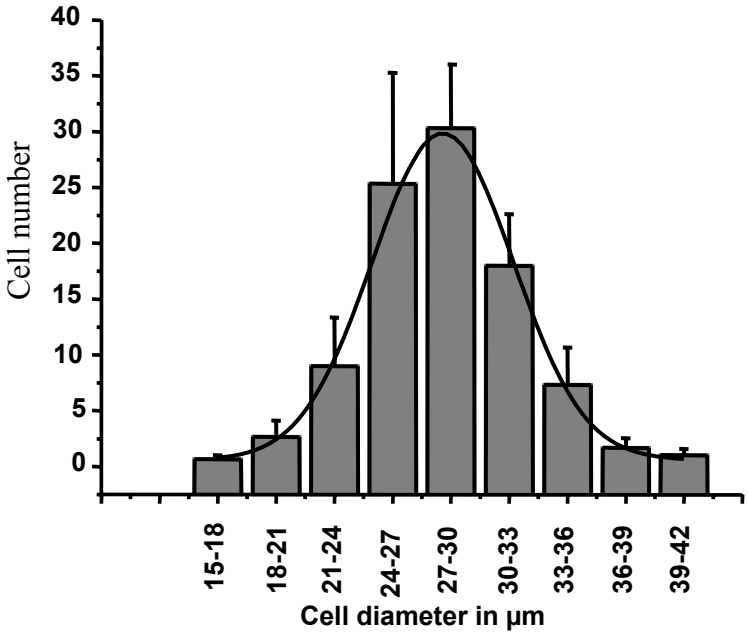


Fig. 33: Left panel shows SPP1 expressing sub-poulation of cells after whole mount DRG *insitu* hybridization. Right panel shows the soma size of the population of SPP1 expressing cells in whole DRGs.

an increase in the mechanical withdrawal threshold in these mutants. Because of the association of SPP1 to integrin and other extra cellular matrix proteins by itself and through CD44 to the cytoskeleton we asked whether this protein could influence the mechanotransduction in a subpopulation of skin sensory afferents.

Age matched SPP1 mutant and control mice were analyzed electrophysiologically using an *in vitro* skin nerve preparation to characterize different sensory neuron types and their physiological properties. All the afferents were tested for stimulus response and Aß fibers were tested for velocity sensitivity. Classification of the units recorded is given in Table 8.

4.3.3 The sensitivity of AM fibers to mechanical stimuli appears increased in SPP1 mutants

The frequency of action potentials generated by the sensory units by increasing displacement stimuli is plotted. Experiments were performed randomly on mutants and controls. It was observed that the stimulus response data obtained from the control animals in the experiments was decreased compared to the control data obtained by another experiment (Christiane Wetzel). So in order to have a better assessment, control data obtained by CW is also plotted together with my data. It was observed that the control data does not vary in different strains (Milenkovic N., et al., Unpublished data). The present data suggests that the stimulus response for AM fibers (Fig. 34) in SPP1 mutant could be increased in comparison to the controls (P <

0.0001, Statistically analysed by two-way RM ANOVA). Also there was no difference observed neither in the stimulus response of other fibers (Example: SA fibers in Fig. 35) nor in the velocity response of Aß fibers. Because of the few number of units recorded further experiments need to be done to confirm these findings.

Receptor type	**Control**			**SPP1 mutant**		
	% Total	CV **m/s**	**vFT** (mN)	**% Total**	CV **m/s**	**vFT** (mN)
Aβ-Fibers RA	25.00 (3/12)	16.32±2.27	1.37 (0.4-3.3)	35.29 (6/17)	15.24±1.36	3.52 (0.4-6.3)
SA	75.00 (8/12)	14.01±2.45	2.41 (1-6.3)	64.71 (11/17)	15.03±0.6	1.43 (0.4-10)
Aδ-Fibers AM	72.73 (8/11)	5.49±0.77	5.025 (2-10.0)	66.67 (6/9)	6.96±1.2	6.82 (2-13)
D-hair	27.27 (3/11)	6.34±2.23	0.4	33.33 (3/9)	6.24±1.25	0.4

Table 8. Numbers and properties of of sensory afferents recorded

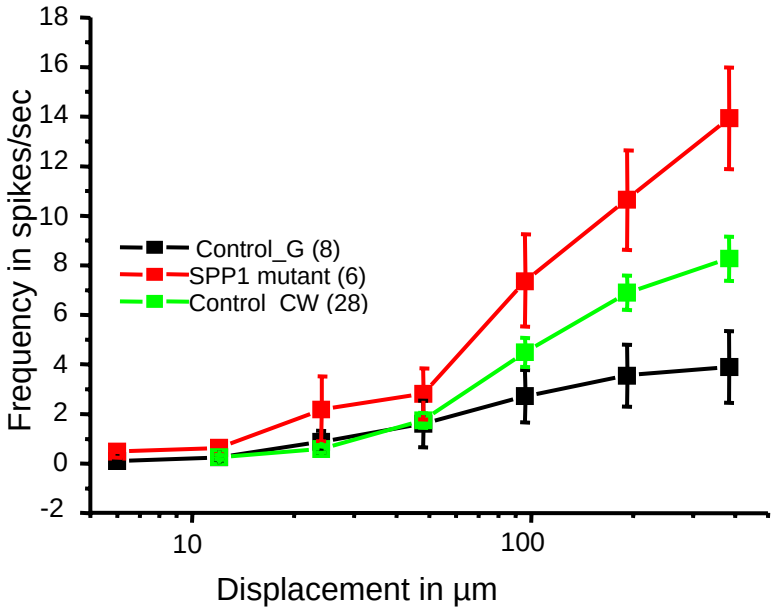


Fig. 34. Stimulus response of AM fibers of SPP1 mutants are increased in comparison with SPP1 controls and the reference control data.

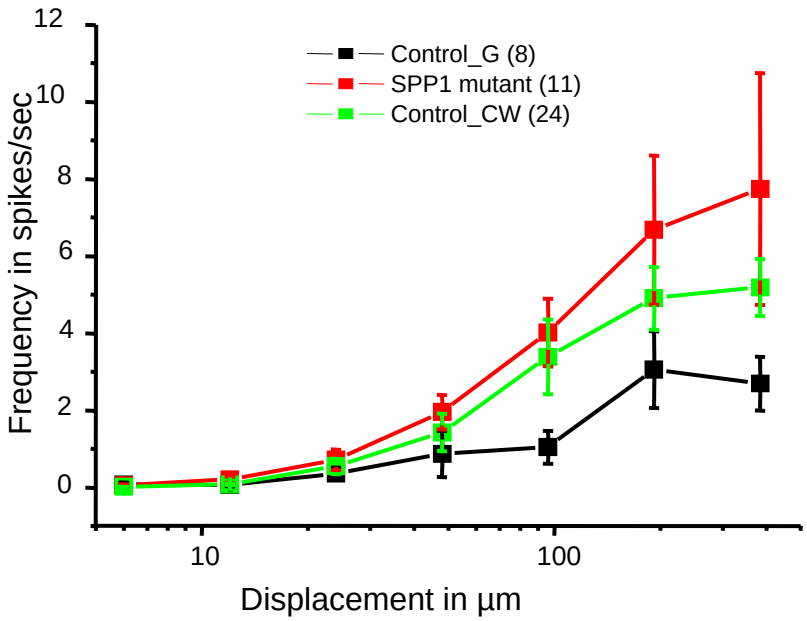


Fig. 35. Stimulus response of SA fibers of SPP1 mutants shows an increasing trend in comparison with control mice and the reference control data.

4.3.4 Results of behavioral experiments

4.3.4.1 Mechanical withdrawal threshold was not changed in SPP1 mutants

To investigate the change in mechanosensation, the force required for a response (paw withdrawal) was determined (Fig.36). There was no change observed for the mechano nociception in SPP1 mutants in comparison to the control animals (T-Test, p = 0.25).

4.3.4.2 Thermal latency was not changed in SPP1 mutants:

Animals were exposed to radiant heat on the hind paw and the latency of withdrawal was determined by an automated device. The latency was measured in seconds (Fig. 37). There was no change observed in SPP1 mutants in the thermal latency compared to control animals (T-Test, p = 0.82).

4.3.4.3 Results of grid based tactile acuity tests:

After observing changes in mechanotransduction using electrophysiology in SPP1 mutants, the behavior of the mutants was investigated using a tactile acuity test. Age matched SPP1 mutants (8) and 16 control animals were tested. Three different grids were used as surface cues with spatial frequencies, 250µm, 500µm and 750µm. After the acclimatization period,

cues were introduced and the activity of animal monitored. The distance travelled, amount of time spent (time visiting), frequency of visits, and activity in the area where the surface cue is

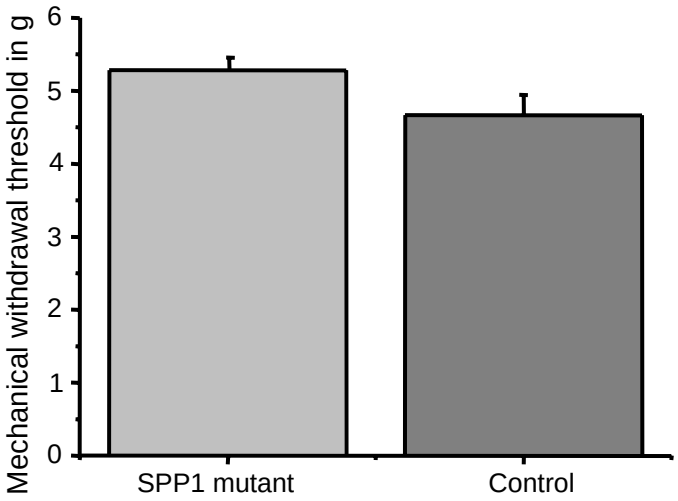


Fig. 36: Mechanical withdrawal threshold in SPP1 mutants and in control animals.

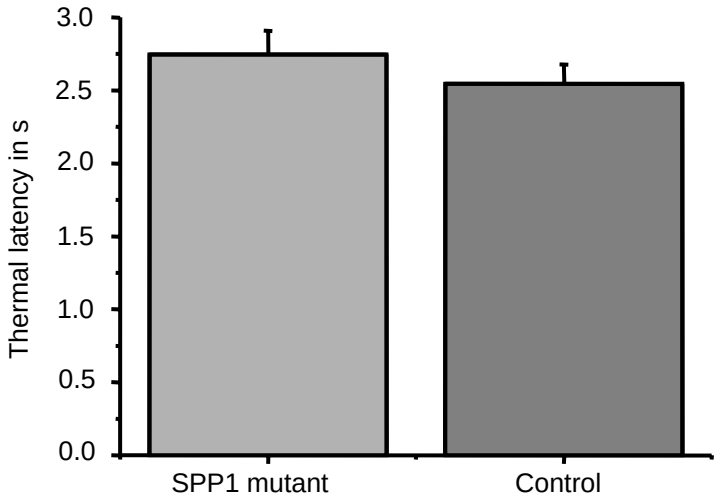


Fig. 37: Thermal latency measured in SPP1 mutants and in control animals using a radiant heat source.

introduced is compared with the area without any cue to assess the behavior of the animals in response to the surface cue. For SPP1 mutants no difference in the distance travelled (Fig. 38, paired T-Test, $p > 0.05$), time spent, number of the visits and the activity when compared to the control area without any cue suggesting that these animals might not have recognized the 250µm grids in comparison with the behavior of control animals. Only 8 mutant animals were used for the studies and so further experiments need to be done to verify the effect.

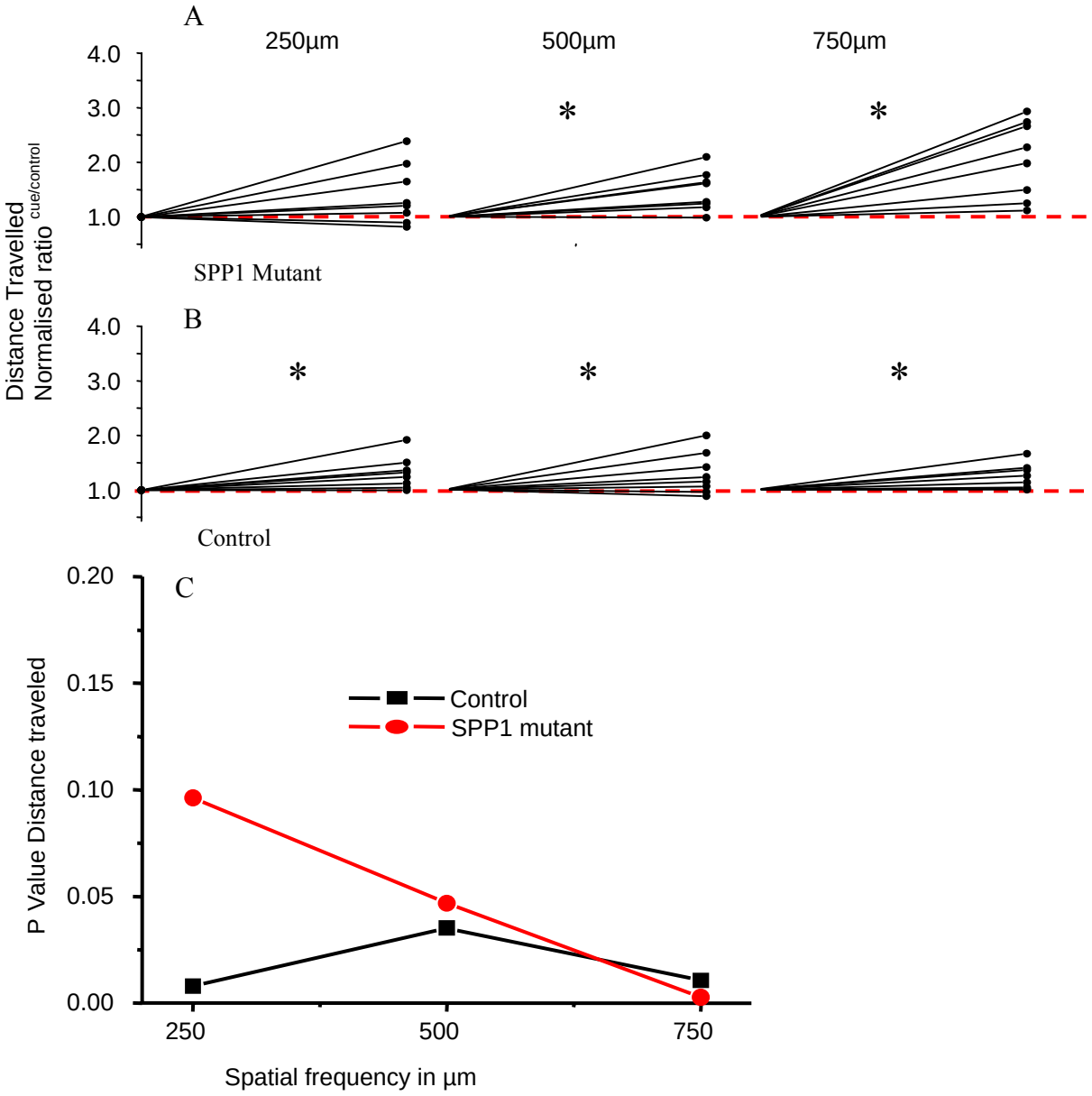


Fig. 38: Distance traveled: A, B) Distance traveled on the area with the surface cue normalized to the control area for SPP1 mutant and control animals respectively. C) P Value (paired T-Test) of distance traveled for control and mutant for its statistical relevance.

4.4 PART IV: Finding molecules which may bind to extra-cellular domain of ASIC3 using a skin phage display:

Acid sensing ion channel 3 (ASIC3), a member of DEG/ENaC is shown to be involved in mechanotransduction (Mogil, et al., 2005, reviewed by Lumpkin and Caterina, 2007, Price, et al., 2001). Our aim was to identify extra cellular matrix (ECM) molecules that could binding to the extra cellular region of the ion channel. Our strategy was to have a phage display library adsorbed on to ASIC3 expressing *X. laevis* oocytes as "biological beads" and do repeated adsorption and amplification to enrich phages that might be binding to the ion channels (Fig.39).

Principle of adsorption:

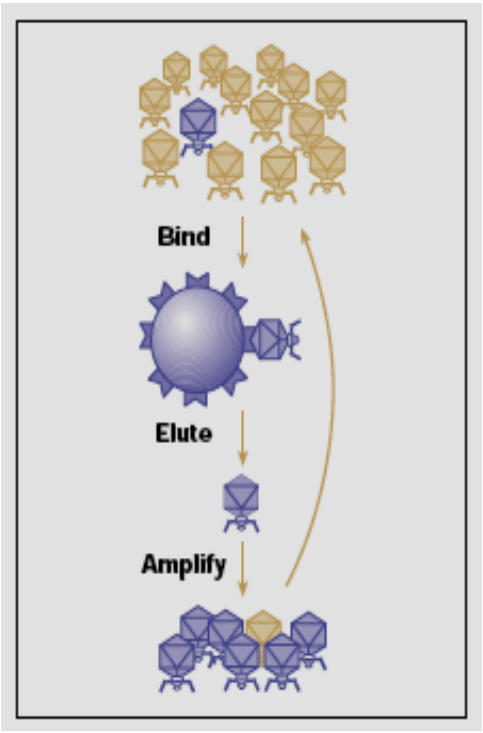


Fig 39. Principle and methodology used for the amplification of phages that bind to ASIC3

Mechanotransduction molecules present in the skin are probably too sparse for normal biochemical methods to be deployed. As we are trying to find molecules, which can bind to the extracellular region of ASIC3, methods like yeast two hybrid screening are not ideal. Moreover, we tried to use the native conformation of ASIC3 using eukaryotic expression system and tried to get the binding protein in part or in full. Oocytes without injecting the

RNA were used as negative controls to identify phages that bind to other surface proteins in the oocyte.

The library was made using T7 phages. Advantages of using this system was that the peptides or proteins displayed on the surface of T7 need not necessarily get secreted through the cell membrane, a prerequisite in filamentous phage assembly. Ease of maintenance and growth and the rapid replication properties were the advantages over either bacteriophage λ or filamentous phage. T7 phage particle was capable of withstanding harsh conditions that inactivate other phages.

4.4.1 Phages were selectively enriched:

After 4 rounds of adsorption, washings and amplification, individual phages were isolated by plating and were PCR amplified. The amplicons were electrophresed to know the size. By the distribution based on the amplicon size, grossly it could be seen that the population of the phages obtained by the adsorption with oocytes expressing ASIC3 were different from phages adsorbed by oocytes not expressing the ion channel (Fig. 40). These were sent for sequencing and inserts identified.

4.4.2 Extra cellular matrix proteins were found to be selectively enriched in the phages adsorbed by ASIC3 expressing oocytes.

After identifying the inserts in the adsorbed phages, only those were identified as positive that were shown to be adsorbed and enriched only by ASIC3 expressing oocytes (Table. 9).

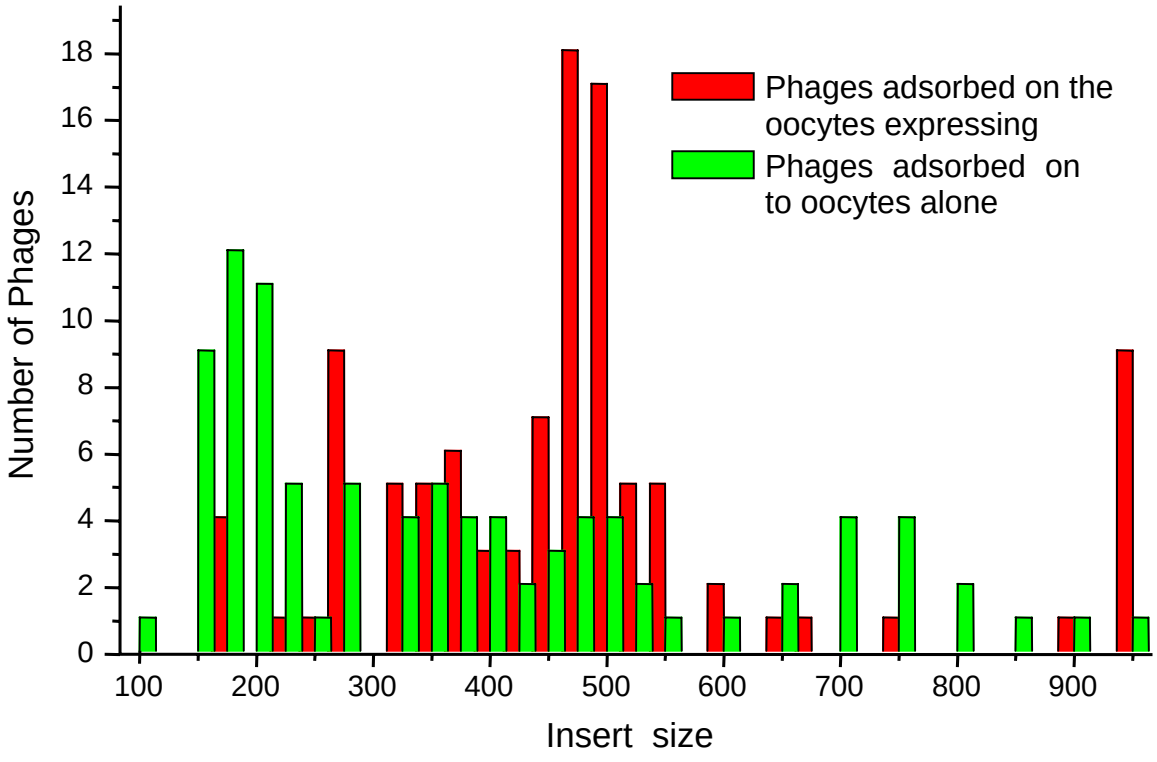


Fig. 40. Insert size of clones in phages enriched by oocytes expressing ASIC3 and normal oocytes.

Sl. No	Total no. of clones	Clones in ASIC3	Clones in Control	Annotation
1	31	31	0	*Mus musculus* keratin 10 (Krt10),
2	3	3	0	*Mus musculus* SET domain containing 2, transcript variant 3 (Setd2).
3	2	2	0	118bp near to 3' region of hypothetical protein similar to secreted acidic cysteine rich glycoprotein (Sparc),
4	1	1	0	PREDICTED: *Mus musculus* ankyrin repeat domain 44, transcript variant 16 (Ankrd44),
5	1	1	0	RAB2, member RAS oncogene family,
6	1	1	0	*Mus musculus* heat shock protein 4 (Hspa4),
7	1	1	0	*Mus musculus* interferon gamma inducible protein 30 (Ifi30),
8	1	1	0	PREDICTED: *Mus musculus* hypothetical protein LOC673353 (LOC673353),
9	1	1	0	*Mus musculus* mRNA for monoglyceride lipase (Mgll gene), transcript 1
10	1	1	0	ZINC FINGER CCCH DOMAIN CONTAINING 13
11	1	1	0	cyclin-dependent kinase 8
12	1	1	0	EPIDERMAL KERATIN 10
13	1	1	0	complement component 1, q subcomponent, alpha polypeptide,
14	1	1	0	*Mus musculus* eukaryotic translation elongation factor 1 alpha 1
15	1	1	0	PREDICTED: *Mus musculus* enhancer of polycomb homolog 2 (Drosophila) (Epc2),
16	1	1	0	*Mus musculus* hexokinase 1, mRNA
17	1	1	0	*Mus musculus* chloride intracellular channel 5,

Table. 9. Genes that were identified to be present only in the population of enriched phages that were adsorbed by ASIC3 expressing oocytes.